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MCL-1 Regulates Mitochondrial Dynamics and Turnover

A dissertation submitted in partial satisfaction of the requirements
for the degree Doctor of Philosophy

in

Biomedical Sciences

by

Alexandra Grady Moyzis

Committee in charge:

Professor Åsa Gustafsson, Chair
Professor Joan Heller Brown
Professor Ju Chen
Professor Nicole Purcell
Professor Deborah Yelon

2020

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The Dissertation of Alexandra Grady Moyzis is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California San Diego

2020

DEDICATION

I dedicate this dissertation to my family, friends, and mentors for all their support and encouragement throughout my life.

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LIST OF ABBREVIATIONS

Ad-	Adenovirus
α MHC	α -myosin heavy chain
AMPK	Adenosine monophosphate(AMP)-activated protein kinase
ANOVA	Analysis of variance
Atg	Autophagy
ATP	Adenosine triphosphate
BAD	BCL-2 associated agonist of cell death
BAX/BAK	BCL2-associated X protein (BAX)/BCL2-antagonistic/killer (BAK)
BCL2	B-cell CLL/lymphoma 2
BCL-2-L13	BCL-2-like 13
BCL-X _L	B-cell lymphoma-extra large
BFL1	BCL-2-related protein A1
β -gal	β -galactosidase
BH	BCL-2 homology
BID	BH3 interacting-domain death agonist
BIK	BCL-2 interacting killer
BIM	BCL-2-like protein 11
BNIP3	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3
BNIP3L/Nix	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 Like
BSA	Bovine serum albumin

CCD	Charged coupled device
cDNA	Complementary DNA
Con	Control
CVD	Cardiovascular disease
DKO	Double knockout
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DRP1	Dynamin related protein 1
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EF	Ejection fraction
EGTA	Ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FCCP	Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone
FIS1	Fission protein 1
FS	Fractional shortening
FUNDC1	FUN14 domain containing 1
GABARAP	Gamma-aminobutyric acid receptor-associated protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein

Glut	Glutamate
GTP	Guanosine triphosphate
h	Hour(s)
H&E	Haematoxylin and eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hsp	Heat shock protein
HW	Heart weight
I/R	Ischemia/reperfusion
IMM	Inner mitochondrial membrane
IMS	Intermembrane space
KO	Knockout
LAD	Left anterior descending artery
LC3	Microtubule-associated protein 1 light chain
LIR	LC3-interacting region
Mal	Malate
MCL-1	Myeloid cell leukemia 1
Mdivi1	Mitochondrial division inhibitor 1
MEF	Mouse embryonic fibroblast
MFF	Mitochondrial fission factor
MFN1/2	Mitofusin 1/2
MI	Myocardial infarction
MiD	Mitochondrial dynamics
MnSOD	Manganese superoxide dismutase

MOI	Multiplicity of infection
MOPS	3-(N-morpholino)propanesulfonic acid
mPTP	Mitochondria permeability transition pore
mtDNA	Mitochondrial DNA
mTOR	Mammalian target of rapamycin
MTS	Mitochondrial targeting sequence
NDP52	Nuclear domain 10 protein 52
NBR1	Neighbor of BRCA1 gene
NOXA	Phorbol-12 myristate-13-acetate-induced protein 1
OCR	Oxygen consumption rate
OMM	Outer mitochondrial membrane
OPA1	Optic atrophy 1
OXPHOS	Oxidative phosphorylation
P62/Sqstm1	Sequestosome 1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEST	Proline, glutamic acid, serine, threonine
pH	Potential of hydrogen
PINK1	PTEN-induced putative kinase 1
PST	PBS plus 5% normal goat serum and 0.2% Tween20
PUMA	P53 upregulated modulator of apoptosis
Pyr	Pyruvate
ROS	Reactive oxygen species

Rot	Rotenone
RNAi	RNA interference
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
S.E.M.	Standard error of the mean
siRNA	Small interfering ribonucleic acid
Succ	Succinate
TAX1BP1	Tax1-binding protein
TEM	Transmission Electron Microscopy
TG	Transgenic
TIM	Translocase of the inner mitochondrial membrane
TL	Tibia length
TOM	Translocase of the outer mitochondrial membrane
Ubl	Ubiquitin like
ULK1	Unc-51 like autophagy activating kinase 1
Un	Untransfected
Vps	Vacuolar protein sorting
WT	Wild type

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VITA

- 2013 Bachelor of Arts, Biology, Scripps College
- 2019 Doctor of Philosophy, Biomedical Sciences, University of California,
San Diego

PUBLICATIONS

Alhoch B, Chen A, Chan E, Elkabti A, Fariña S, Gilbert C, Kang J, King B, Leung K, Levy J, Martin E, Mazer B, McKinney S, Moyzis A, Nurimba M, Ozaki M, Purvis-Roberts K, Rothman JM, Raju S, Selassie C, Smith O, Ticus J, Edwards-Gilbert G, Negritto MC, Wang R, Tang Z. Comparative Genomic Screen in Two Yeasts Reveals Conserved Pathways in the Response Network to Phenol Stress. *G3 (Bethesda)* 9(3):639-650, 2019.

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ABSTRACTS

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AWARDS

UCSD BMS Retreat: Best Research Talk Award (2018)

International Society for Heart Research-North American Section Poster Award (2018)

International Society for Heart Research-North American Section Travel Award (2018)

NIH F31 Predoctoral Fellowship (2017-2019)

American Heart Association Predoctoral Fellowship (Jan 2016-2017)

“Best of BCVS” Poster Award, American Heart Association (2015)

Institutional Cardiology Training Grant 5T32HL007444-32, University of California, San Diego (2014-2016)

Keck Summer Research Fellowship, Scripps College (2011 and 2012)

Norris Summer Research Fellowship, Scripps College (2010)

ABSTRACT OF THE DISSERTATION

MCL-1 Regulates Mitochondrial Dynamics and Turnover

by

Alexandra Grady Moyzis

Doctor of Philosophy in Biomedical Sciences

University of California San Diego, 2020

Professor Åsa Gustafsson, Chair

Myeloid cell leukemia-1 (MCL-1) is an anti-apoptotic protein that is highly expressed in the myocardium and plays a critical role in maintaining cardiac homeostasis. Cardiac-specific MCL-1-knockout mice develop rapid cardiac dysfunction and cardiomyopathy but show little activation of apoptotic cell death. Instead, loss of MCL-1 resulted in atypical mitochondrial morphology and function. This suggests that

besides its anti-apoptotic role, MCL-1 may have broader functions in regulating mitochondrial dynamics and function. MCL-1 localizes to two different mitochondrial locations, both on the outer mitochondrial membrane (MCL-1_{OM}) and a shorter cleaved form resides in the mitochondrial matrix (MCL-1_{Matrix}). Cardiac-specific ablation of MCL-1 resulted in the loss of MCL-1 at both of these distinct mitochondrial locations, so it is unknown what their respective functional contributions are. My studies have focused on MCL-1 on the outer mitochondrial membrane (MCL-1_{OM}).

I have found that MCL-1_{OM} regulates DRP-1-mediated fission as an adaptive response to stress. I have also developed a novel MCL-1_{OM} transgenic mouse model and observed that MCL-1_{OM} overexpression results in enhanced mitochondrial fission *in vivo* as well. Additionally, MCL-1 overexpression facilitates mitochondrial clearance in response to stress. My data suggests that MCL-1 does not act as a mitophagy receptor itself, but likely induces turnover through the mitophagy receptor BNIP3. Finally, MCL-1 inhibits general autophagy in response to energetic stress, but does not exert this effect under circumstances that induce oxidative stress and cellular damage. Taken together, these findings suggest that in addition to its anti-apoptotic role, MCL-1_{OM} has additional functions in regulating DRP1-mediated mitochondrial fission and facilitating mitochondrial clearance via the mitophagy receptor BNIP3. My dissertation also extends concerns about potential cardiotoxicity for chemotherapeutics targeting MCL-1.

CHAPTER 1: INTRODUCTION

1.1 The BCL-2 Family and MCL-1

The BCL-2 family proteins are important regulators of the intrinsic pathway of apoptosis at the mitochondria. This family is composed of both pro-apoptotic and anti-apoptotic members that either antagonize or promote the apoptotic cell death pathway (Kale et al., 2018). Pro-apoptotic members include BAX and BAK, whereas anti-apoptotic members include BCL-2, BCL-X_L, MCL-1, BCL-W, and BFL1/A1 (Kale et al., 2018). The anti-apoptotic members bind to BAX and BAK in order to prevent their activation (Shamas-Din et al., 2013). There are also pro-apoptotic BH3-only proteins including BAD, BID, BIK, BIM, NOXA, PUMA, BNIP3, and NIX/BNIP3L that can disrupt this interaction by binding to the BH domains of the anti-apoptotic proteins themselves in order to release BAX and BAK (Gustafsson and Gottlieb, 2007; Kale et al., 2018). Once BAX and BAK are released, they oligomerize in the outer mitochondrial membrane, which leads to outer membrane permeabilization (MOMP) and subsequent release of pro-apoptotic factors such as cytochrome c and AIF (Volkman et al., 2014). This activates caspases and allows the programmed apoptotic cell death cascade to progress (Volkman et al., 2014).

Anti-apoptotic BCL-2 family proteins have been extensively studied because they are upregulated in a variety of human cancers (Hata et al., 2015). In fact, MCL-1 was first identified as a protein upregulated during the differentiation of ML-1 human myeloid leukemia cells (Kozopas et al., 1993) and it is often a marker of poor prognosis and chemotherapeutic resistance (Xiang et al., 2018). Elevated MCL-1 levels are also

associated with breast and lung cancers, with one study finding MCL-1 increased in as many as 36% and 54% of specimens, respectively (Beroukhi et al., 2010). This is because increased MCL-1 levels prevent cells from undergoing apoptosis when they should, and evading cell death is one of the hallmarks of cancer (Hanahan and Weinberg, 2011). Thus, the anti-apoptotic BCL-2 family members are attractive therapeutic targets and many inhibitors have already been developed that are directed toward them (Campbell and Tait, 2018). Additionally, several selective MCL-1 inhibitors have been synthesized (Xiang et al., 2018). Three of these selective MCL-1 inhibitors, AMG176, MIK665/S64315, and AZD5991, are currently in clinical trials (Xiang et al., 2018). However, MCL-1 expression is not restricted to cancer cells and a greater understanding of MCL-1's function in cells and tissues is necessary to continue pursuing development of these therapeutics and prevent unwanted side-effects.

While each of the BCL-2 family members share anti-apoptotic activity, knockout studies have revealed that MCL-1 is distinctly important at an early developmental stage. Global knockout of MCL-1 is embryonic lethal by day 4 (Rinkenberger et al., 2000). In contrast, while BCL-X_L is necessary for brain development, BCL-X_L-knockout mice survive to embryonic day 13.5 (Motoyama et al., 1995). Finally, BCL-2 knockout mice are viable but show growth retardation, renal failure, and apoptosis of the lymphocytes (Veis et al., 1993). MCL-1 is also structurally distinct from its anti-apoptotic family members. It lacks a BH4 domain and has an elongated N-terminus with the presence of two PEST (proline, glutamic acid, serine, and threonine) sequences that allow for rapid proteosomal degradation (Thomas et al., 2010) (Figure 1.1). This makes MCL-1 more tightly regulated in response to stress than BCL-2 and BCL-X_L. Thus,

while there is some redundancy in function, the anti-apoptotic BCL-2 family proteins also have distinct physiological niches.

1.2 MCL-1's Function in the Heart

MCL-1 is essential for ensuring the proper function and survival of a variety of cell lineages and tissues (Arbour et al., 2008; Csepregi et al., 2018; Dzhagalov et al., 2008; Dzhagalov et al., 2007; Opferman et al., 2005; Opferman et al., 2003; Vick et al., 2009). MCL-1 is also highly expressed in the heart (Wu et al., 1997) and our group and others have shown that MCL-1 is required for normal cardiac function (Thomas et al., 2013; Wang et al., 2013). The importance of MCL-1 in maintaining homeostasis in the heart is evident whether MCL-1 is knocked out during embryogenesis (Wang et al., 2013) or in adult mice (Thomas et al., 2013). MCL-1-deficient hearts show signs of severe contractile dysfunction, hypertrophy, and fibrosis, ultimately leading to a significant reduction in lifespan (Thomas et al., 2013; Wang et al., 2013). Given that MCL-1 is an anti-apoptotic protein, it could be expected that these cardiac defects occur as a result of increased apoptosis. However, results from the two studies differ regarding the importance of apoptosis. Thomas et al. found no detectable increase in apoptosis, despite employing a variety of techniques and assays to assess it (Thomas et al., 2013). Wang et al. were able to detect increased apoptosis in the MCL-1 knockout hearts, albeit at very low levels (Wang et al., 2013). Furthermore, Wang et al. showed functional rescue upon concurrent knockout of MCL-1 with the pro-apoptotic proteins BAX and BAK (Wang et al., 2013). However, BAX and BAK have also been

implicated in regulating necrotic cell death (Whelan et al., 2012), so the specific role of apoptosis in this case remains unclear.

Regardless, both studies clearly demonstrated that MCL-1 also exhibits functional roles beyond apoptosis. This is consistent with global MCL-1 knockout mice, which intriguingly, do not show signs of increased apoptosis (Rinkenberger et al., 2000). Upon doing transmission electron microscopy, Thomas et al. observed swollen mitochondria and ruptured myocytes, which are suggestive of necrotic, rather than apoptotic, cell death (Thomas et al., 2013). These hearts also showed increased inflammation and increased lactate dehydrogenase (LDH) activity in plasma, which are other indicators of necrosis (Thomas et al., 2013). Crossing MCL-1-knockout mice with mice lacking cyclophilin D, an essential component of the mPTP and regulator of necrotic cell death (Baines et al., 2005), delayed the development of heart failure (Thomas et al., 2013). This suggests that MCL-1 does not directly control mPTP opening, but rather loss of MCL-1 leads to mitochondrial dysfunction, subsequently activating this process (Thomas and Gustafsson, 2013). Wang et al. also observed loss of myofibrils and mitochondria that are rounded, disorganized, and dispersed with disrupted cristae in MCL-1-ablated hearts (Wang et al., 2013). These structural defects also corresponded to deficits in mitochondrial respiration in these mice (Wang et al., 2013). Interestingly, while BAX/BAK double knockout preserved cardiac function in MCL-1 knockout mice, it did not prevent mitochondrial abnormalities from occurring (Wang et al., 2013). Both of these studies provide evidence for additional, previously uncharacterized, roles of MCL-1 extending beyond apoptosis to broader implications in

maintaining mitochondrial homeostasis. This is particularly important in terminally differentiated tissues such as the heart that possess limited regenerative capacity.

1.3 MCL-1 Localizes to Two Distinct Mitochondrial Locations

Recently it was uncovered that MCL-1 localizes to two distinct mitochondrial locations, the outer mitochondrial membrane (MCL-1_{OM}) and the matrix (MCL-1_{Matrix}) (Huang and Yang-Yen, 2010; Perciavalle et al., 2012) (Figure 1.2). MCL-1 contains a mitochondrial targeting sequence at its N-terminus, which gets cleaved off upon import into the matrix (Perciavalle et al., 2012). However, it is currently unknown what kind of post-translational modification could signal import of MCL-1. Given that knockout studies in the heart have ablated MCL-1 at both mitochondrial locations, it is unclear whether loss of one or both leads to the observed cardiac defects (Thomas et al., 2013; Wang et al., 2013). Studies suggest unique roles for MCL-1_{OM} versus MCL-1_{Matrix}. MCL-1_{OM} retains MCL-1's anti-apoptotic activity and is required for protection against apoptotic cell death, whereas MCL-1_{Matrix} has been implicated in regulating mitochondrial fusion, energetics, and metabolism (Escudero et al., 2018; Huang and Yang-Yen, 2010; Perciavalle et al., 2012). However, their exact functional contributions are still being examined. Interestingly, MCL-1_{OM} and MCL-1_{Matrix} respond differently to stress. For instance, subjecting mice to myocardial infarction (MI) reveals that MCL-1 on the outer membrane is rapidly degraded four hours after MI and later restored at twenty-four hours (Thomas et al., 2013). This is in contrast to MCL-1 in the matrix, which is maintained at four hours and spared from degradation and increases at twenty-four hours (Thomas et al., 2013). However, their expression patterns also need to be

monitored in response to additional types of stressors to determine if MCL-1 on the outer membrane is still more susceptible to degradation. Thus, these two localizations of MCL-1 are implicated in distinct roles, but their exact functional contributions, specifically in regard to protection against cell death in the heart require further elucidation.

1.4 Mitochondrial Fission and Fusion

Mitochondria are very dynamic organelles, constantly undergoing fission and fusion to maintain homeostasis and shape their networks in response to stress. When mitochondria undergo fission they fragment into shorter organelles, whereas fusion causes the mitochondria to become elongated (Figure 1.3). The balance of these two processes is crucial to maintaining homeostasis in the heart and defects in either result in severe functional consequences (Chen et al., 2015; Chen et al., 2011; Ikeda et al., 2015; Papanicolaou et al., 2012; Song et al., 2017; Song et al., 2015b). Mitochondrial fission is regulated by the large dynamin-like GTPase dynamin-related protein 1 (DRP1), which translocates from the cytosol to the mitochondria to induce scission (Nan et al., 2017). Additional regulators of mitochondrial fission include MFF, FIS1, and MiD49/51, which have been reported to function as receptors for DRP1 on the outer mitochondrial membrane (Liu and Chan, 2015; Loson et al., 2013; Palmer et al., 2013). Mitofusin 1 and 2 (MFN1 and MFN2) on the outer mitochondrial membrane and optic atrophy 1 (OPA1) in the inner mitochondrial membrane are the primary regulators of mitochondrial fusion (Cipolat et al., 2004; Santel and Fuller, 2001). Both processes are necessary to ensure cardiac homeostasis, as cardiomyocyte-specific deletion of DRP1

results in unopposed fusion that ultimately leads to cardiac dysfunction and death (Kageyama et al., 2014; Shirakabe et al., 2016; Song et al., 2015b).

Interestingly, mitochondrial dynamics have also been linked to mitophagy, as it is thought that mitochondria undergo asymmetric fission in which the damaged portion of the mitochondria is selectively degraded, whereas the functional portion fuses with a healthy mitochondrion and persists (Twig et al., 2008). Given this, mitochondrial fission has been shown to facilitate mitophagy (Burman et al., 2017; Kageyama et al., 2014; Lee et al., 2011; Shirakabe et al., 2016; Song et al., 2015a; Twig et al., 2008), whereas fusion protects the mitochondria from being cleared (Gomes et al., 2011; Rambold et al., 2011). The effect of disruptions in the natural balance of fission and fusion proteins on mitophagy has been demonstrated *in vivo* with cardiac-specific DRP1, MFN1, and MFN2 triple knockout mice (Song et al., 2017). Complete disruption of mitochondrial dynamics in the hearts of these mice led to accumulation of a mixture of fragmented and fused mitochondria, defects in mitochondrial turnover, and ultimately development of cardiac hypertrophy (Song et al., 2017). This suggests that disruption of mitochondrial dynamics, even in a balanced manner, is detrimental to the heart.

While many of the major regulators of mitochondrial dynamics have already been identified, new proteins and pathways continue to be discovered due to the intricacy and complexity of this process. For example, the BCL-2 family proteins are suggested to play a part in regulating mitochondrial morphology and function (Berman et al., 2009; Karbowski et al., 2006; Sheridan et al., 2008). However, whether this is coupled with or distinct from their role in apoptosis is still being investigated. Multiple studies indicate a close association between mitochondrial dynamics and apoptosis. The role of

mitochondrial fission remains contested as it has been shown to prevent BAX-mediated outer membrane permeabilization (Renault et al., 2015) but inhibition of DRP1 also delays cytochrome c release, linking fission to apoptosis (Montessuit et al., 2010; Suen et al., 2008). Additionally, studies suggest that the role of fission in the heart is context-dependent and varies with different stressors. While fission in response to pathological stressors, such as ischemia or doxorubicin exposure, is maladaptive and associated with mitochondrial dysfunction (Disatnik et al., 2013; Gharanei et al., 2013) fission in response to physiological stimuli, such as exercise, leads to enhanced mitochondrial function as an adaptation to increased energy demand (Coronado et al., 2018). It has been reported that MCL-1 is also involved in regulating mitochondrial dynamics (Morciano et al., 2016; Rasmussen et al., 2018). However, how MCL-1 regulates mitochondrial dynamics in the heart, particularly in relation to protection against cell death, is still unexplored.

1.5 Autophagy

Autophagy is an evolutionarily conserved process in which cellular components are recycled (Figure 1.4). Autophagy occurs in a number of discrete steps that are regulated by a variety of autophagy-related (Atg) proteins (Sciarretta et al., 2018). Canonical autophagy is initiated by ULK1, which then activates BECLIN 1 by phosphorylating it at serine 14 (Kundu et al., 2008; Russell et al., 2013). The BECLIN1/VPS34/VPS15/ATG14L complex initiates formation of the autophagosome through the nucleation step (Sciarretta et al., 2018). Next, the ATG5/ATG16/ATG12 complex and light chain 3 (LC3) protein are involved in elongating the autophagosome

membrane (Sciarretta et al., 2018). The autophagosome then sequesters the damaged cellular components inside a mature double membrane vesicle. The final step is fusion of the autophagosome with a lysosome to form an autophagolysosome where the cellular components are degraded by the acid hydrolases (Kriegenburg et al., 2018). Autophagy is crucial for maintaining cardiac homeostasis and as an adaptation in response to stress (Billia et al., 2011; Endo et al., 2015; Kubli et al., 2013; Nakai et al., 2007; Nishino et al., 2000; Santulli, 2018). The BCL-2 family proteins act as negative regulators of autophagy by sequestering and inhibiting BECLIN1 (Maiuri et al., 2007; Pattingre et al., 2005). While MCL-1 interacts with BECLIN1 and is suggested to inhibit autophagy in cortical neurons (Germain et al., 2011), its role in regulating autophagy in the heart has not been characterized.

1.6 PINK1/PARKIN vs. Receptor-Mediated Mitophagy

Mitophagy refers to the selective removal of mitochondria via the autophagy pathway. Mitophagy can either be facilitated by the PINK1/PARKIN pathway or be mediated by one of the selective mitophagy receptors (Figure 1.5). The PINK1/PARKIN pathway is the most well characterized mitophagy pathway to date. In this process, mitochondrial depolarization acts as a signal for the serine/threonine kinase (PTEN)-induced kinase 1 (PINK1) to accumulate on the outer mitochondrial membrane, instead of completing its normal import into the mitochondria where it undergoes proteolysis and subsequent degradation (Jin et al., 2010). PINK1 accumulation leads to recruitment of the E3 ubiquitin ligase PARKIN from the cytosol to the mitochondria. PARKIN mediates K63-linked ubiquitination of proteins on the outer membrane to label the

mitochondrion for degradation (Narendra et al., 2008). There have been several proposed mechanisms for how PINK1 is able to recruit PARKIN. First, PINK1 has enhanced activity after being phosphorylated at Ser495 by AMPK α 2 (Wang et al., 2018), and then activated PINK1 phosphorylates Mitofusin 2 (MFN2), which functions as a mitochondrial receptor for Parkin on the outer mitochondrial membrane (Chen and Dorn, 2013). For mitophagy to proceed, PINK1 also mediates phosphorylation of ubiquitin at Serine 65 (Kane et al., 2014; Koyano et al., 2014) and the ubiquitin like (Ubl) domain in PARKIN at Serine 65 (Kondapalli et al., 2012). Another report suggests that phospho-ubiquitin at the mitochondria leads to PARKIN recruitment, and that subsequent PINK1 phosphorylation allows PARKIN to be activated (Gladkova et al., 2018). After the mitochondria have been labeled for degradation, various adaptor proteins physically link the ubiquitinated mitochondria to the forming autophagosome membranes. The tethering is done by adaptor proteins, which contain both ubiquitin-binding and LC3-interacting region (LIR) motifs, so that they can connect the cargo to the autophagosome (Johansen and Lamark, 2011). The adaptor proteins are degraded with the cargo they labeled. Known adaptor proteins include: p62/sequestome 1 (p62), neighbor of BRCA1 gene 1 (NBR1), optineurin, nuclear domain 10 protein 52 (NDP52), and Tax1-binding protein 1 (TAX1BP1) (Geisler et al., 2010; Lamark et al., 2009; Lazarou et al., 2015; Moore and Holzbaur, 2016).

Mitochondrial clearance can also occur via receptor-mediated mitophagy. These receptors can bind directly to the autophagosome in a PINK1/PARKIN-independent manner to facilitate mitophagy. Mitophagy receptors are proteins that contain a transmembrane domain and are anchored in the outer mitochondrial membranes.

Another key aspect of mitophagy receptors is that they possess an LIR motif that allows them to bind directly to LC3, without the need for ubiquitin and adaptor proteins (Hanna et al., 2012). There are several different mitophagy receptors that have been discovered. These include BNIP3, NIX, FUNDC1, and BCL2-L-13 (Hanna et al., 2012; Liu et al., 2012; Murakawa et al., 2015; Novak et al., 2010). Many of these proteins also have other functions in the cell, often allowing for more efficient coordination of mitochondrial degradation with other relevant processes. For instance, several of them also have roles in regulating mitochondrial fission, which is often thought to precede mitophagy (Lee et al., 2011; Murakawa et al., 2015). Two of the most well studied mitophagy receptors are BNIP3 and BNIP3L/NIX. Like MCL-1, BNIP3 and BNIP3L/NIX are also members of the BCL-2 family. Although they were originally identified as pro-apoptotic BH3-only proteins, they are now better known for their role in regulating mitophagy in a variety of cell types (Bellot et al., 2009; Dorn, 2010; Kubli et al., 2007; Ma et al., 2012; Rikka et al., 2011; Sandoval et al., 2008). BNIP3 knockout mice accumulate dysfunctional mitochondria in their livers (Glick et al., 2012) and BNIP3 and NIX-deficient mice also accumulate dysfunctional mitochondria in their hearts with age (Dorn, 2010), suggesting that these mitophagy receptors are important for regulating mitochondrial clearance. BNIP3 and NIX also have the capacity to directly activate autophagy in cells, likely through disruption of the interaction between BCL-2 and BECLIN1 (Zhang and Ney, 2009). As new mitophagy receptors continue to be discovered, it is very possible that there are even more proteins that can occupy this role.

1.7 Apoptosis, Mitochondrial Dynamics, and Mitophagy in Heart Failure

Accumulation of damaged and dysfunctional mitochondria has been implicated in the development of heart failure. Given that many studies have established important links between apoptosis, mitochondrial dynamics, and mitophagy in maintaining homeostasis in the heart, there is likely also interplay in these processes in heart failure. Apoptosis has been suggested as a crucial step in the progression of heart failure (Kang and Izumo, 2000). Due to the fact that cardiomyocytes are terminally differentiated, even low levels of apoptosis can result in significant and permanent damage in the heart (Kirshenbaum et al., 1996; Kirshenbaum and Schneider, 1995; Wencker et al., 2003). Studies suggest that the rate of occurrence of apoptosis varies widely among different models of heart failure (Kang and Izumo, 2000). Interestingly, myocyte apoptosis itself can induce heart failure, as transgenic mice with ligand-activatable cardiac-specific overexpression of caspase-8 manifest spontaneous apoptosis in myocytes that leads to a dilated cardiomyopathy (Wencker et al., 1999). Thus, limiting myocyte loss by inhibiting apoptosis could have potentially beneficial effects in treating heart failure. Findings in line with this sentiment include a study that showed that overexpression of the anti-apoptotic protein BCL-2 was able to effectively reduce myocardial ischemia/reperfusion injury by decreasing apoptosis in cardiac myocytes (Brocheriou et al., 2000). Other studies have confirmed that elevated levels of BCL-2 and BCL-X_L protect against myocardial reperfusion injury (Chen et al., 2001; Huang et al., 2003; Imahashi et al., 2004). Caspase inhibitors have also been able to reduce apoptosis in the area of risk and reduce infarct size during I/R (Holly et al., 1999; Yaoita et al., 1998). However, the interplay between apoptosis and other cell death

pathways as well as how beneficial therapeutic intervention of apoptosis will ultimately be varies widely in different contexts of heart failure.

While studies of mitochondrial dynamics in heart failure are fairly limited, mitochondria are reported to be small and dysfunctional during heart failure (Chen and Knowlton, 2011). Consistent with these findings, the fusion protein OPA1 is decreased in both human and rat models of heart failure (Chen et al., 2009). Another study found that while there was no change in OPA1, fission and fusion dynamics were instead disrupted and shifted toward increased fission by decreased MFN2 and increased FIS1 levels (Javadov et al., 2011). Further studies are necessary to clarify whether increased fission is a cause or consequence of heart failure. However, there is some evidence that decreasing mitochondrial fission may have cardioprotective benefits, as treatment of adult mouse cardiomyocytes with Mdivi1, which is a DRP1 inhibitor, reduced cell death after simulated I/R and led to reduced myocardial infarct size *in vivo* (Ong et al., 2010). Additionally, it was reported that DRP1-heterozygous mice were protected from Doxorubicin-induced cardiac damage (Catanzaro et al., 2019).

Mitophagy, which has close ties to both mitochondrial dynamics and apoptosis, has also been investigated in heart failure models. PARKIN-mediated mitophagy is protective after myocardial infarction (MI) since PARKIN-knockout mice exhibited reduced survival and accumulation of dysfunctional mitochondria (Kubli et al., 2013). Mice deficient in the mitophagy receptor FUNDC1 in the heart also showed increased susceptibility to MI and reduced survival (Wu et al., 2017). However, since FUNDC1 deletion also disrupted other processes, such as mitochondrial dynamics, it is difficult to assess the exact contribution of mitophagy in this case. The study does suggest an

intrinsic link between mitochondrial fission and mitophagy, as they were both found to be dependent on the fission protein FIS1 (Wu et al., 2017). Shirakabe et al., showed that mitophagy was also protective in response to pressure-overload induced heart failure and found that this was dependent upon DRP1 (Shirakabe et al., 2016). This suggests that even though inhibiting fission is protective in myocardial I/R, fission may actually be required in order to facilitate mitophagy in response to other models of heart failure, such as chronic hemodynamic stress. The interplay between fission and mitophagy in response to heart failure needs additional clarification to determine potential cardioprotective effects. Further investigation is also necessary to determine the role of different mechanisms of mitophagy in response to heart failure.

1.8 Rationale and Specific Aims of the Thesis

The anti-apoptotic BCL-2 family protein MCL-1 is essential for maintaining mitochondrial integrity and function in the adult heart. Cardiac-specific deletion of MCL-1 leads to rapid mitochondrial dysfunction, loss of myocytes and lethal cardiomyopathy. Surprisingly, ablation of MCL-1 does not result in apoptotic cell death, but rather cells show signs of necrotic cell death such as mitochondrial swelling, cellular rupture and release of intracellular contents into extracellular space. This suggests that in addition to its traditional anti-apoptotic role, MCL-1 has essential but yet unidentified roles in maintaining mitochondrial function in cardiac myocytes. MCL-1 exists in two distinct locations in myocytes: one form is localized to the outer mitochondrial membrane (MCL-1_{OM}) and a shorter cleaved form exists in the mitochondrial matrix (MCL-1_{Matrix}). However, the specific functional contributions of these two forms remain elusive since

both proteins are absent in MCL-1 deficient mice. Based on my preliminary data, I will explore the hypothesis that, in addition to sequestering pro-apoptotic proteins, **MCL-1_{OM} protects against cell death by facilitating DRP1-mediated mitochondrial fission and removing aberrant mitochondria via autophagy.** This hypothesis will be tested with two specific aims.

Aim 1: To delineate the function of MCL-1_{OM} in regulating mitochondrial fission.

Aim 2: To evaluate the role of MCL-1_{OM} in facilitating mitophagy.

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Parts of Chapter1 are coauthored/unpublished material. Moyzis, Alexandra; Lally, Navraj; Najor, Rita; Gustafsson, Åsa. Chapter 1, Introduction. Chapter 2, Experimental Methods and Materials. Chapter 6, The Effect of Mcl-1 Overexpression on Autophagy.

Chapter 7, Discussion. The dissertation author was the primary investigator and author of this paper.

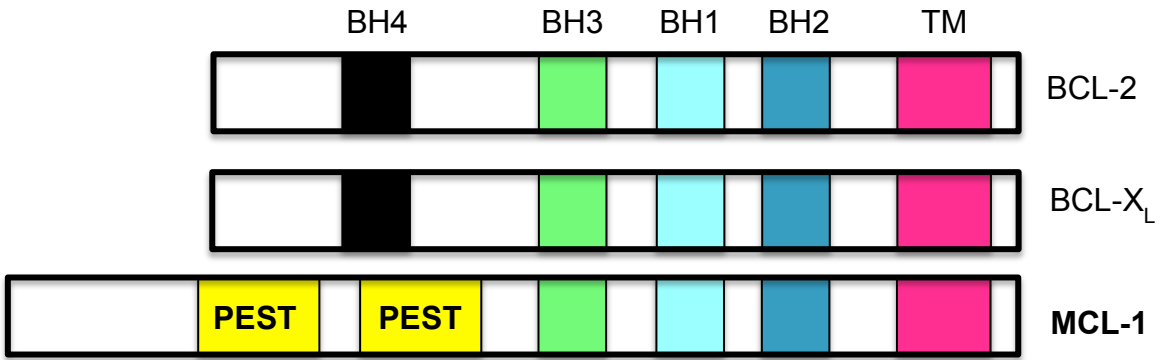


Figure 1.1 MCL-1 is structurally distinct from the other anti-apoptotic BCL-2 family members.

MCL-1, BCL-2, and BCL-X_L are the main anti-apoptotic BCL-2 family members. They all contain conserved BCL-2 homology (BH) domains that allow them to bind to and sequester pro-apoptotic proteins. However, MCL-1 is unique because it lacks a BH4 domain and has an elongated N-terminus with two PEST (proline, glutamic acid, serine, and threonine) sequences that allow for rapid proteosomal degradation.

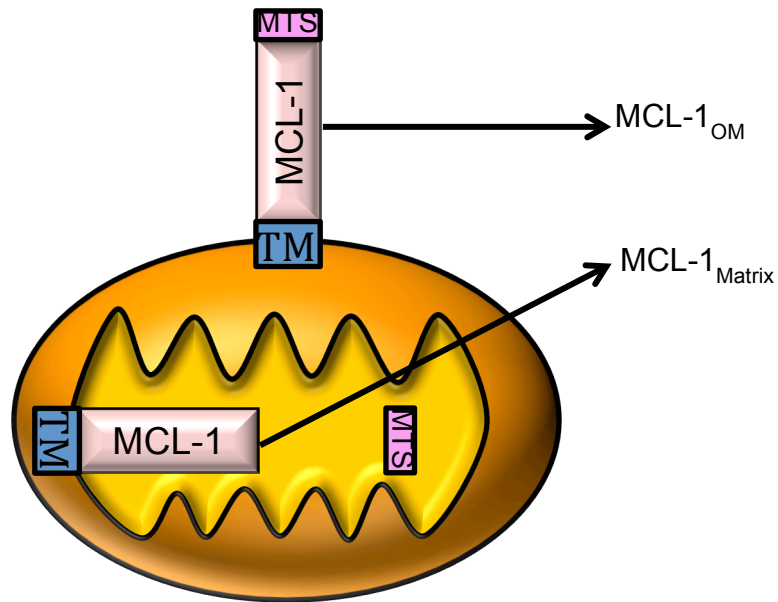


Figure 1.2. MCL-1 localizes to two distinct mitochondrial locations.

MCL-1 resides both on the outer mitochondrial membrane (MCL-1_{OM}) and in the mitochondrial matrix (MCL-1_{Matrix}). MCL-1 contains a mitochondrial targeting sequence (MTS) at its N-terminus that gets cleaved off upon import into the matrix.

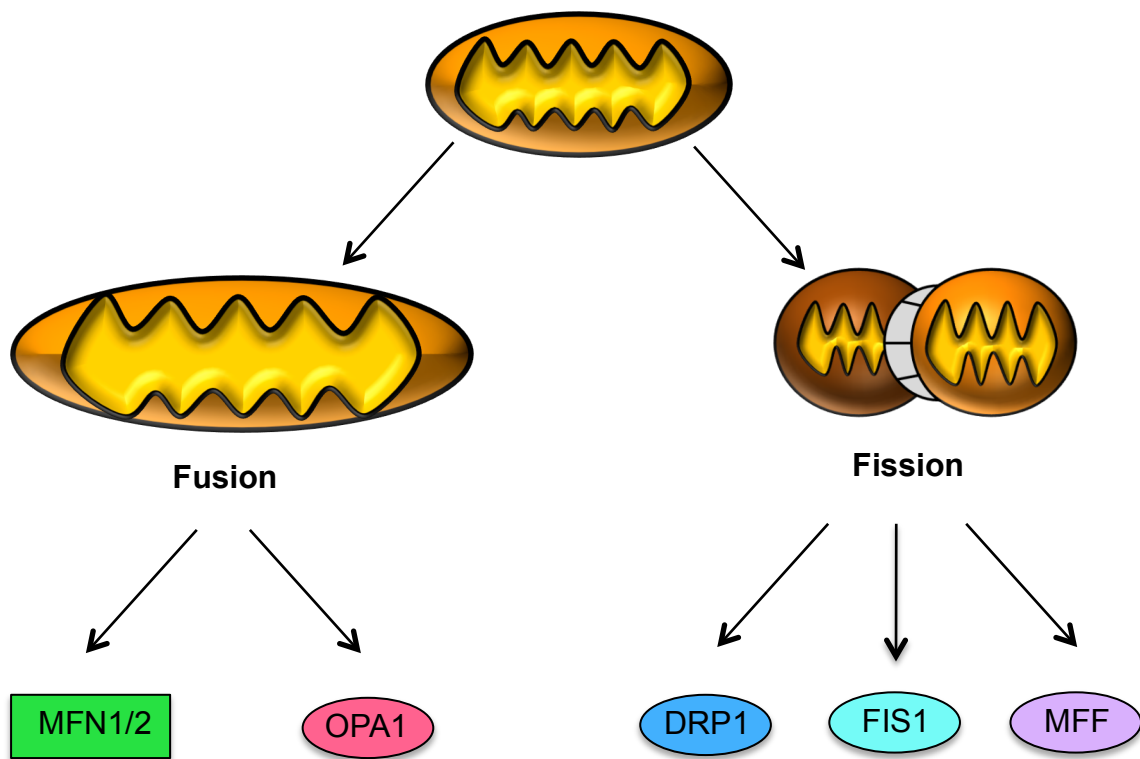


Figure 1.3. Mitochondria are dynamic organelles.

Mitochondria are constantly undergoing the processes of fission and fusion in order to maintain cellular homeostasis and in response to stress. Fusion causes individual mitochondrion to merge together to become elongated and is regulated by MFN1 and MFN2 on the outer mitochondrial membrane and OPA1 in the inner mitochondrial membrane. Fission results in the mitochondria fragmenting into shorter organelles and is regulated by DRP1, FIS1, and MFF.

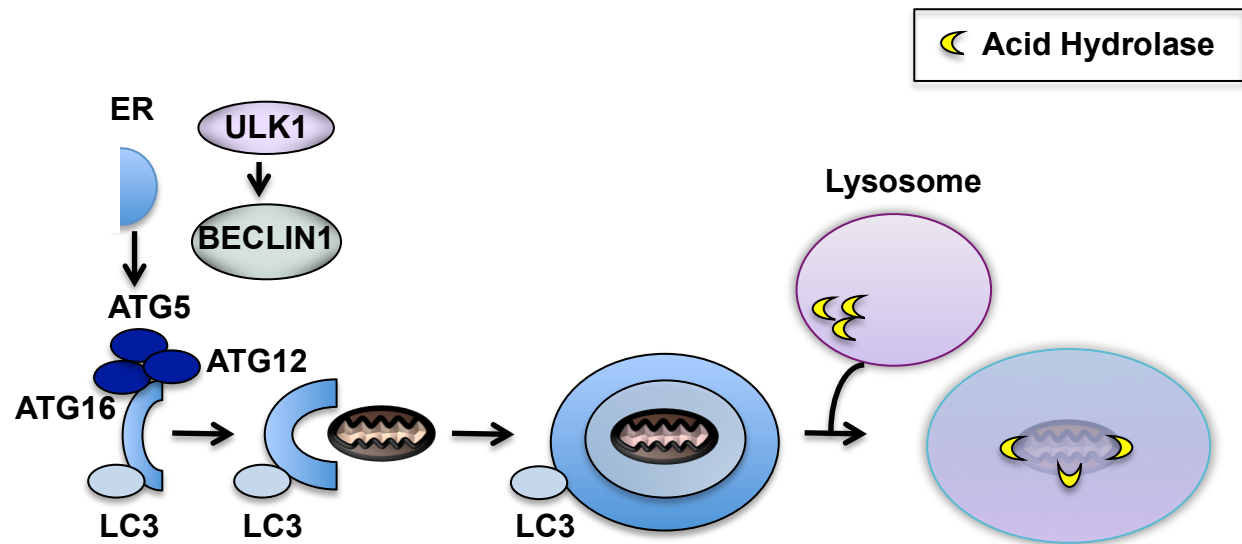


Figure 1.4. Overview of Autophagy.

Autophagy proceeds through a variety of discrete steps. Autophagy is initiated by ULK1, which then activates BECLIN1. The autophagosome membrane is derived from the endoplasmic reticulum (ER). ATG5/ATG12/ATG16 and LC3 facilitate elongation of the membrane. The autophagosome then sequesters the mitochondrion inside a double-membrane vesicle. The autophagosome fuses with a lysosome and the mitochondrion is degraded by the acid hydrolases.

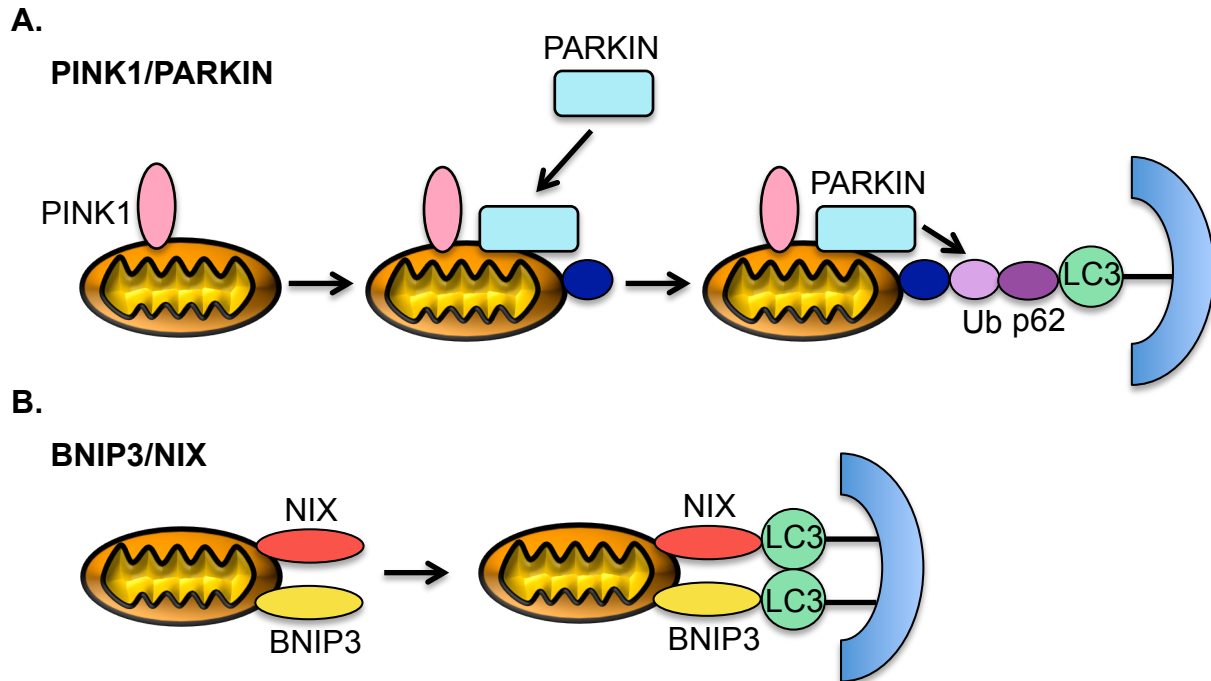


Figure 1.5. Mitophagy can proceed through PINK1/PARKIN or Receptor-mediated pathways.

A. Upon loss of mitochondrial membrane potential, PINK1 accumulates on the OMM surface. PINK1 recruits PARKIN, which ubiquitinates OMM proteins. Adaptor proteins, such as p62 then connect the ubiquitinated mitochondrion to the autophagosome by binding to LC3. **B.** Some proteins, such as BNIP3 and NIX, can function as autophagy receptors independently of PINK1 and PARKIN on mitochondria by binding directly to LC3 on the autophagosome. Both pathways result in the autophagic sequestration of the mitochondrion, fusion with a lysosome, and degradation of the organelle.

CHAPTER 2: EXPERIMENTAL METHODS AND MATERIALS

Antibodies

The following antibodies were used for immunofluorescence (IF) and Western blot (WB) experiments: ACTIN (1:1000; GeneTex, GTX109638), BAK (1:500, Millipore Upstate, 06-536), BAX (1:1000, Cell Signaling, 2772), BCL-2 (1:1000, BD Biosciences, 610539), BCL-X_L (1:1000, Santa Cruz, sc-8392), BNIP3 (1:500, Sigma, B7931), COX IV S1 (IF 1:100, Invitrogen, #459600), DRP1 (1:1000, BD Biosciences, 611113), FIS1, (1:1000, Abcam, ab96764), GAPDH (1:1000; GeneTex, GTX627408), HSP70 (1:1000, Thermo Fisher Scientific, MA3-028), LC3 (1:1000; Cell Signaling, 4108), MCL-1 (1:1000; Rockland, 600-401-394), MFF (1:1000, Proteintech, 17090-1-AP), MFN1 (1:1000; Santa Cruz Biotechnology, sc-50330), MFN2 (1:1000; MilliporeSigma, M6319), MitoProfile Total OXPHOS Rodent WB Antibody cocktail (1:1000; Abcam/MitoSciences, MS604), MnSOD (1:1000; Millipore, 06-984), OPA1 (1:1000, BD Biosciences, 612607), p62 (1:1000; Abcam, ab56416), TIM23 (1:1000; BD Biosciences, 611222), TOM20 (WB 1:1000 and IF 1:100; Santa Cruz, sc-11415), and TUBULIN (WB 1:1000; Sigma, T6074).

cDNA Constructs

MCL-1 WT, OM, and Matrix constructs were provided by Dr. Joseph Opferman from St. Jude Children's Research Hospital (Perciavalle et al., 2012). The MCL-1_{OM} construct replaces arginines 5 and 6 with alanines. The MCL-1_{Matrix} construct lacks the N-terminal 67 amino acids and is fused with the N-terminal 58 amino acids of

Neurospora crassa ATP-synthase (Pfanner et al., 1987). The HA-MCL-1-BH3-mutant (G198E D199A) replaces glycine 198 with glutamic acid and aspartic acid 199 with alanine and was synthesized by Genscript. The MCL-1-BCL-2 Chimera replaces MCL-1's BH1 (aa 234-253), BH2 (aa 285-300), and BH3 (aa 190-204) domains with those from BCL-2 (aa 133-152, 184-199, and 190-204) and was synthesized by Genscript. The HA-MCL-1-LIR1 mutant (W242A, I245A) replaces tryptophan 242 with alanine and isoleucine 245 with alanine and was synthesized by Genscript. The HA-MCL-1-LIR2 mutant (F227A, V230A) replaces phenylalanine 227 with alanine and valine 230 with alanine and the HA-MCL-1-LIR3 mutant (F299A, V302A) replaces phenylalanine 299 with alanine and valine 302 with alanine and were generated using PCR-based site directed mutagenesis. The HA-MCL-1-LIR123 mutant (W242A, I245A, F227A, V230A, F299A, V302A) combined all of the mutations for the individual LIR mutants previously listed and was purchased from Genscript. The Myc-BNIP3 construct has previously been described (Kubli et al., 2008). The GFP-LC3 construct has previously been described (Hanna et al., 2012).

Animal Protocols and Generation of Mouse lines

8-12 week-old WT and MCL-1_{OM} transgenic mice on a C57Bl/6 background were used for experimental procedures. Both male and female mice were used in all experiments. WT littermates were used as controls in the experiments. MCL-1_{OM} transgene mice were generated at the UCSD Mouse Transgenic Core facility and overexpress MCL-1_{OM} under the transcriptional control of the cardiomyocyte-specific α -myosin heavy chain (α -MHC) promoter. All protocols were carried out in accordance

with institutional guidelines and approved by the Institutional Animal Care and Use Committee of the University of California, San Diego. Mice were subjected to fasting by removal of food but were permitted water for the indicated amounts of time. Food was removed at the same time (at the beginning of the light cycle) for each experiment. For the *in vivo* autophagy flux experiments, mice were injected with saline or 80 mg/kg chloroquine by intraperitoneal (IP) injection. Hearts were collected two hours later and protein levels were assessed by Western blot analysis. For the *in vivo* swimming experiments, mice were pre-trained for 5 minutes on day 1, 5 minutes on day 2, 10 minutes on day 3 and then the actual experiments were conducted on day 4. For the swimming time course, the mice swam for the indicated amount of time, ranging from 15 minutes to 60 minutes and the hearts were collected immediately after. For the forced swimming experiment, weights (7.5% body weight) were attached to the tail stem of the mice and the mice swam until exhausted (as assessed by 5 seconds continuously under water) and the time they spent swimming was recorded.

Cells and Culture Conditions

WT Mouse Embryonic Fibroblasts (MEFs) were generously provided by Dr. Noboru Mizushima (The University of Tokyo, Japan). MEFs were maintained in culture media (DMEM (Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies), 100 U ml⁻¹ penicillin (Gemini) and 100 µg ml⁻¹ streptomycin (Gemini)), and cultured in a 5% CO₂ atmosphere at 37 °C. For glucose deprivation experiments, cells were cultured in glucose-free media (Gibco, 11966-025). Hypoxia experiments were performed by placing cells in MEF culture media supplemented

with 20 mM HEPES pH 7.4 (Gibco, 15630-080) and placing the dishes in a 5% CO₂ atmosphere at 37 °C for normoxic controls, or in hypoxic pouches (GasPak EZ, BD Biosciences), equilibrated to 95% N₂, 5% CO₂ for hypoxic experimental conditions. For experiments with Rapamcyin and mitochondrial toxins, drugs were added to MEF media (100 nM Rapamycin; 1 μM Rotenone or 25 μM FCCP) along with corresponding amounts of DMSO for controls.

Transient Transfections and siRNA Knockdown

Cells were transiently transfected with DNA using Eugene 6 Transfection Reagent (Promega) for imaging experiments or Lipofectamine 2000 (Invitrogen) for Western blot experiments according to the manufacturer's instructions. Drp1 knockdown was performed by transfecting 50 nM Mission siRNA Universal Negative Control #1 (Sigma, SIC001) or Drp1 small interfering RNA (siRNA) (Sigma, SASI_Mm01_000125369) with Lipofectamine RNAiMax Transfection Reagent (Invitrogen) for 72 hours, according to the manufacturer's instructions. For knockdown experiments plus adenoviral infection, Drp1 was knocked down for 48 hours and the adenoviral infection was done for the last 24 hours.

Adenoviral Infections

Cells were infected with adenoviruses in DMEM+2% heat-inactivated serum for 3 hours and rescued with growth media. Experiments were performed 21 hours later for a total infection time of 24 hours. Ad-HA-MCL-1 WT, OM, Matrix, and BH3-Mutant viruses were generated as previously described (Graham and Prevec, 1995).

Immunofluorescence

Cells were fixed with 4% paraformaldehyde (Ted Pella Inc.) in Dulbecco's PBS without calcium chloride and magnesium chloride (PBS) (Gibco, 14200-075), permeabilized with 0.2% Triton X-100 in PBS and blocked in 5% normal goat serum. Cells were incubated with primary antibodies (1:100, 4 °C, overnight) in 5% normal goat serum in PBS, rinsed with PBS, incubated with secondary antibodies (1:200, 37 °C, 1 h) and counter-stained with Hoechst 33342. Fluorescence images were captured using a Carl Zeiss Axio Observer Z1 fitted with a motorized Z-stage with a × 63 Plan-Apochromat (oil immersion) objective. Z-stacks of red and green fluorescence separated by 0.6 µm along the z-axis were acquired with an ApoTome using a high-resolution AxioCam MRm digital camera and Zeiss AxioVision 4.8 software (Carl Zeiss). Fluorescence images were also collected using a Nikon Eclipse microscope (60x objective). Mitochondrial morphology was assessed based on counts of 200 cells per dish repeated for three separate experiments. All scoring of morphology was done unblinded with respect to sample identity. Mitophagy was assessed by analyzing co-localization between GFP-LC3 puncta and TOM20-labeled mitochondria and quantifications were based on counts of at least 30 cells per dish repeated for three separate experiments.

Cell Death Assays

For the staurosporine cell death assays, MEFs were plated in 12-well plates and infected with Ad-β-gal or Ad-HA-MCL-1 or Ad-HA-MCL-1-BH3 Mutant for 24 hours and then treated with vehicle (DMSO; Sigma) or 1 µM staurosporine (Sigma) for 24 hours. For the glucose-deprivation cell death assays, MEFs were plated in 24-

well plates, and cells with DRP1 knocked down were infected with Ad- β -gal or Ad-Ha-MCL-1 and incubated with DMEM with or without glucose for 24 hours. To assess viability, cells were stained with YO-PRO-1 Iodide (1:1,000; Life Technologies) plus Hoechst 33342 (1:1000; Invitrogen) for 20 min. Cell death was assessed as the number of YO-PRO-1-positive cells divided by total number of Hoechst 33342-positive cells as previously described (Kubli et al., 2007).

Western Blotting and Co-immunoprecipitation Experiments

Cells were lysed in ice-cold 50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100 and Complete protease inhibitor cocktail (Roche). Cell lysates were passed through a 27G needle four times and then incubated on ice for 45 minutes. Lysates were cleared by centrifugation at 20,000 g for 20 minutes at 4 °C. For tissue samples, hearts were homogenized by polytron in detergent-free lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, plus complete Protease Inhibitor cocktail (Roche)). Triton X-100 buffer was then added to the homogenates at a final concentration of 1% and incubated on ice for 45 minutes. Finally, the homogenates were centrifuged at 20,000 g for 20 minutes and the supernatant was collected as the whole heart lysate. Protein concentration for both cells and heart lysates was quantified using Bradford assay. Proteins were separated on Invitrogen NuPAGE Bis-Tris gels and transferred to nitrocellulose membranes. Membranes were incubated with the indicated antibodies and proteins were detected using a ChemiDoc XRS+ System (Bio-Rad). Band densitometry quantification was performed using the Image Lab software (Bio-Rad). Immunoprecipitation experiments were performed as previously described (Hanna et al., 2012). Briefly, samples were pre-

cleared with protein A PLUS-agarose (Santa Cruz Biotechnology, sc-2001) for thirty minutes, and then incubated with 2.5 µg anti-MCL-1 antibody (Rockland, 600-401-394) or anti-Myc antibody (Sigma, M4439) overnight. Immunocomplexes were captured with protein A PLUS-agarose beads for an additional two-hour incubation, washed in PBS, eluted in 2X sample buffer, and analyzed by Western blot.

Mitochondrial Isolation for Cells

Cells were transfected with MCL-1_{WT} or MCL-1_{OM} using Lipofectamine 2000 (Invitrogen) for 24 hours. Cells were then collected using a cell scraper and incubated in isolation buffer (220 mM mannitol, 70 mM sucrose, 1 mM EDTA and 10 mM HEPES at 7.4 pH) for 45 min on ice. Cells were homogenized using an Eppendorf pestle and centrifuged for 10 min at 1,000 g to pellet unbroken cells. The supernatant was collected and centrifuged for 15 min at 14,000 g to collect the heavy membrane fraction. After removal of the supernatant, the remaining pellet was resuspended in isolation buffer and separated by SDS-PAGE.

Mitochondrial Isolation/Subcellular Fractionation For Tissue

Hearts were minced in homogenization buffer containing 250 mM sucrose, 5 mM KH₂PO₄, 2 mM MgCl₂, 10 mM MOPS, pH 7.4, 1 mM EGTA, 0.1% fatty acid-free BSA, protease inhibitor cocktail (Roche). Next, a Polytron at 11,000 rpm was used to homogenize the hearts, followed by 3–4 strokes using the Potter-Elvehjem Teflon tissue grinder to further homogenize the tissue. Whole Heart homogenates were centrifuged twice at 600 g for 5 minutes at 4°C to remove nuclear contamination and debris. The

supernatant was collected and transferred to another tube and centrifuged at 6000 *g* for 10 minutes at 4°C. The resulting supernatant contained the crude cytosolic fraction, and the pellet contained the mitochondrial fraction. The mitochondrial pellet was washed in homogenization buffer and centrifuged at 6000 *g* for 10 minutes at 4°C; this wash step was performed 3 times. The resulting mitochondria pellet was resuspended and lysed in Triton X-100 lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, and protease inhibitor cocktail (Roche). Protein concentrations for whole heart lysates and mitochondrial fractions were determined by Bradford assay. For Proteinase K experiments, isolated mitochondria were incubated with 200 ng/μl of Proteinase K for the indicated periods of time to digest the outer mitochondrial membrane before sample preparation.

Mitochondrial Respiration Measurements of Isolated Mitochondria

Intact mitochondria were isolated from WT and MCL-1_{OM} TG mice, and mitochondrial respiration was measured using the Seahorse XF96 Analyzer (Seahorse Bioscience – Agilent Technologies) following a modified protocol originally optimized in the Seahorse XF24 Analyzer (Rogers et al., 2011). Hearts were homogenized using a Polytron followed by a Potter-Elvehjem Teflon tissue grinder in buffer containing 70 mM sucrose, 210 mM mannitol, 5 mM HEPES, 1 mM EGTA, and 0.5% fatty acid-free BSA. The homogenate was centrifuged at low speed (600 *g* for 5 minutes at 4°C). This step was repeated twice. The supernatant was collected and centrifuged at 6,000 *g* for 10 minutes at 4°C to obtain a mitochondrial pellet. The pellet was washed in buffer and centrifuged at 6,000 *g* for 10 minutes at 4°C, and this wash step was performed twice. Finally, the pellet was resuspended in a small volume (~20 μl) of buffer, and the protein

concentration was determined by Bradford assay. Mitochondria were plated on a 96-well microplate at densities of 1 μ g per well. Assay media (70 mM sucrose, 220 mM mannitol, 10 mM KH_2PO_4 , 5 mM MgCl_2 , 2 mM HEPES, 1 mM EGTA, 0.2% BSA, and 4 mM ADP) containing different substrates were used to assess glucose metabolism (10 mM pyruvate and 1 mM malate) and glutamine (10 mM glutamate and 10 mM malate), and complex II–dependent oxygen consumption (10 mM succinate and 2 μ M rotenone). Oligomycin (2 μ M) was added to inhibit ATP synthase followed by 3 successive additions of 1.5 μ M FCCP to obtain maximal respiration rate. Data were analyzed using Wave for Desktop (Seahorse Bioscience – Agilent Technologies).

mtDNA Content

Genomic DNA was extracted using GenElute Mammalian Genomic DNA Miniprep Kit (Sigma) and PCR-amplified with TaqMan Universal Master Mix II. 18S rRNA was used as a control for nuclear DNA, and D-loop was used for mtDNA quantitation using primer sequences previously described (Life Technologies-Thermo Fisher Scientific) (Woodall et al., 2019).

Echocardiography

Echocardiography was performed as previously described using a Vevo770 In Vivo Micro-Imaging System with an RMV707B 15–45 MHz imaging transducer (VisualSonics Inc.) (Kubli et al., 2013). Briefly, mice were maintained under light anesthesia through a nose cone (0.5%–1% isoflurane, 98%–99.5% O_2). Mice were

placed in a supine position on a recirculating water warming pad while acquiring measurements. Cardiac parameters were quantified using the VisualSonics software.

TEM and Morphometric Analysis

Transmission electron microscopy was performed on heart sections as previously described (Woodall et al., 2019). Briefly, adult male and female mouse hearts were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer; post-fixed in 1% osmium tetroxide; then treated with 0.5% tannic acid, 1% sodium sulfate; cleared in 2-hydroxypropyl methacrylate; and embedded in LX112 (Ladd Research). Sections were mounted on copper slot grids coated with parlodion and stained with uranyl acetate and lead citrate for examination on a Philips CM100 electron microscope (FEI). Quantification was done by analyzing 300 mitochondria from two hearts using 14 different sections at 7900 X magnification.

Histological Analysis and Immunofluorescence

Histology was performed as previously described (Woodall et al., 2019). Briefly, hearts were perfused with 200 mM KCl to induce arrest at diastole before harvesting. Next, hearts were fixed in 10% neutral buffered formalin paraformaldehyde for 24 hours, dehydrated in 70% ethanol and processed in a tissue processor (Thermo Scientific STP 120). After being processed, the hearts were embedded in paraffin and cut into sections using a microtome (Leica Biosystems). Sections from 3 different hearts were then stained with haematoxylin and eosin (H&E) and imaged.

Myocardial Infarction

Mice were subjected to myocardial infarction by permanently ligating the left anterior descending coronary artery as described previously (Kubli et al., 2013). Briefly, 8–10-week-old WT mice were anesthetized with isoflurane, intubated, and ventilated. Pressure-controlled ventilation (Harvard Apparatus) was maintained at 9 cm of H₂O. An 8-0 silk suture was placed around the left anterior descending coronary artery and then tightened. The suture was left in place, and the animal was immediately closed up. Hearts were then collected at the indicated time points.

Statistics

All values are expressed as mean $\pm$ SEM. Student's 2-tailed *t* test was used to compare 2 sets of data. For data comparing more than 2 groups, statistical analyses were performed using 1-way or 2-way ANOVA, followed by Dunnett's or Tukey's multiple comparison test (GraphPad Prism 7). *P* values of less than 0.05 were considered statistically significant.

Parts of Chapters 2 are currently being prepared for submission for publication. Moyzis, Alexandra; Lally, Navraj; Leon, Leonardo; Shires, Sarah; Orogo, Amabel; Liang, Wenjing; Najor, Rita; Gustafsson, Åsa. "Mcl-1 on the outer mitochondrial membrane promotes Drp1-mediated mitochondrial fission and affects exercise capacity in the heart." The dissertation author was the primary investigator and author of this paper.

Parts of Chapters 2 are coauthored/unpublished material. Moyzis, Alexandra; Lally, Navraj; Najor, Rita; Gustafsson, Åsa. Chapter 1, Introduction. Chapter 2, Experimental Methods and Materials. Chapter 6, The Effect of Mcl-1 Overexpression on

Autophagy. Chapter 7, Discussion. The dissertation author was the primary investigator and author of this paper.

CHAPTER 3: MCL-1 FACILITATES DRP1-MEDIATED FISSION AS AN ADAPTIVE RESPONSE TO STRESS

3.1 Introduction

MCL-1 is integral for survival in the heart (Thomas et al., 2013; Wang et al., 2013). While studies have suggested roles for MCL-1 beyond regulating apoptosis (Perciavalle et al., 2012; Thomas et al., 2013), these have not been fully elucidated. Furthermore, the exact functional contributions of MCL-1 at the two distinct mitochondrial locations remain unclear. Here, I wanted to investigate other potential roles for MCL-1 at the mitochondria. I overexpressed MCL-1 and assessed its effect on mitochondrial morphology and function. Given that mitochondria are dynamic organelles, I wanted to evaluate if MCL-1 could influence mitochondrial morphology as well. Furthermore, I wanted to determine if MCL-1's additional roles were distinct from or coupled to MCL-1's anti-apoptotic activity.

3.2 Results

3.2.1 MCL-1_{OM} Induces Perinuclear Clustering of the Mitochondria

Given that MCL-1 is a mitochondrial protein, I first wanted to investigate whether MCL-1 overexpression affects mitochondrial morphology. To determine the effect of MCL-1 on mitochondrial morphology in a system where I could more easily visualize changes in the network, I overexpressed adenoviruses encoding either β -gal or MCL-1 in mouse embryonic fibroblasts (MEFs). I then stained the cells with an antibody against the mitochondrial protein TOM20 and used immunofluorescence microscopy to

monitor changes. While the cells overexpressing β -gal exhibited normal filamentous mitochondrial networks, MCL-1 overexpression led to a significant increase in the percentage of cells with mitochondria that were clustering in the perinuclear region (Figure 3.1A and B).

Next, I wanted to assess if MCL-1 at one or both distinct mitochondrial locations could facilitate these morphological changes to induce perinuclear clustering. To do this, in addition to overexpressing MCL-1_{WT}, I also overexpressed MCL-1_{OM}, which resides specifically on the outer mitochondrial membrane and MCL-1_{Matrix}, which localizes to the mitochondrial matrix (Figure 3.2A). MCL-1_{OM} has mutated arginines 5 and 6 to alanines in order to prevent import and restrict localization to the outer membrane. As it is currently unknown what regulates MCL-1 import, MCL-1_{Matrix} is fused to the ATP-synthase mitochondrial targeting sequence to allow it to localize specifically to the matrix. Interestingly, I found that overexpression of MCL-1_{WT} and MCL-1_{OM}, but not MCL-1_{Matrix} led to perinuclear clustering of the mitochondria (Figure 3.2B). This suggests that these morphological changes are arising specifically as a result of MCL-1 on the outer mitochondrial membrane and that MCL-1 within the matrix is unable to exert these effects. To determine if MCL-1 overexpression caused a reduction in overall mitochondrial content, I assessed levels of the mitochondrial proteins TIM23 and MnSOD, but found that these were not significantly changed (Figure 3.3A and B). This indicates that the mitochondrial content is maintained but redistributed and clustered in the perinuclear region.

3.2.2 MCL-1-induced Perinuclear Clustering Requires a Functional BH3 Domain

Given that MCL-1's anti-apoptotic function is attributed to its location on the outer membrane, I wanted to determine if its role in inducing morphological changes in mitochondria was related to this function. To examine whether MCL-1's ability to induce perinuclear clustering was associated with its anti-apoptotic activity, I mutated MCL-1's BH3 domain, which is required for MCL-1's anti-apoptotic function (Clohessy et al., 2006). Specifically, I mutated glycine 198 to glutamic acid and aspartic acid 199 to alanine in order to disrupt MCL-1's ability to antagonize pro-apoptotic proteins through its BH3 domain (MCL-1-BH3-mutant; Figure 3.4A). To confirm disruption of MCL-1's anti-apoptotic activity, cell viability was assessed in MEFs overexpressing MCL-1 or the MCL-1-BH3-mutant in response to treatment with staurosporine (Figure 3.4B). While MCL-1 was able to protect against staurosporine-induced cell death, the MCL-1-BH3-mutant showed a significant reduction in protection, confirming that disruption of MCL-1's BH3 domain affects its anti-apoptotic function (Figure 3.4B)

Next, I assessed the effect of overexpressing the MCL-1-BH3-mutant on mitochondrial morphology in MEFs. I stained cells with TOM20 to label the mitochondria and visualized the mitochondrial networks using immunofluorescence microscopy. Unexpectedly, the MCL-1-BH3-mutant abrogated MCL-1's ability to induce perinuclear clustering (Figure 3.5A and B), suggesting that it requires a functional BH3 domain. Additionally, to evaluate whether this function is conserved among other anti-apoptotic BCL-2 family members, I also generated a MCL-1-BCL2 chimera, in which MCL-1's BH1, BH2, and BH3 domains are replaced with those of BCL-2 (MCL-1-BCL2-Chimera). While the BH domains between MCL-1 and BCL-2 are highly conserved, they are not identical, allowing me to potentially identify unique residues responsible for

MCL-1's ability to induce perinuclear clustering. MEFs were transfected with HA, HA-MCL-1, or HA-MCL-1-BCL2-Chimera and stained with HA to identify cells that were transfected and anti-COX IV to label mitochondria. However, the MCL-1-BCL-2-Chimera was still able to induce perinuclear clustering of mitochondria to an extent similar to that of MCL-1 (Figure 3.6A and B). This indicates that the presence of a functional BH3 domain is sufficient for induction of perinuclear clustering.

3.2.3 MCL-1-induced Perinuclear Clustering is DRP1-Dependent

In addition to clustering in the perinuclear region, the mitochondria in the MCL-1-overexpressing MEFs also exhibited a more punctate morphology, which is characteristic of mitochondrial fission (Tilokani et al., 2018). To assess the role of fission in the perinuclear clustering, I wanted to investigate the contribution of the key mitochondrial fission regulator DRP1. When the large GTPase DRP1 translocates to the mitochondria from the cytosol, it induces division of the mitochondria into two separate portions (Smirnova et al., 2001). First, I assessed DRP1 levels in MEFs and found that DRP1 increased at the mitochondria in MCL-1_{WT} and MCL-1_{OM} overexpressing cells (Figure 3.7A). Moreover, MCL-1 was immunoprecipitated from MEFs, and I found an increase in the interaction between MCL-1 and endogenous DRP1 with MCL-1 overexpression (Figure 3.7B). To determine if the perinuclear clustering caused by MCL-1 overexpression was DRP1-dependent, I used siRNA to knock down DRP1 in MEFs. I confirmed that DRP1 was successfully knocked down by Western blot analysis (Figure 3.8A). I then transfected MEFs with either control or DRP1 siRNA and subsequently infected them with Ad-βgal or Ad-MCL-1 and assessed

the effect on mitochondrial morphology. I used fluorescence microscopy with staining for TOM20 to label the mitochondria and found that DRP1 knockdown led to a significant reduction in the percentage of cells exhibiting perinuclear clustering of the mitochondria (Figure 3.8B and C). These findings indicate that DRP1 facilitates MCL-1-induced perinuclear clustering.

3.2.4 Endogenous MCL-1 and DRP1 Interact as an Adaptive Response to Stress

Based on my data suggesting that MCL-1-mediated morphological changes were DRP1-dependent, I wanted to further explore the role of MCL-1 in DRP1-mediated fission. Specifically, I wanted to examine the relationship between endogenous MCL-1 and DRP1 in response to stressors known to induce mitochondrial fission in MEFs. Fission has been reported to occur in response to a variety of stimuli, particularly ones that lead to disruptions in mitochondrial energetics (Toyama et al., 2016; Youle and van der Bliek, 2012). I assessed induction of mitochondrial fission in response to glucose deprivation, treatment with the complex I inhibitor rotenone, treatment with the mitochondrial uncoupler FCCP, and hypoxia. I confirmed that each of these stressors resulted in a significant shift in the mitochondrial morphology toward fragmentation (Figure 3.9A-D). Next, I wanted to determine how these fission-promoting stimuli affected the interaction between endogenous levels of MCL-1 and DRP1. To do this, I immunoprecipitated MCL-1 from MEFs, and examined the interaction between MCL-1 and DRP1. I confirmed that there is increased interaction between endogenous MCL-1 and DRP1 in MEFs in response to each of these stressors, although the temporal response varies based on the type of stressor (Figure 3.10A-D).

Next I wanted to determine the functional implications of increased association between MCL-1 and DRP1 in response to fission-promoting stimuli, as fission has been reported to be both protective and detrimental in response to stress (Coronado et al., 2018; Youle and van der Bliek, 2012). Therefore, I assessed cell viability following glucose deprivation, which is known to induce mitochondrial fragmentation (Rambold et al., 2011). MEFs were transfected with either control or DRP1 siRNA and then infected with Ad- β -gal or Ad-MCL-1. Cell viability was assessed for each condition after glucose deprivation. MCL-1 overexpression protected against cell death in response to glucose deprivation, but this protection was abrogated when DRP1 was knocked down using siRNA (Figure 3.11). These findings suggest that MCL-1 and DRP1 interact to promote mitochondrial fission as an adaptive response to stress.

3.3 Discussion

My findings indicate that MCL-1 on the outer mitochondrial membrane has a role in regulating mitochondrial dynamics in addition to its anti-apoptotic function. Specifically, my data suggests that MCL-1 is involved in facilitating DRP1-mediated mitochondrial fission in cells. Given the complex nature and intrinsic links between many mitochondrial processes, important mitochondrial proteins such as MCL-1 are presumably involved in regulating more than one function. Overexpression of MCL-1_{OM}, but not MCL-1_{Matrix}, induced perinuclear clustering of the mitochondria, indicating that MCL-1 must localize to the outer mitochondrial membrane to perform this function. My data suggests that this is likely because MCL-1 facilitates changes in mitochondrial morphology through DRP1, which binds to the mitochondria on the outer membrane.

Specifically, I found that overexpression of MCL-1 resulted in increased DRP1 levels at the mitochondria, an increased interaction between MCL-1 and DRP1, and the morphological changes I observed were attenuated upon knockdown of DRP1. Interestingly, MCL-1 facilitated mitochondrial fission without altering mitochondrial content, suggesting that the mitochondrial network is being reorganized around the perinuclear region. Microtubules play a crucial role in the maintenance of organelle shape and distribution, and it has been reported that hyperacetylation of microtubules contributes to the recruitment of DRP1 to the mitochondria in response to stress (Perdiz et al., 2017). It is possible that MCL-1 is coordinating with microtubules to facilitate a redistribution of the mitochondria in a manner that will help to recruit DRP1 and protect against stress. Determining whether microtubule inhibitors abrogate MCL-1's ability to induce morphological changes will provide further insight into the underlying mechanism of MCL-1-mediated fission.

Most studies have indicated that increased mitochondrial fission leads to apoptosis (Youle and van der Bliek, 2012). However, my data suggests that fission elicits an anti-apoptotic response instead. I discovered that mutating MCL-1's conserved BH3 domain associated with its anti-apoptotic activity (Clohessy et al., 2006) abrogated MCL-1-mediated perinuclear clustering of the mitochondria. Given that the MCL-1-BH3-mutant showed reduced cell viability in response to the apoptotic stressor staurosporine, these morphological changes may be involved in conferring protection. Additionally, since the MCL-1-BCL-2-Chimera could still induce mitochondrial perinuclear clustering, this provides further evidence that it may be related to the BCL-2 family's conserved anti-apoptotic activity. However, additional studies are required to

determine the exact nature of the interplay between MCL-1-mediated mitochondrial fission and its anti-apoptotic function.

Interestingly, I also confirmed that endogenous MCL-1 and DRP1 interact in response to fission-promoting stimuli. This indicates that MCL-1's involvement in mitochondrial fission has a physiological basis and is not merely an effect of overexpression. While the interaction between MCL-1 and DRP1 is fairly rapid, there is some variability based on the type of stress. This is consistent with previous reports that stressors that disrupt mitochondrial metabolism or electron transport chain inhibitors induce rapid fragmentation (Hung et al., 2018; Toyama et al., 2016), whereas, fragmentation occurs at a later time point during hypoxia (Zhang et al., 2018). However, in each case the interaction between MCL-1 and DRP1 either preceded or corresponded to alterations in the mitochondrial networks in favor of fission. In contrast to previous reports indicating that fission is maladaptive (Youle and van der Bliek, 2012), my findings suggest that DRP1-mediated fission facilitated by MCL-1 is protective during nutrient deprivation. However, it will be necessary to assess cell death in response to other fission-inducing stressors to determine if this is a specific response to nutrient deprivation or a more widely conserved adaptation. Overall, my findings demonstrate that MCL-1 can facilitate DRP1-mediated mitochondrial fission as an adaptive response to stress.

Parts of Chapter 3 are currently being prepared for submission for publication. Moyzis, Alexandra; Lally, Navraj; Leon, Leonardo; Shires, Sarah; Orogo, Amabel; Liang, Wenjing; Najor, Rita, Gustafsson, Åsa. "Mcl-1 on the outer mitochondrial membrane

promotes Drp1-mediated mitochondrial fission and affects exercise capacity in the heart.” The dissertation author was the primary investigator and author of this paper.

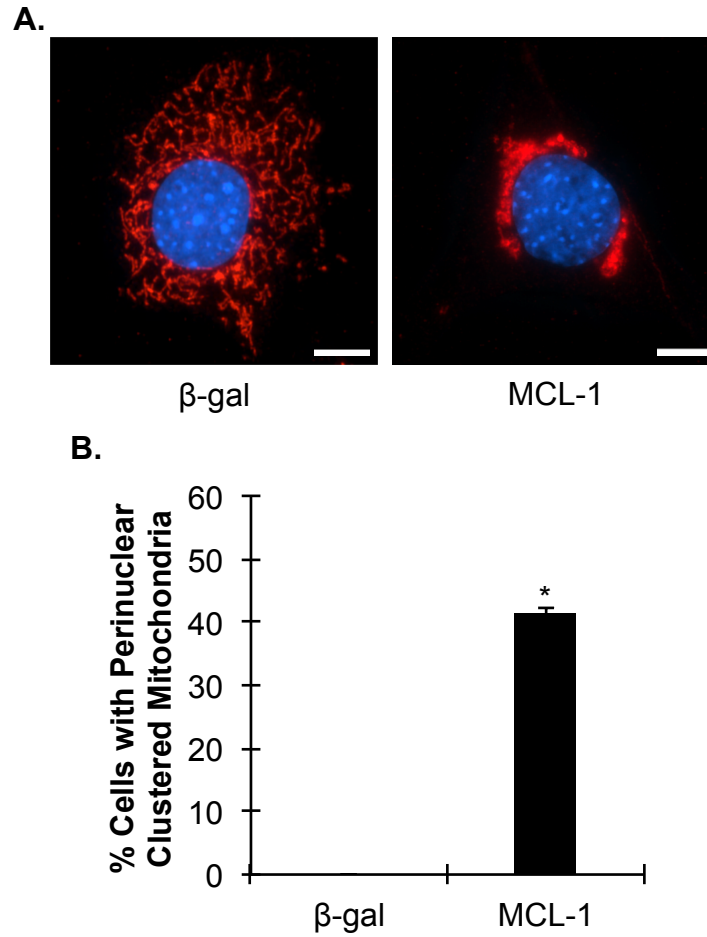
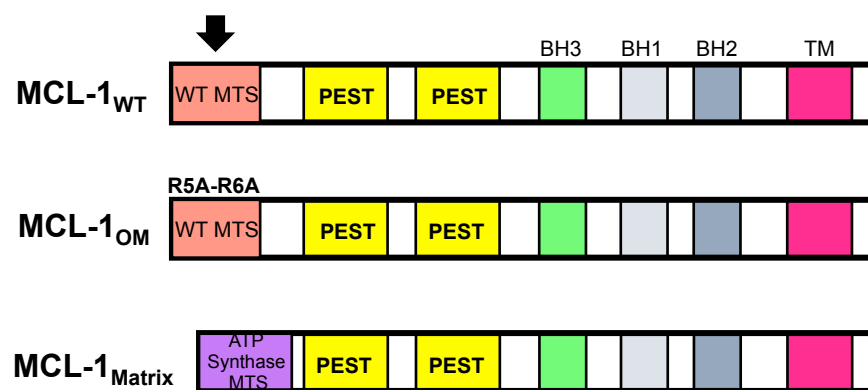


Figure 3.1. Overexpression of MCL-1 induces perinuclear clustering of mitochondria in MEFs.

A. MEFs were infected with Ad-β-gal or Ad-HA-MCL-1 and stained for the mitochondrial marker TOM20 (red). Scale bar: 10 μm. **B.** Quantitation of the percentage of cells exhibiting perinuclear clustering of the mitochondria (*p<0.05, n=3, 200 cells were counted for each n).

A.



B.

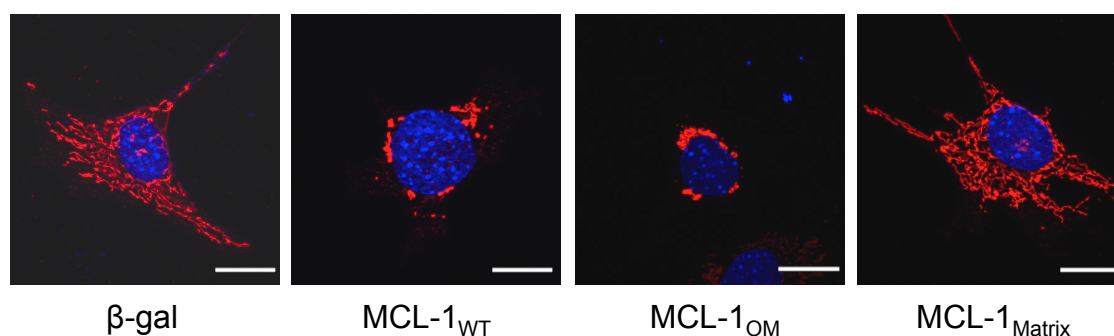
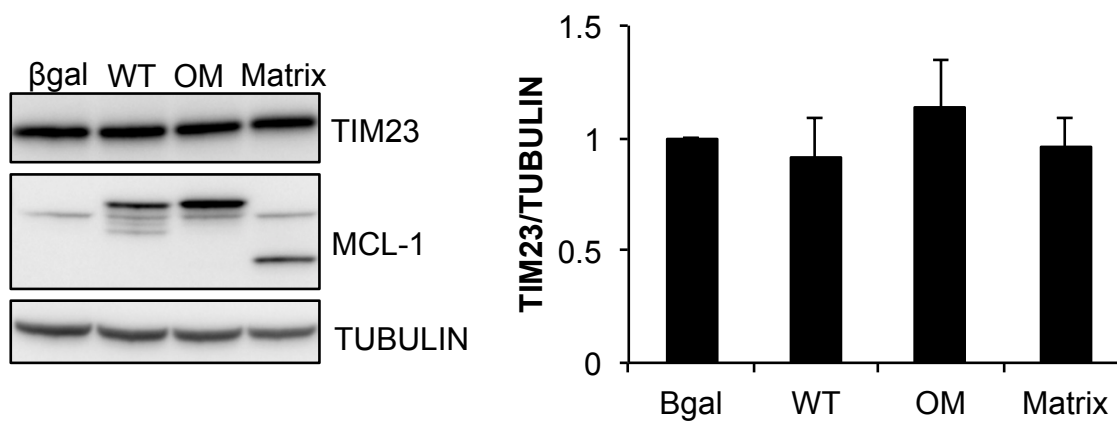


Figure 3.2. Overexpression of MCL-1_{WT} and MCL-1_{OM}, but not MCL-1_{Matrix} induce perinuclear clustering of the mitochondria.

A. Schematics showing the three MCL-1 constructs: MCL1-_{WT}, MCL-1_{OM}, and MCL-1_{Matrix}. MTS = mitochondrial targeting sequence. **B.** MEFs were infected with Ad-β-gal, Ad-HA-MCL-1, Ad-Myc-MCL-1OM, or Ad-HA-MCL-1Matrix and stained for TOM20 as a mitochondrial marker (red). Scale bar: 20 μm.

A.



B.

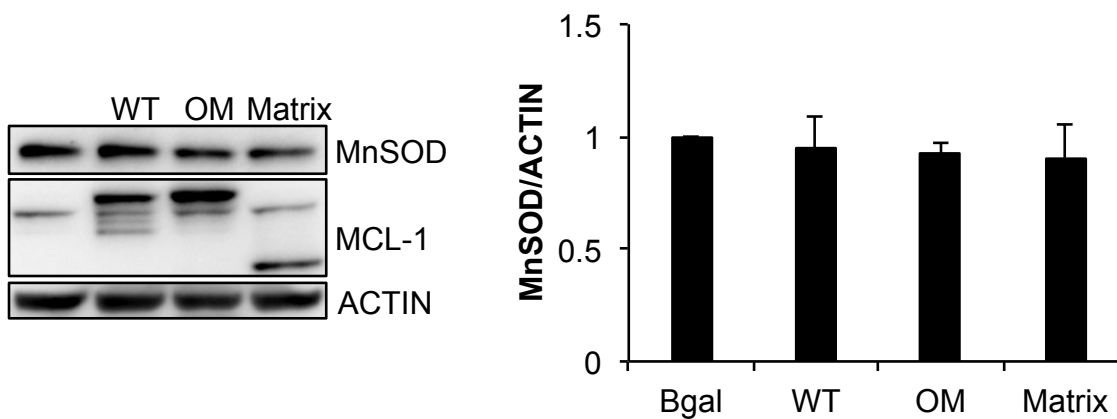


Figure 3.3. MCL-1 overexpression does not lead to changes in mitochondrial content.

A. Representative Western blot and quantitation assessing levels of the mitochondrial protein TIM23 (n=3). **B.** Representative Western blot and quantitation assessing levels of the mitochondrial protein MnSOD (n=3).

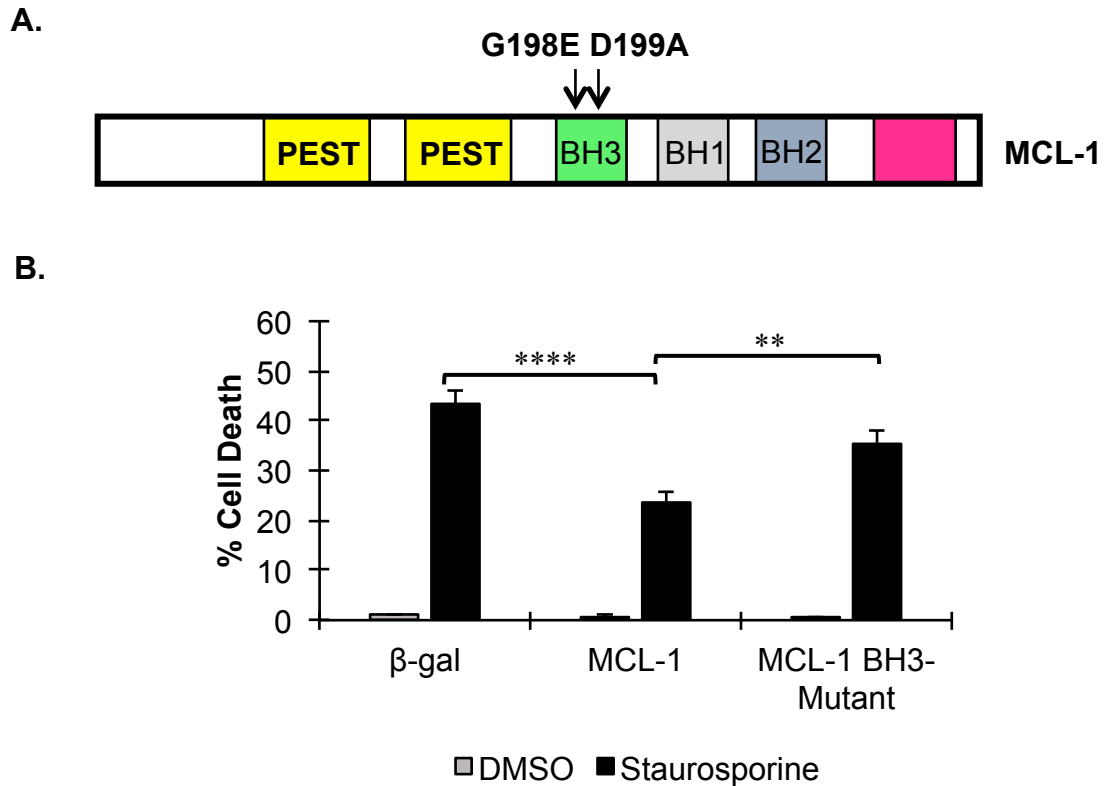
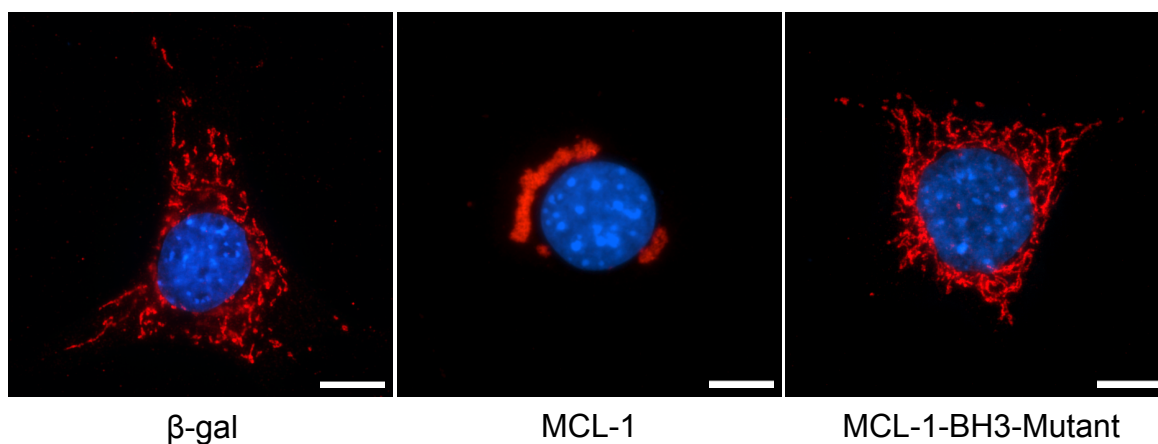


Figure 3.4. Mutation of MCL-1's BH3 domain disrupts its ability to protect against cell death in response to staurosporine.

A. Schematic representation of the MCL-1 BH3-mutant generated by mutating two conserved residues (G198E and D199A) in MCL-1's BH3 domain (MCL-1-BH3-Mutant).

B. Quantitation of cell death after treatment with staurosporine. MEFs were infected with Ad-β-gal, Ad-HA-MCL-1, Ad-HA-MCL-1-BH3-mutant and subsequently treated with 1 μM staurosporine for 24 hours (** $p < 0.01$; **** $p < 0.0001$; $n = 3$).

A.



B.

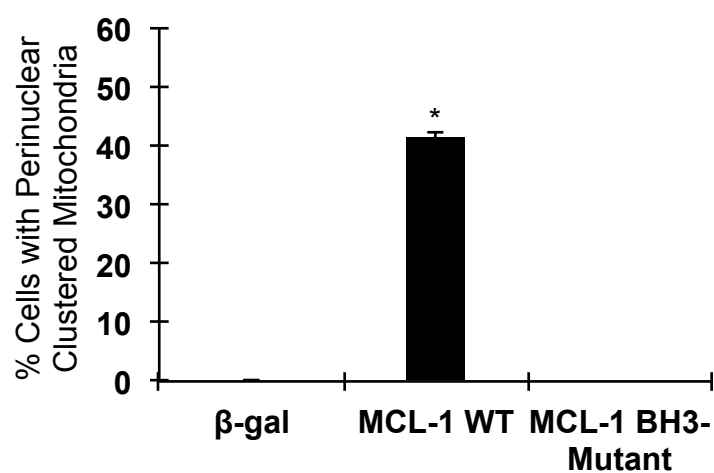


Figure 3.5. Mutating MCL-1's BH3 domain abrogates perinuclear clustering of the mitochondria.

A. MEFs were infected with Ad-β-gal, Ad-HA-MCL-1, Ad-HA-MCL-1-BH3-mutant and stained for TOM20 (red) as a mitochondrial marker. Scale bar: 10 μm **B.** Quantitation of the percentage of cells exhibiting perinuclear clustered mitochondria (*p<0.05, n=3, 200 cells were counted for each n).

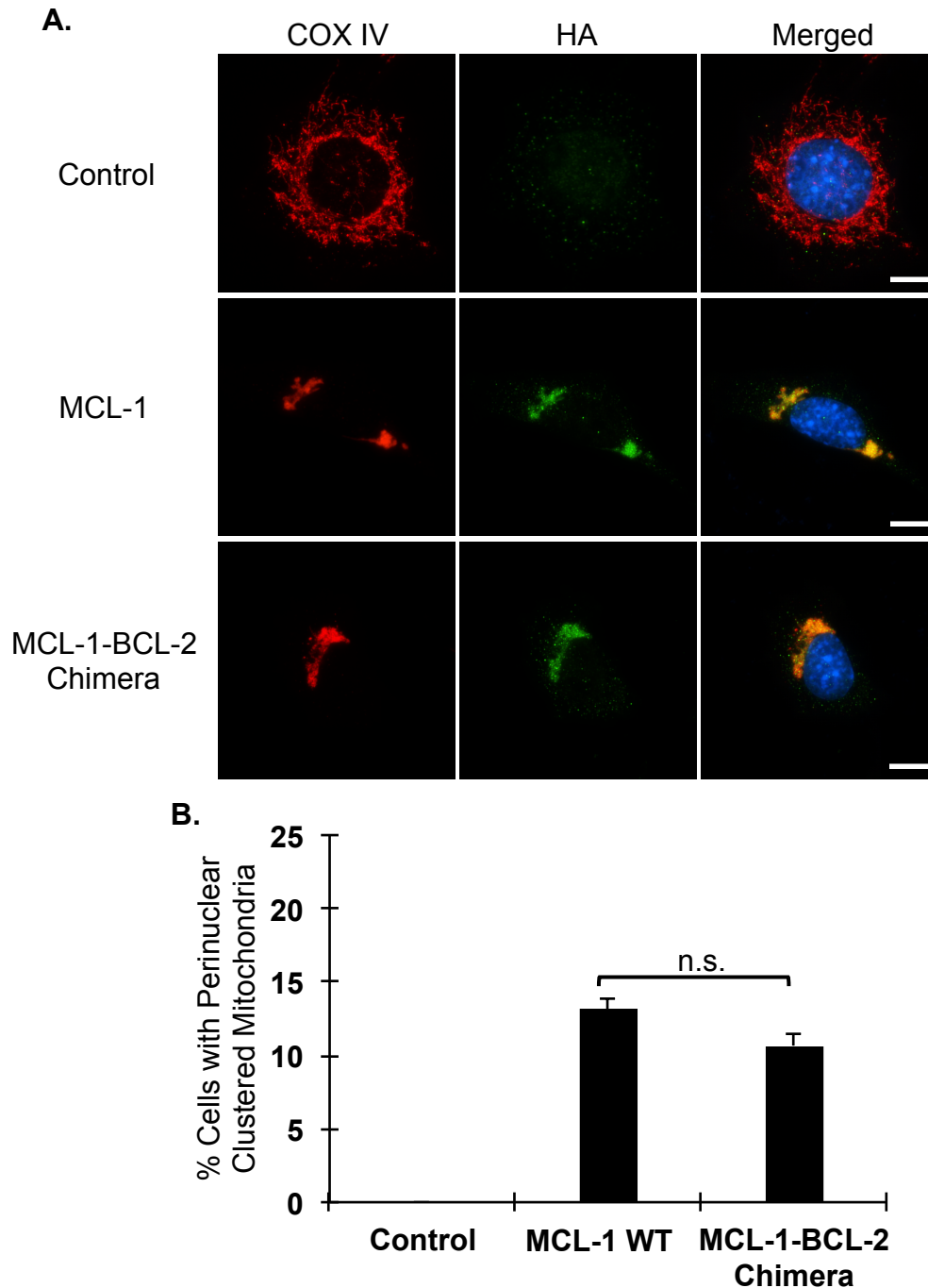
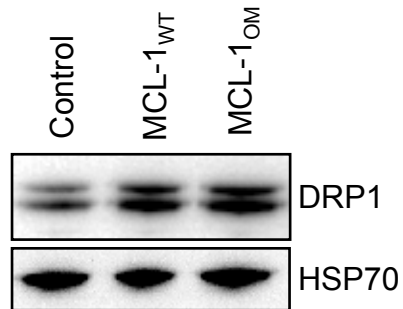


Figure 3.6. MCL-1-induced perinuclear clustering of mitochondria requires a functional BH3 domain.

A. A MCL-1-BCL-2 chimera was generated by replacing all of MCL-1's BH domains with those from its anti-apoptotic family member BCL-2 (HA-MCL-1-BCL-2 Chimera). MEFs were transfected with HA, HA-MCL-1 or HA-MCL-1-BCL2 Chimera and stained for HA (green) as a marker for overexpressed MCL-1 and COX IV (red) as a mitochondrial marker. Scale bar: 10 μ m B. Quantitation of the percentage of cells exhibiting perinuclear clustered mitochondria (n.s., not significant; n=3).

A.

Mitochondrial Fraction



B.

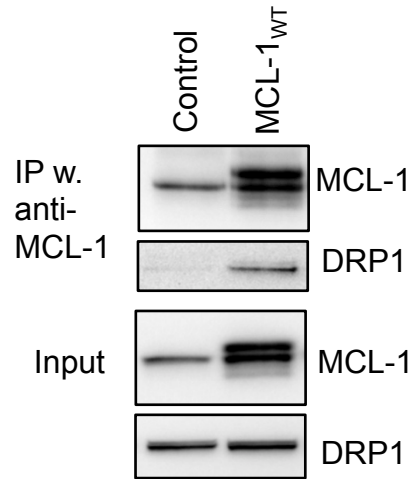


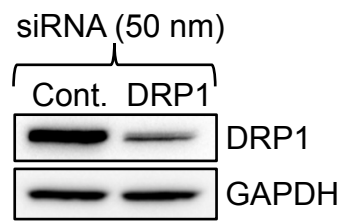
Figure 3.7. MCL-1 overexpression leads to increased DRP1 levels at the mitochondria.

A. Representative Western blot showing increased DRP1 levels at the mitochondria in MEFs overexpressing MCL-1_{WT} or MCL-1_{OM} constructs. **B.** Immunoprecipitation of MCL-1 reveals increased interaction between MCL-1 and endogenous DRP1 when MCL-1 is overexpressed in MEFs.

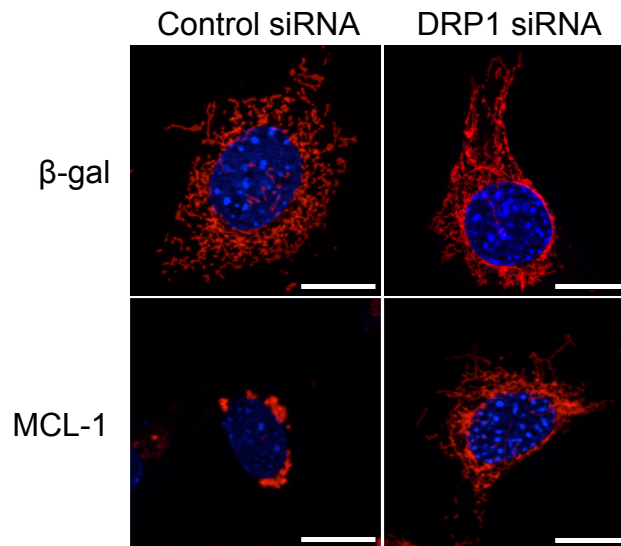
Figure 3.8. MCL-1-induced perinuclear clustering of mitochondria is DRP1-dependent.

A. MEFs were transfected with 50 nM control or Drp1 siRNA and DRP1 levels were assessed via Western blot after 72 hours. **B.** MEFs were transfected with 50 nM control or DRP1 siRNA and subsequently infected with either Ad- β -gal or Ad-HA-MCL-1 and stained with the mitochondrial marker TOM20. Scale bar: 20 μ m. **C.** Quantitation of the percentage of cells exhibiting clustered mitochondria (* $p < 0.05$, $n = 3$, 200 cells were counted for each n).

A.



B.



C.

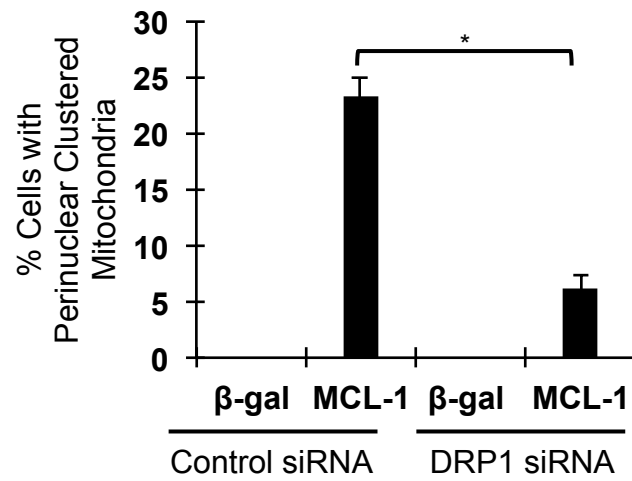
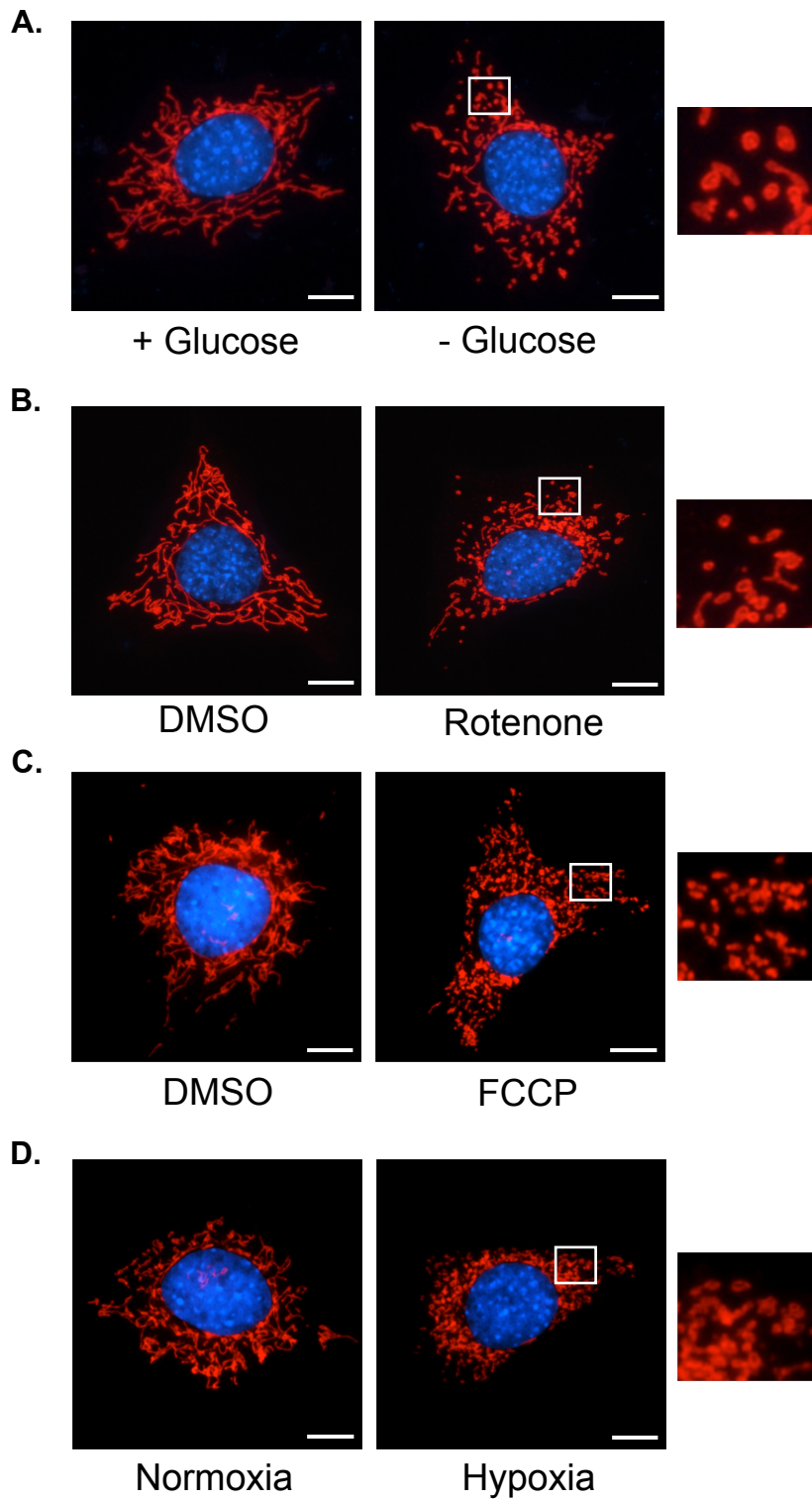


Figure 3.9. Stressors resulting in increased mitochondrial fragmentation. Mitochondria become fragmented in response to **A.** Glucose and serum deprivation (2 hours), **B.** treatment with 1 μ M rotenone (2 hours), **C.** treatment with 25 μ M FCCP (1 hour), and **D.** hypoxia (6 hours). MEFs were stained with TOM20 as a mitochondrial marker. Scale bar: 10 μ m.



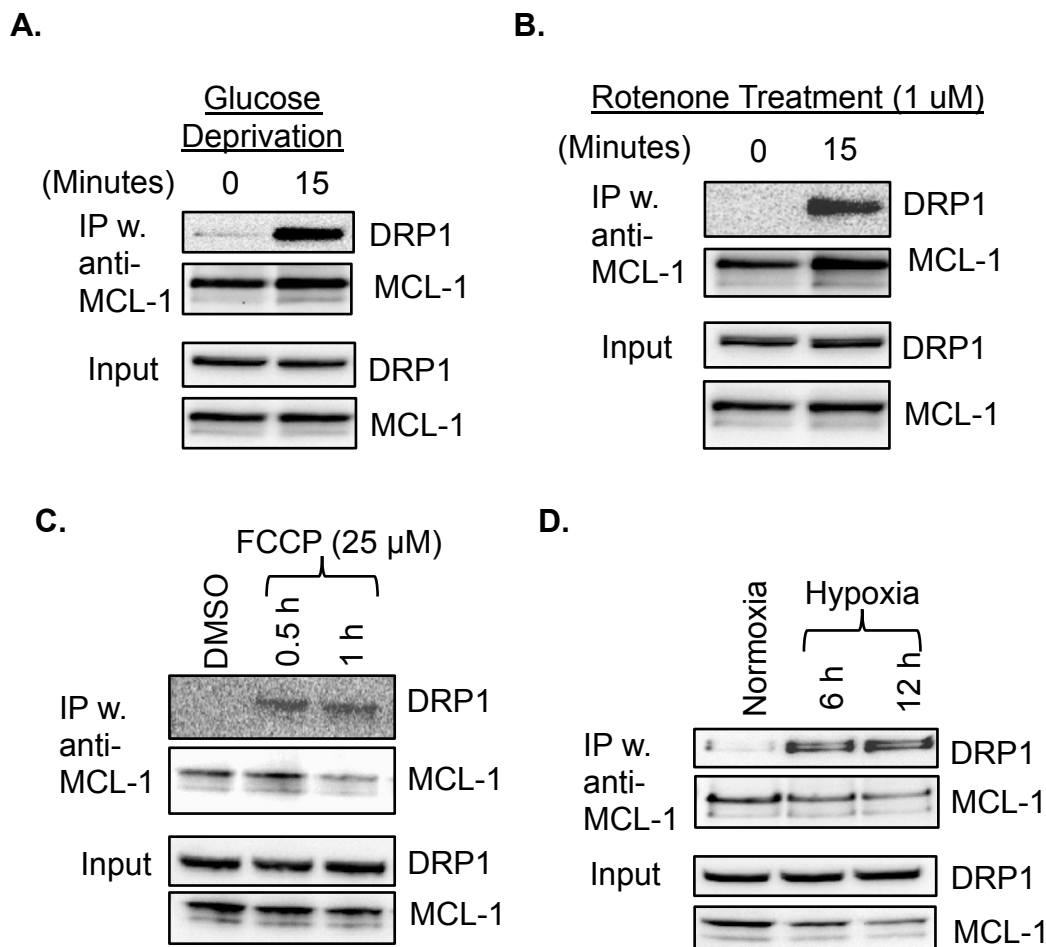


Figure 3.10. Endogenous MCL-1 and DRP1 interact in response to various fission-promoting stimuli.

MEFs were subjected to **A.** Glucose and serum deprivation, **B.** treatment with 1 μ M rotenone, **C.** treatment with 25 μ M FCCP, and **D.** hypoxia for the indicated amounts of time. MCL-1 was immunoprecipitated in order to assess the interaction between endogenous MCL-1 and DRP1.

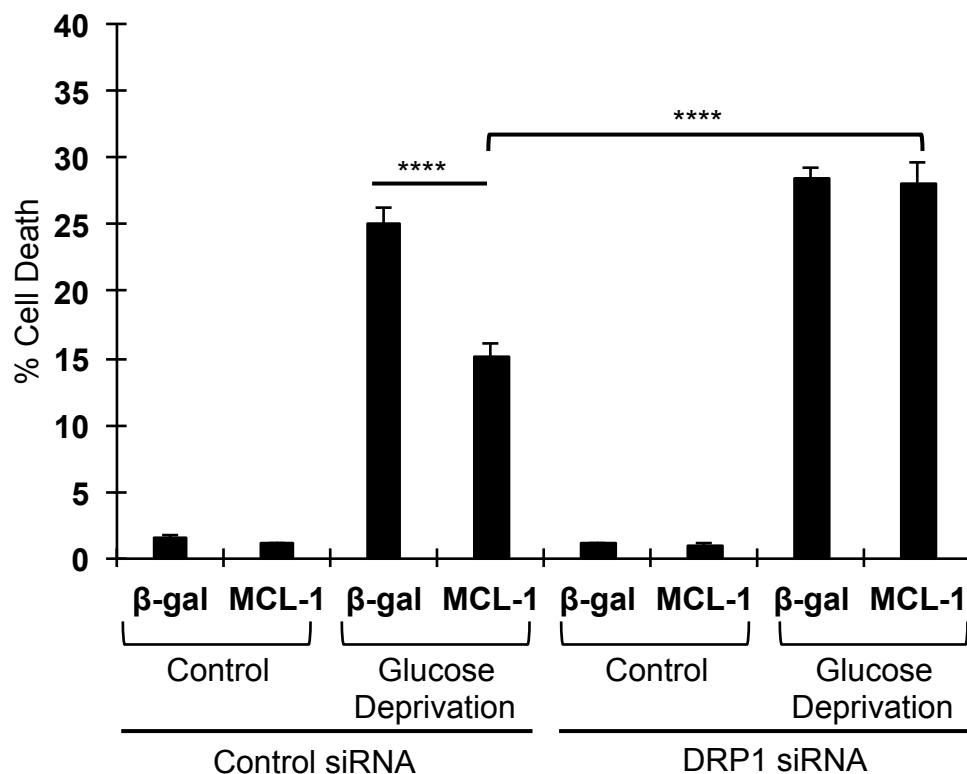


Figure 3.11. MCL-1's ability to protect against glucose-deprivation-induced cell death is abrogated when Drp1 is knocked down.

MEFs were transfected with 50 nM control or DRP1 siRNA and subsequently infected with either Ad-β-gal or Ad-HA-MCL-1. Cells were subjected to glucose deprivation for 24 hours and cell death was assessed. (**** $p < 0.0001$; $n = 3$).

CHAPTER 4: THE EFFECT OF MCL-1_{OM} OVEREXPRESSION ON MITOCHONDRIAL FISSION *IN VIVO*

4.1 Introduction

In order to extend my *in vitro* findings, I also wanted to assess the effect of MCL-1_{OM} overexpression *in vivo*, particularly in relation to mitochondrial dynamics. The mitochondrial fission regulator DRP1 is crucial for maintaining homeostasis in the heart *in vivo* and cardiac-specific deletion results in unopposed fusion and lethal cardiomyopathy (Song et al., 2015b). This indicates that the disruption of the balance between fission and fusion has adverse effects in the heart. However, further investigation revealed that disruption of mitochondrial dynamics, even in a balanced manner, is still detrimental. Although cardiac-specific DRP1, MFN1, and MFN2-triple knockout mice were viable, they accumulated a mixture of fragmented and fused mitochondria in the heart and developed a unique pathological form of cardiac hypertrophy (Song et al., 2017). This suggests that modifying expression of any protein involved in regulating mitochondrial dynamics will cause changes in the heart. MCL-1 is required to maintain normal mitochondrial and cardiac function (Thomas et al., 2013; Wang et al., 2013). However, the role of MCL-1_{OM} in the heart *in vivo* has never been explored. Thus, I wanted to determine if MCL-1_{OM} overexpression would lead to increased fission and result in any alterations in cardiac or mitochondrial function under baseline conditions and in response to stress.

4.2 Results

4.2.1 Characterization of α -MHC-MCL-1_{OM} Transgenic Mice

To examine the role of MCL-1_{OM} *in vivo*, I first confirmed that endogenous MCL-1 resides on both the outer mitochondrial membrane and in the mitochondrial matrix in the hearts of wild type mice (Figure 4.1). To do this, I incubated isolated mitochondria with proteinase K, which rapidly degrades proteins on the outer mitochondrial membrane. After verifying that MCL-1 localizes to both mitochondrial locations, I generated cardiac-specific MCL-1_{OM} transgenic mice under the alpha-MHC (α -MHC) promoter. I obtained three founder lines (MCL-1_{OM} TG) (Figure 4.2A). Line #30 was born at the expected Mendelian ratio and used for further characterization. I confirmed that the MCL-1_{OM} transgene localized specifically to the outer mitochondrial membrane by isolating the mitochondria and subjecting them to treatment with proteinase K (Figure 4.2B). The MCL-1_{OM} TG mice showed no significant differences in heart weight/body weight or heart weight/tibia length ratios (Figure 4.2C). Histological analysis using haematoxylin and eosin (H&E) staining revealed no significant differences in the WT versus MCL-1_{OM} TG hearts (Figure 4.2D). The MCL-1_{OM} TG mice also showed no changes in cardiac function or structure as assessed by echocardiographs. The MCL-1_{OM} TG mice had similar ejection fraction (EF), fractional shortening (FS), left ventricular internal diameter end-systole (LVID (s)), and left ventricular internal diameter end-diastole (LVID (d)) as WT littermates (Figure 4.3A and B). Next, I determined the effect of MCL-1_{OM} overexpression on other BCL-2 family members. However, the MCL-1_{OM} TG mice showed no significant alterations in anti-apoptotic BCL-2 and BCL-X_L (Figure 4.4A and B). Pro-apoptotic BAX and BAK were also unchanged in hearts overexpressing MCL-

1_{OM} (Figure 4.4). Taken together, this data indicates that elevated MCL-1_{OM} has no significant effect on cardiac structure or function under baseline conditions.

4.2.2 DRP1 Levels are Increased at the Mitochondria in MCL-1_{OM} TG Mice

Although there were no changes in cardiac function in the MCL-1_{OM} TG mice, I wanted to further analyze their hearts at the ultrastructural level. I used transmission electron microscopy (TEM) to assess whether MCL-1_{OM} overexpression would cause structural changes or alterations in mitochondrial morphology in the heart. Interestingly, I observed that many of the mitochondria in the MCL-1_{OM} TG mouse hearts were smaller than those from the WT mice, resulting in a significant decrease in the mean mitochondrial area (Figure 4.5A and B). These results are indicative of enhanced mitochondrial fission, which is known to lead to smaller mitochondria. Based on these findings, I assessed the levels of mitochondrial fission and fusion proteins in the MCL-1_{OM} TG hearts. However, there were no significant differences in the overall levels of the major mitochondrial fission proteins DRP1 and FIS1 (Figure 4.6A and B) and fusion proteins OPA1, MFN1, and MFN2 (Figure 4.7A and B) in the MCL-1_{OM} TG mice. Of note, the levels of MFF, which is reported to act as a mitochondrial receptor for DRP1, were significantly reduced in these mice (Figure 4.6B). DRP1 normally localizes to the cytosol, but its recruitment to the mitochondria induces mitochondrial fission (Nan et al., 2017). Thus, even though the overall levels of DRP1 remained unchanged, I wanted to assess DRP1 levels specifically at the mitochondria. Consistent with the observed decrease in mitochondrial size, I found that DRP1 levels were significantly increased at the mitochondria in the hearts of MCL-1_{OM} TG mice (Figure 4.8A). I also examined whether MCL-1 interacts with DRP1 at the mitochondria. I immunoprecipitated MCL-1

and detected an increased interaction between MCL-1 and DRP1 in the MCL-1_{OM} TG hearts (Figure 4.8B). These findings indicate an important relationship between MCL-1 on the outer mitochondrial membrane and DRP1 in regulating mitochondrial dynamics in the heart.

4.2.3 MCL-1_{OM} Overexpression has no Effect on Mitochondrial Content or Function

Mitochondrial dynamics are closely associated with mitochondrial bioenergetics and mitophagy (Mishra and Chan, 2016; Twig et al., 2008). Next, I wanted to determine whether increased DRP1 levels and smaller mitochondria would affect mitochondrial content and function. To evaluate mitochondrial function, I used the Seahorse XFP analyzer and measured respiration in isolated mitochondria using different substrates to assess glucose metabolism (pyruvate/malate and glutamate/malate) and complex II-dependent oxygen consumption (succinate/rotenone). However, there were no significant alterations in mitochondrial respiration in response to any of the substrates tested (Figure 4.9), indicating that even though the mitochondria are smaller in MCL-1_{OM} TG hearts, they are not functionally compromised. Next, I wanted to determine if MCL-1_{OM} overexpression led to differences in mitochondrial content. I assessed mitochondrial DNA (mtDNA) content, but I found no significant differences between the MCL-1_{OM} TG and WT mice. I also utilized the OXPHOS proteins, TIM23, and TOM20 to assess mitochondrial protein levels. A decrease in protein levels is indicative of fewer mitochondria. I did not detect any significant differences in the OXPHOS proteins or TOM20, but I observed a slight, albeit significant, increase in TIM23 levels in the MCL-

1_{OM} TG mice (Figure 4.10A-D). Taken together, these findings suggest that MCL-1_{OM} overexpression has little effect on mitochondrial content or function.

4.2.4 MCL-1_{OM} TG Mice Have Reduced Exercise Capacity

It has been reported that DRP1 facilitates adaptive physiological fragmentation in response to exercise (Coronado et al., 2018; Moore et al., 2019). While there were no functional changes in the hearts of MCL-1_{OM} TG mice under baseline conditions, I wanted to investigate if MCL-1_{OM} overexpression would have an effect in response to stress. To evaluate the role of mitochondrial fission in response to swimming in the hearts of WT mice, I did a time course looking at DRP1 levels at the mitochondria. Mice were pre-trained for 3 days prior to performing the endurance experiment. My data showed that DRP1 levels increased at the mitochondria in the hearts of WT mice after 60 minutes of swimming (Figure 4.11A). Since MCL-1_{OM} TG mice already have increased DRP1 levels at their mitochondria, I wanted to determine how this would affect their exercise capacity. To investigate this, I pre-trained the mice for 3 days to acclimate them to the water and then subjected them to a forced swimming test (Wei et al., 2017). This test adds weights equivalent to 7.5% of the mouse's body weight and forces them to continuously swim until exhaustion. Intriguingly, I found that the MCL-1_{OM} TG mice showed a significant reduction in the amount of time that they were able to swim compared to the WT mice (Figure 4.11B). This indicates that increased MCL-1_{OM} expression in the heart leads to reduced exercise capacity and suggests that enhanced DRP1 levels at the mitochondria and corresponding mitochondrial fission are maladaptive in response to exercise-induced cardiac stress.

4.3 Discussion

These findings are consistent with my *in vitro* data showing that MCL-1 and DRP1 interact and regulate mitochondrial fission. To my knowledge, this is the first time that the relationship between MCL-1 and DRP1 is suggested to have physiological relevance *in vivo* in the heart. While I observed increased DRP1 levels at the mitochondria and corresponding morphological changes in the MCL-1_{OM} TG mice, these alterations did not affect baseline mitochondrial or cardiac function. This indicates that increased mitochondrial fission at the observed levels is not inherently detrimental to the heart. However, the MCL-1_{OM} TG mice exhibited compromised exercise capacity, suggesting that enhanced MCL-1-DRP1-mediated fission is maladaptive in response to stress.

My data strongly supports that MCL-1 positively regulates DRP1-mediated fission. Given that MCL-1_{OM} overexpression alone is able to recruit increased DRP1, MCL-1_{OM} may act as receptor for DRP1 at the mitochondria. Additional evidence that MCL-1 functions as a DRP1 receptor includes that it was previously reported that mitochondria from MCL-1-deficient hearts lack DRP1 (Thomas et al., 2013). However, whether MCL-1_{OM} interacts with DRP1 directly or is part of a larger complex still needs to be determined. Specifically, assessing the relationship between MCL-1_{OM} and the other putative DRP1 receptors FIS1, MFF, and MiD49/51 would be informative. MCL-1_{OM} overexpression did not result in any differences in levels of the DRP1 receptor FIS1, but it did significantly reduce the levels of MFF. Given that MFF also resides on the outer mitochondrial membrane and recruits DRP1 to the mitochondria, it is possible that increased MCL-1_{OM} levels caused a compensatory reduction in MFF. Interestingly,

it has been reported that deletion of either DRP1 or MFF in cells led to reduced expression, shorter half-lives and increased ubiquitination of MCL-1 and MiD49 through MARCH5 (Cherok et al., 2017). These results further reinforce the possibility that MCL-1_{OM} acts as a DRP1 receptor. Mechanistically, it is also possible that MCL-1_{OM} is involved in facilitating post-translational modifications of DRP1, which have been shown to regulate DRP1's recruitment to the mitochondria (Zemirli et al., 2018).

In contrast to previous reports suggesting that increased DRP1 at the mitochondria was beneficial in response to exercise (Coronado et al., 2018; Moore et al., 2019), I observed a significant decrease in the amount of time that the MCL-1_{OM} TG mice were able to swim compared to the WT mice. A possible explanation for this discrepancy in findings is that exercise capacity can be affected by the type of exercise, the amount of pre-training, and can vary in different mouse lines (Avila et al., 2017). For instance, other studies were done using treadmills and often focused on endurance exercise rather than shorter energetic responses. This is consistent with my finding that WT mice take an hour to recruit DRP1 in response to normal swimming, suggesting that starting with increased DRP1 at the mitochondria in models of exercise that require rapid energy response is maladaptive. Recent studies in skeletal muscle indicate that exercise leads to enhanced mitochondrial fusion as an adaptive response to stress (Arribat et al., 2019; Axelrod et al., 2019). If this is also true in the heart, it could help explain why the MCL-1_{OM} TG mice are unable to swim as long as the WT mice. Taken together, these findings suggest that enhanced fission may be adaptive for longer periods of exercise, but starting out with fragmented mitochondria is disadvantageous. Given that mitochondrial dynamics have also been closely linked with other important

cellular processes, such as bioenergetics and mitophagy, the role of these in response to stress also needs to be evaluated. Further investigation is required to determine if it is enhanced mitochondrial fission or some other effect of MCL-1_{OM} overexpression on the heart that causes a significant reduction in the amount of time the MCL-1_{OM} TG mice can swim. Exploring other forms of exercise and additional stressors to determine the effect of MCL-1_{OM} overexpression will be important moving forward too.

Parts of Chapter 4 are currently being prepared for submission for publication. Moyzis, Alexandra; Lally, Navraj; Leon, Leonardo; Shires, Sarah; Orogo, Amabel; Liang, Wenjing; Najor, Rita, Gustafsson, Åsa. "Mcl-1 on the outer mitochondrial membrane promotes Drp1-mediated mitochondrial fission and affects exercise capacity in the heart." The dissertation author was the primary investigator and author of this paper.

Proteinase K (200 ng/ul)

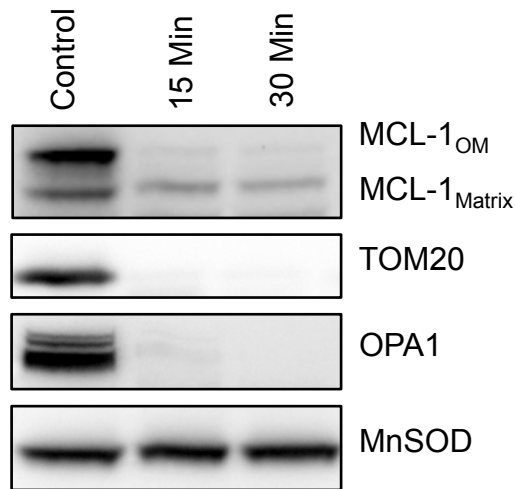


Figure 4.1. MCL-1 localizes to both the outer mitochondrial membrane and the mitochondrial matrix in the hearts of WT mice.

Mitochondria were isolated from WT mouse hearts and subjected to treatment with 200 ng/μl of proteinase K for either 15 or 30 minutes. Proteinase K degraded the longer form of MCL-1 on the outer mitochondrial membrane (MCL-1_{OM}) along with the outer membrane protein TOM20 and the inner membrane protein OPA1. In contrast, the faster migrating form of MCL-1 (MCL-1_{Matrix}) and the matrix protein MnSOD were protected against the digestions. This confirms that MCL-1 localizes to both outside and inside the cardiac mitochondria.

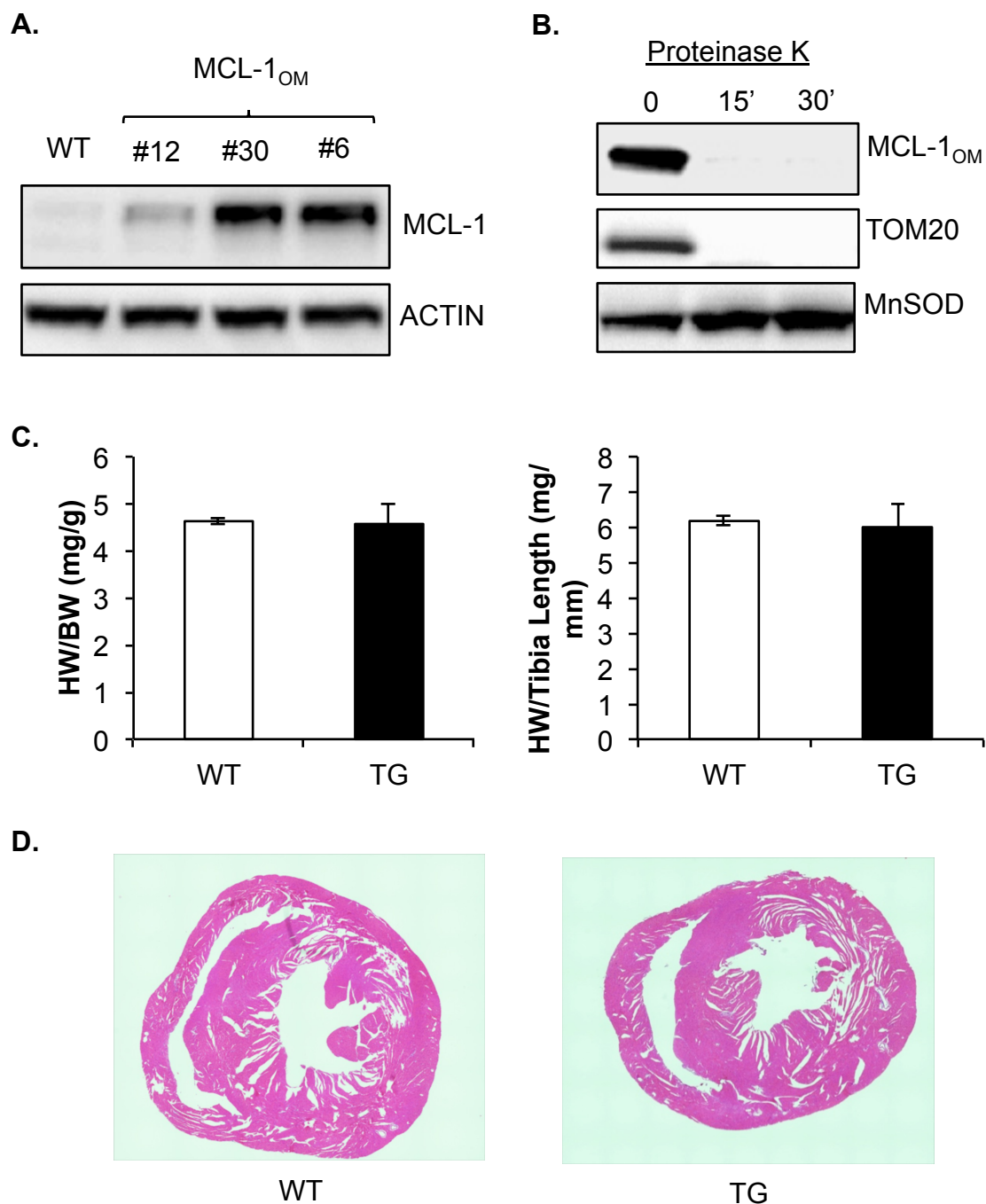


Figure 4.2. Characterization of α -MHC MCL-1_{OM} transgenic mice.

A. Representative Western blot showing the expression levels of the three generated MCL-1_{OM} transgenic mouse lines. **B.** The MCL-1_{OM} transgene localizes specifically to the outer membrane of the mitochondria. Mitochondria were isolated from a MCL-1_{OM} TG mouse heart and treated with 200 ng/ μ l proteinase K to degrade proteins on the outer mitochondrial membrane. **C.** Heart weight/body weight and heart weight/tibia length ratios comparing WT and MCL-1_{OM} TG mice (n=8). **D.** Histological analysis using haematoxylin and eosin (H&E) staining of WT and MCL-1_{OM} TG hearts.

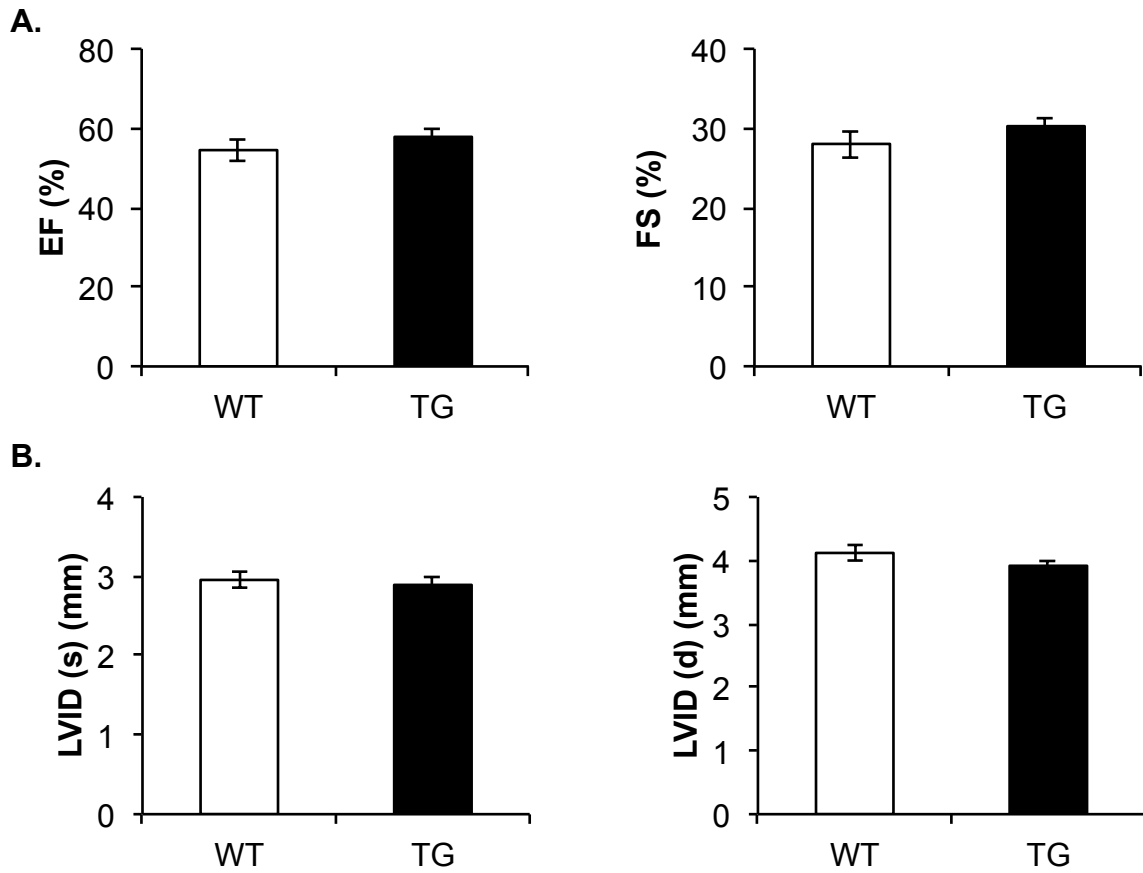


Figure 4.3. MCL-1_{OM} TG mice show no alterations in cardiac function.

Echocardiographic analysis showed similar **A.** ejection fraction (EF) and fractional shortening (FS) and **B.** left ventricular internal dimension at systole (LVID (s)) and diastole (LVID (d)) in WT and MCL-1_{OM} TG hearts (n=8).

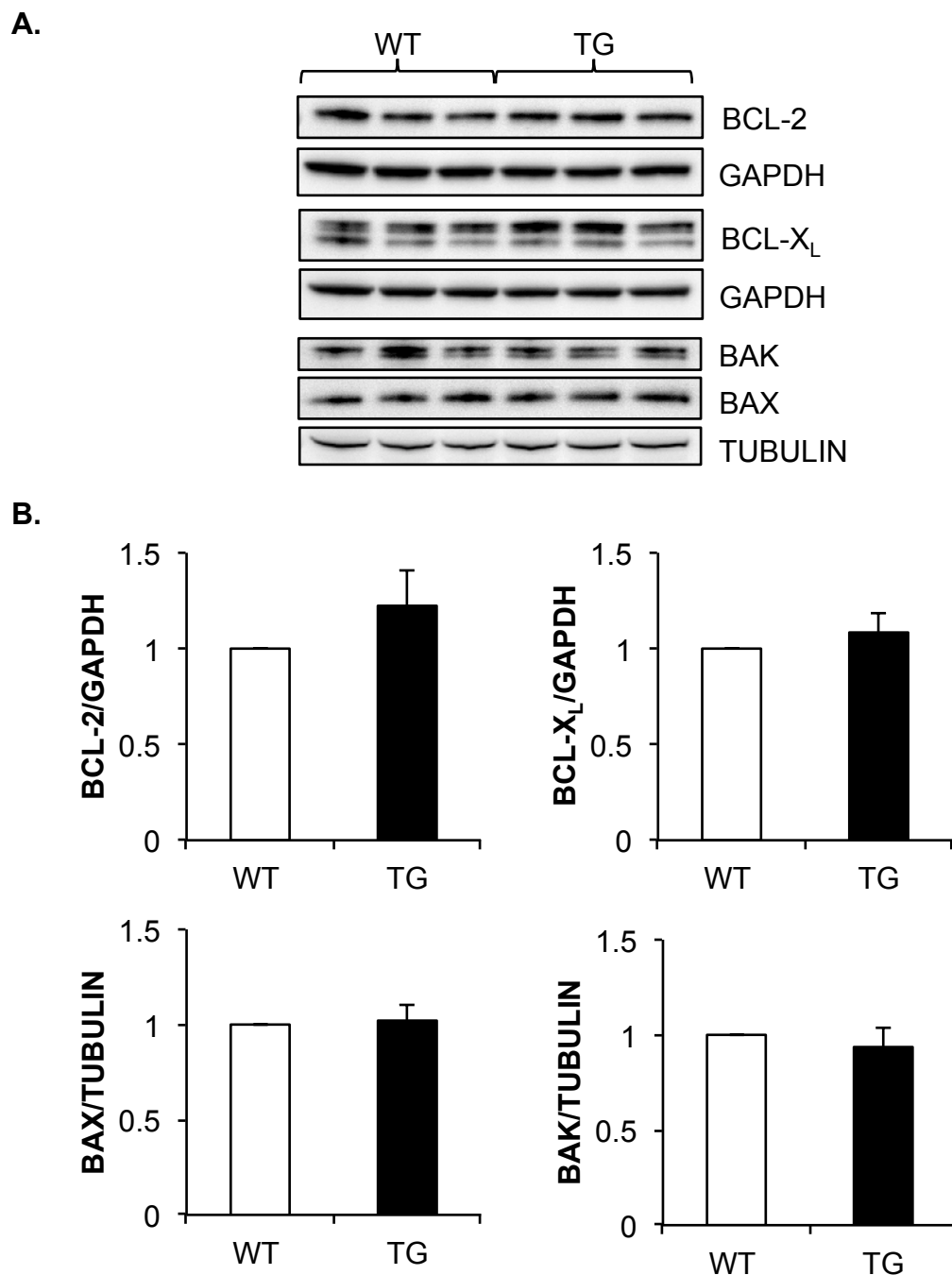


Figure 4.4. There are no significant alterations in other BCL-2 family proteins in MCL-1_{OM} TG hearts.

A. Representative Western blots showing BCL-2 family proteins in the hearts of WT and MCL-1_{OM} TG mice **B.** Quantitation of BCL-2, BCL-X_L, BAX and BAK levels (n=5-6).

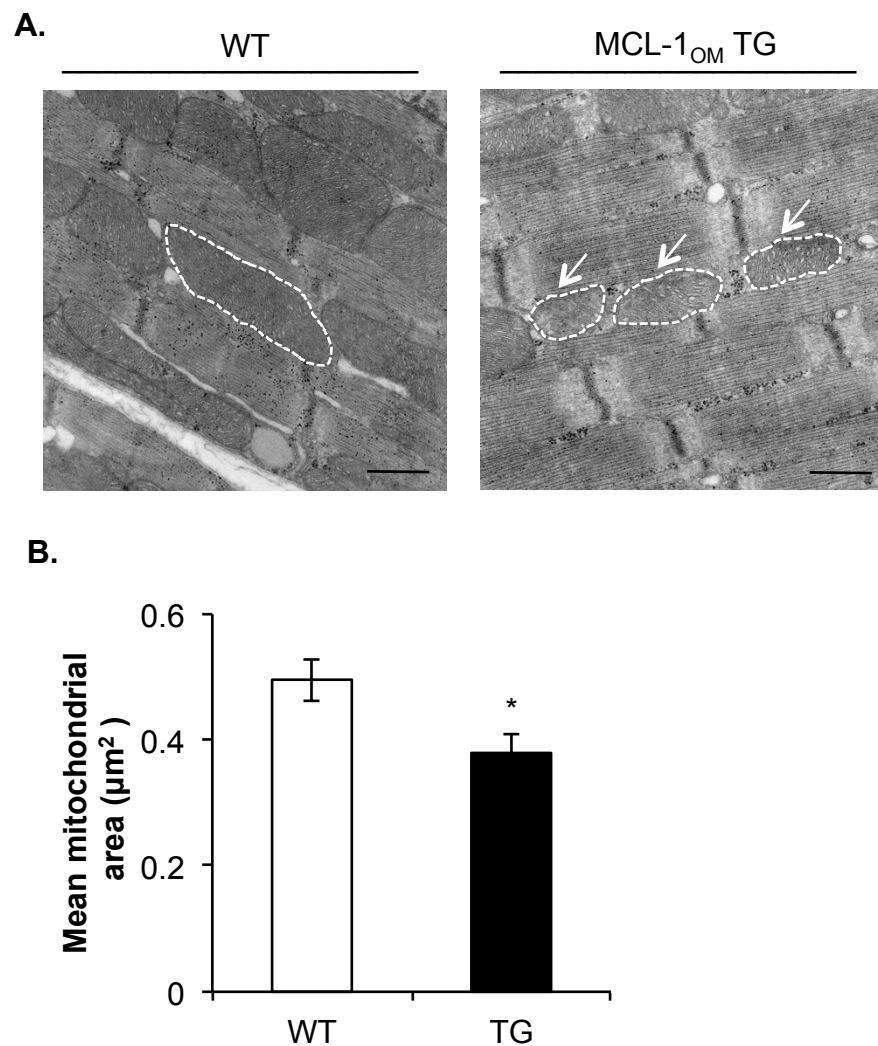
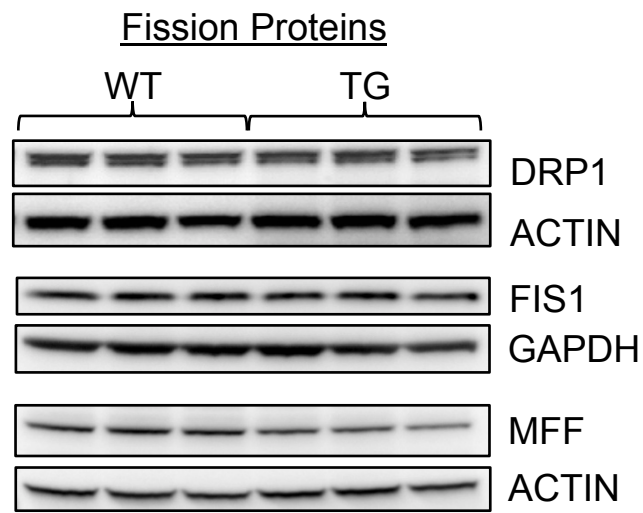


Figure 4.5. Mean mitochondrial area is reduced in MCL-1_{OM} TG mice.

A. Representative images of mitochondria in WT and MCL-1_{OM} TG mouse hearts taken using transmission electron microscopy (TEM) (scale = 0.5 μm) **B.** Quantitation of mean mitochondrial area (μm²) (*p<0.05).

A.



B.

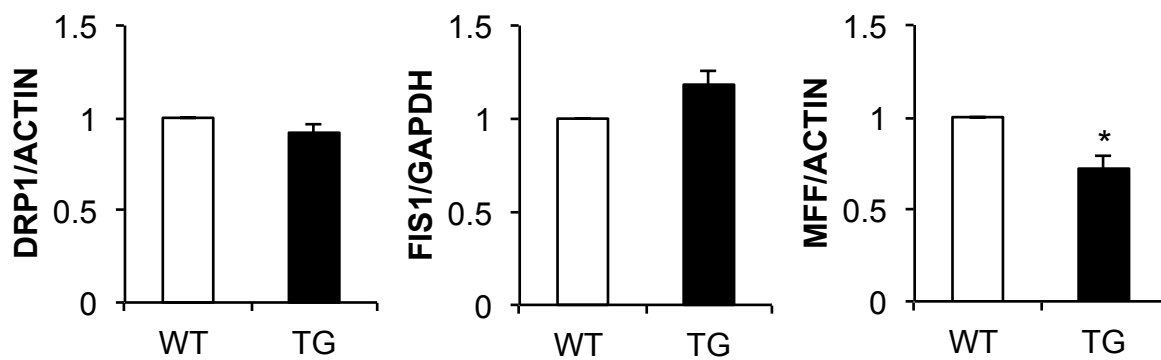


Figure 4.6. Analysis of mitochondrial fission proteins in MCL-1_{OM} TG hearts.

A. Representative Western blots showing mitochondrial fission proteins in WT and MCL-1_{OM} TG hearts. **B.** Quantitation of DRP1, FIS1, and MFF (n=5-6, *p<0.05).

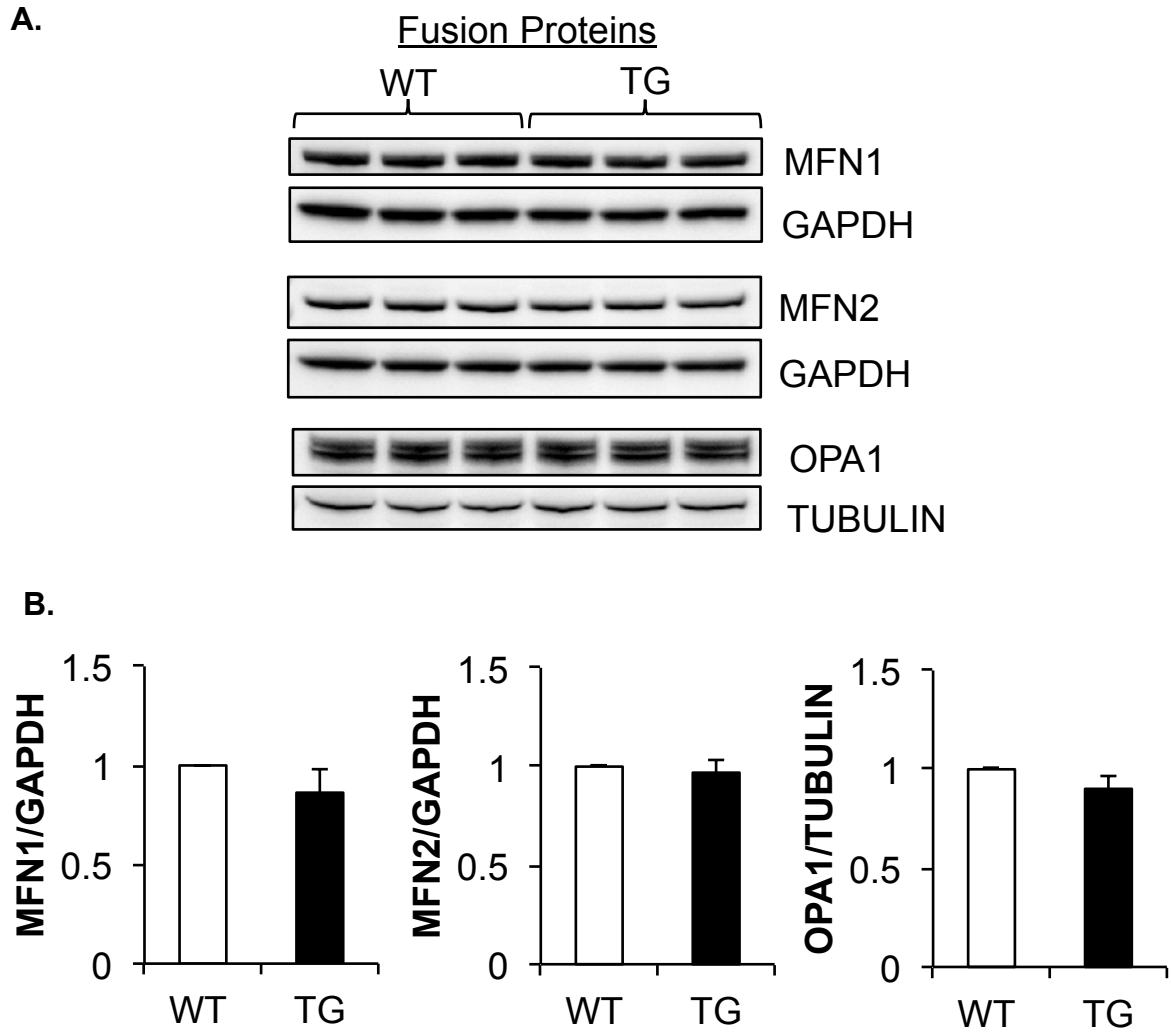


Figure 4.7. Analysis of mitochondrial fusion proteins in MCL-_{OM} TG hearts.

A. Representative Western blots showing mitochondrial fusion proteins in WT and MCL-_{1OM} TG hearts **B.** Quantitation of MFN1, MFN2, and OPA1 (n=5-6).

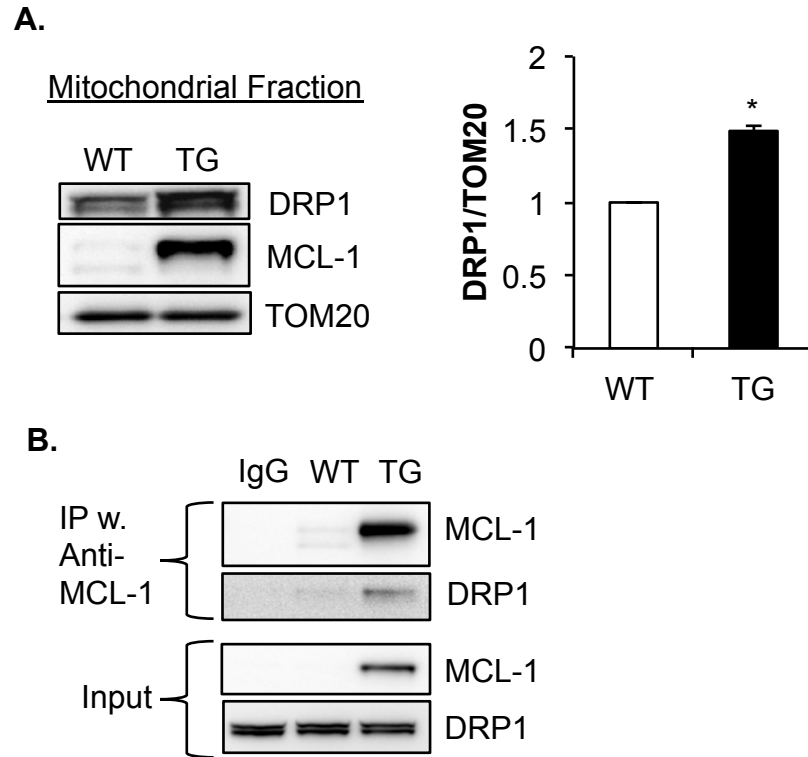


Figure 4.8. DRP1 levels increase at the mitochondria in MCL-1_{OM} TG hearts.

A. Representative Western blot and quantitation of DRP1 levels at the mitochondria in WT and MCL-1_{OM} TG hearts (n=4, *p<0.05). **B.** Immunoprecipitation of MCL-1 reveals an increased interaction between MCL-1 and DRP1 in MCL-1_{OM} TG hearts.

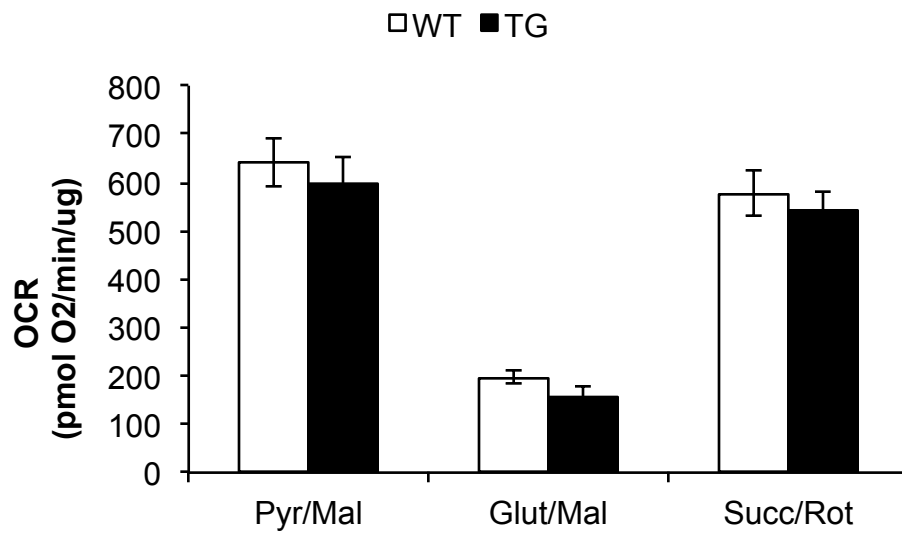


Figure 4.9. There are no significant differences in mitochondrial function in MCL-1_{OM} TG mice.

Mitochondrial respiration was assessed using a Seahorse XFP analyzer in response to three substrates: pyruvate/malate, glutamate/malate, and succinate/rotenone (n=6).

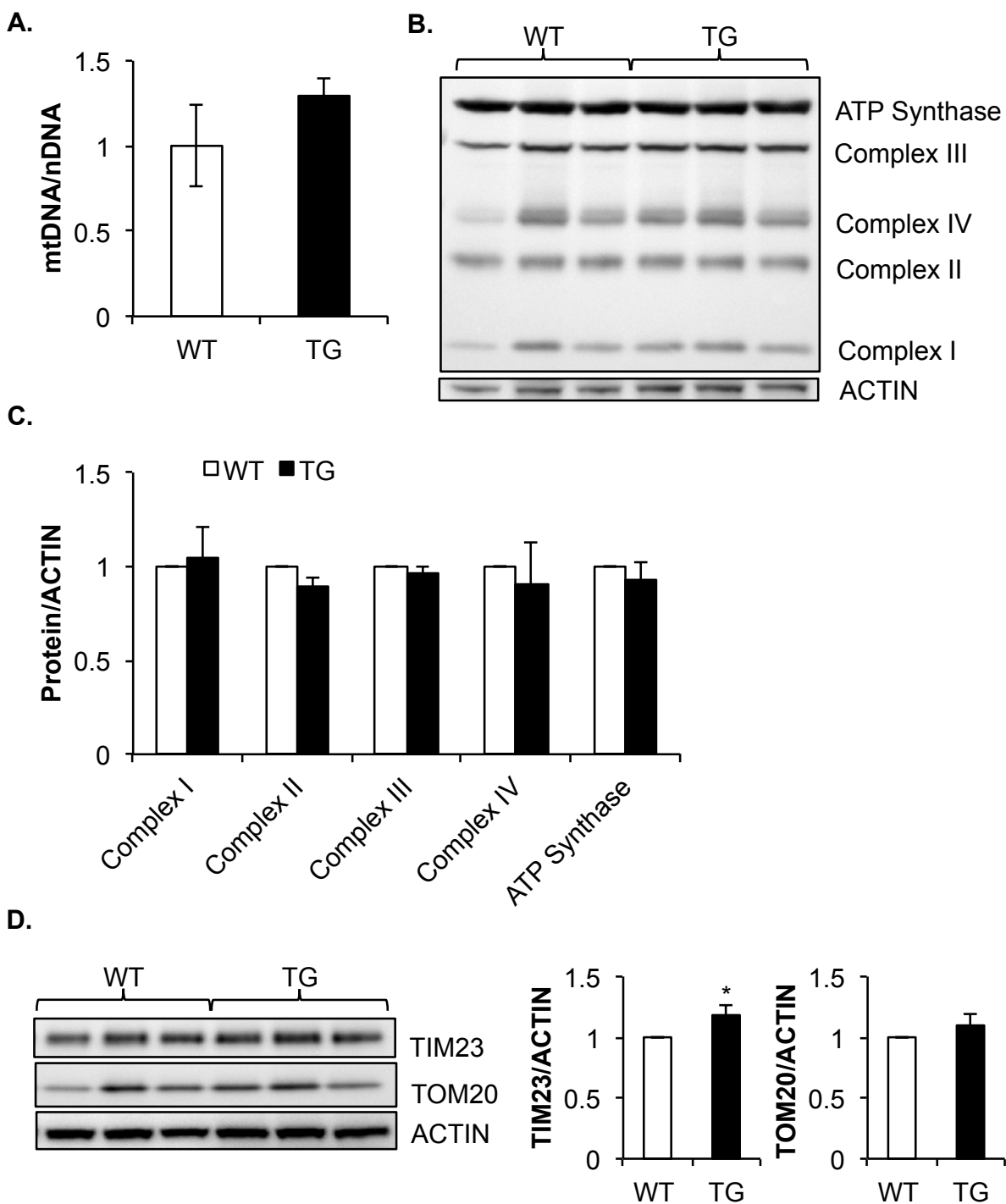


Figure 4.10. There are no differences in mitochondrial content in MCL-1_{OM} TG mice.

A. Quantitation of mitochondrial DNA (mtDNA) content in WT and MCL-1_{OM} TG mice (n=4). **B.** Representative Western blot showing levels of OXPHOS proteins in WT and MCL-1_{OM} TG mice. **C.** Quantitation of mitochondrial OXPHOS proteins (n=5-6). **D.** Representative Western blots and quantitation of mitochondrial markers TIM23 and TOM20 (n=5-6, *p<0.05).

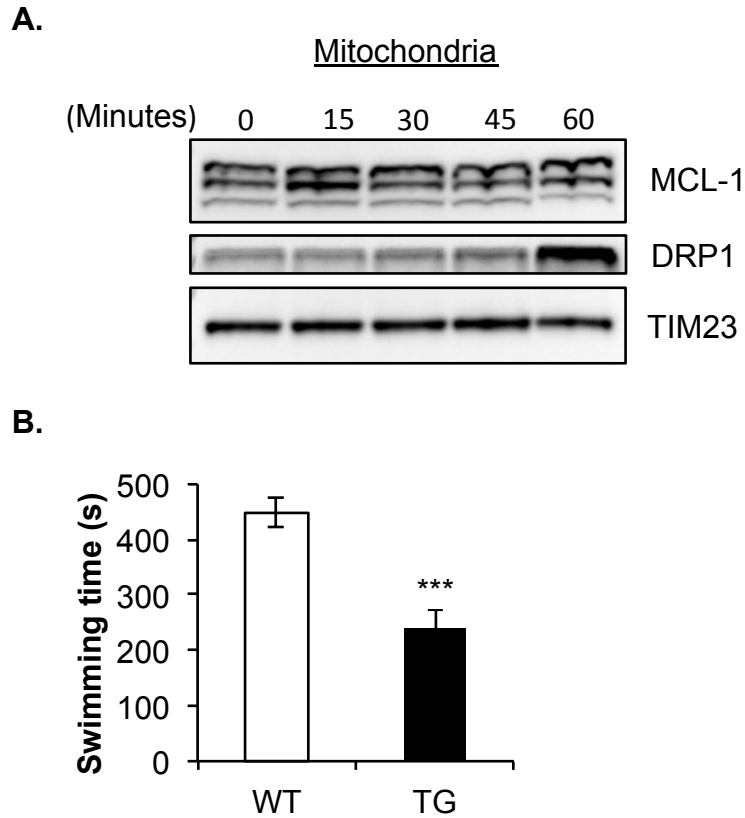


Figure 4.11. MCL-1_{OM} TG mice exhibit reduced exercise capacity in response to the forced swimming test.

A. Representative Western blot showing DRP1 recruitment to mitochondria in response to a swimming time course in WT mice. **B.** Quantitation of swimming time in seconds of WT and MCL-1_{OM} TG mice in response to the forced swimming test (n=7, ***p<0.001).

CHAPTER 5: MCL-1 FACILITATES THE REMOVAL OF DAMAGED MITOCHONDRIA VIA THE MITOPHAGY RECEPTOR BNIP3

5.1 Introduction

Many studies have indicated a link between mitochondrial dynamics and mitophagy. It has been reported that DRP1-mediated mitochondrial fission precedes and facilitates mitophagy (Burman et al., 2017; Kageyama et al., 2014; Lee et al., 2011; Shirakabe et al., 2016; Song et al., 2015a; Twig et al., 2008). Alternatively, mitochondrial fusion has been demonstrated to protect against mitochondrial clearance (Gomes et al., 2011; Rambold et al., 2011). Based on my previous findings that MCL-1 induces DRP1-mediated mitochondrial fission, I wanted to explore whether MCL-1 could facilitate mitophagy of damaged mitochondria. If MCL-1 has dual roles in regulating both mitochondrial fission and clearance this could allow for more efficient removal of damaged mitochondria. Mitophagy can be mediated by both the PINK1/PARKIN pathway and through selective receptors that contain LC3-interacting region (LIR) motifs that allow them to bind directly to the autophagosome (Sciarretta et al., 2018). Here, I investigated whether MCL-1 could facilitate mitophagy and explored the underlying mechanism.

5.2 Results

5.2.1 MCL-1 Promotes Mitophagy

To investigate if MCL-1 has a role in facilitating mitochondrial clearance, I assessed the effect of MCL-1 overexpression in response to treatment with the potent

mitochondrial uncoupler FCCP in MEFs. Since MEFs do not have detectable levels of PARKIN (Ding et al., 2010), they are unable to efficiently clear their mitochondria in response to mitochondrial uncouplers. To assess levels of endogenous MCL-1 in response to FCCP, I treated MEFs with DMSO or 25 μ M FCCP for 2, 4, and 6 hours. Using Western blot analysis, I found that endogenous MCL-1 is rapidly degraded in response to FCCP treatment (Figure 5.1). To determine if MCL-1 overexpression could induce mitophagy, I infected MEFs with adenoviruses encoding β -gal or MCL-1 and treated them with DMSO or 25 μ M FCCP for 24 hours. I assessed mitochondrial clearance by observing levels of the mitochondrial protein TIM23. Interestingly, MCL-1 overexpression led to a significant reduction in TIM23 levels after treatment with FCCP, indicative of enhanced mitochondrial clearance (Figure 5.2A and B). This data suggests that MCL-1 overexpression is able to promote mitochondrial clearance in a PARKIN-independent manner.

Given that MCL-1 was able to induce clearance in the absence of PARKIN, I wanted to investigate the underlying mechanism. Mitophagy receptors are able to facilitate mitochondrial clearance by directly interacting with and binding to LC3 on autophagosomes through their conserved LC3 interacting region (LIR) motifs (Birgisdottir et al., 2013). These LIR motifs are characterized by the presence of a tryptophan, phenylalanine, or tyrosine residue, followed by any two amino acid residues and then either a leucine, isoleucine, or a valine residue (W/F/Y-X-X-L/I/V) (Johansen and Lamark, 2011). To investigate the possibility that MCL-1 could be acting as a mitophagy receptor, I screened MCL-1's amino acid sequence and identified three potential conserved LIR motifs (242-WGRI-245; 227-FSRV-230; 299-FFHV-302) (Figure

5.3). To determine if these LIR motifs were functional, I generated four different MCL-1 mutants with point mutations in the different LIR motifs. Specifically, I generated a mutant with tryptophan 242 and isoleucine 254 mutated to alanine (MCL-1-LIR1), a mutant with phenylalanine 227 and valine 230 mutated to alanine (MCL-1-LIR2), a mutant with phenylalanine 299 and valine 302 mutated to alanine (MCL-1-LIR3), and in case the different motifs could functionally compensate for each other, I also generated a mutant with all six aforementioned point mutations (MCL-1-LIR123; Figure 5.3). To evaluate mitophagy, I used fluorescence microscopy to assess co-localization between mitochondria and LC3-positive autophagosomes in MEFs overexpressing MCL-1. MEFs were transfected with GFP-LC3 and either HA, HA-MCL-1, HA-MCL-1-LIR2, or HA-MCL-1-LIR3 and in a separate experiment transfected with GFP-LC3 and either HA, HA-MCL-1, HA-MCL-1-LIR1, and HA-MCL-1-LIR123. MEFs were then treated with DMSO or 25 μ M FCCP for 2 hours, fixed and stained with TOM20 antibody as a mitochondrial marker. I confirmed that overexpression of MCL-1 led to a significant increase in co-localization between GFP-LC3 positive autophagosomes and mitochondria, indicative of mitophagy (Figures 5.4A and B and 5.5A and B). I found that individual mutation of MCL-1's potential LIR motifs did not have a significant effect on MCL-1-mediated mitophagy (Figures 5.4A and B and 5.5A and B). Interestingly, only the MCL-1-LIR123 mutant had a small, but significant, reduction in co-localization between LC3 puncta and mitochondria (Figure 5.5A and B). Based on these findings, I evaluated the interaction between the MCL-1-LIR123 mutant and LC3 II using co-immunoprecipitation. I transfected MEFs with HA, HA-MCL-1, or HA-MCL-1-LIR123 and treated them with DMSO or 25 μ M FCCP for 1 hour. I immunoprecipitated MCL-1

and found that the MCL-1-LIR123 mutant did not show reduced interaction with LC3 II compared to MCL-1 (Figure 5.6). Taken together, this data suggests that MCL-1 likely does not directly interact with LC3 on the autophagosome via its LIR motifs to promote mitophagy.

5.2.2 MCL-1 Promotes Mitochondrial Clearance in Response to Hypoxia Independently of its Anti-Apoptotic Activity

My previous data suggested that MCL-1 was likely not functioning as a mitophagy receptor, so I wanted to investigate the possibility that it was facilitating clearance through interaction with a known mitophagy receptor on the outer mitochondrial membrane instead. The well-known mitophagy receptors BNIP3 and NIX reside in the outer mitochondrial membrane and can promote mitophagy in response to stress induced by hypoxia (Bellot et al., 2009; Hanna et al., 2012; Novak et al., 2010). Based on these findings, I wanted to determine if MCL-1 could also promote mitophagy in response to hypoxia. MEFs were infected with adenoviruses encoding β -gal or MCL-1 and subjected to normoxia or hypoxia for 24 hours. I used Western blot analysis of the mitochondrial marker TIM23 to assess mitochondrial content. MCL-1 overexpression led to a significant reduction in TIM23 levels in response to hypoxia, confirming that MCL-1 can enhance hypoxia-mediated mitophagy (Figure 5.7A and B).

Next, I wanted to determine if MCL-1's ability to induce mitophagy in response to hypoxia was related to its anti-apoptotic activity. Since I had previously found that MCL-1-induced perinuclear clustering of mitochondria required a functional BH3-domain, I also wanted to assess whether it was necessary for MCL-1 to induce mitophagy. To

explore this, MEFs were infected with Ad- β -gal, Ad-MCL-1, or Ad-MCL-1-BH3-mutant and subjected to normoxia or hypoxia for 24 hours. Mitochondrial clearance was assessed by monitoring levels of the mitochondrial protein TIM23 using Western blot analysis. Similar to Ad-MCL-1, overexpression of the MCL-1-BH3-mutant resulted in a significant reduction in TIM23 levels in response to hypoxia (Figure 5.8A and B). This suggests that MCL-1's ability to facilitate mitochondrial clearance does not require a functional BH3 domain and is independent of its anti-apoptotic activity.

5.2.3 MCL-1 Interacts with the Mitophagy Receptor BNIP3

Since MCL-1 was able to enhance hypoxia-mediated mitophagy and BNIP3 is upregulated in response to hypoxia (Bellot et al., 2009; Drake et al., 2017), I wanted to investigate whether MCL-1 may be interacting with BNIP3 to facilitate mitochondrial clearance. To confirm that BNIP3 was upregulated in MEFs in response to hypoxia, MEFs were subjected to control or hypoxia for 2, 4, 8, 12, or 24 hours. Levels of MCL-1 and BNIP3 were assessed by Western blot analysis. BNIP3 levels were significantly increased following 8, 12, and 24 hours of hypoxia, whereas MCL-1 levels were significantly reduced after 24 hours of hypoxia (Figure 5.9A and B).

Next, I wanted to determine if I could detect an interaction between MCL-1 and BNIP3 during hypoxia. MEFs were transfected with controls, MCL-1, and BNIP3 and subjected to normoxia or hypoxia for 2, 4, 6, 8, or 12 hours. An antibody against Myc was used to immunoprecipitate BNIP3, and MCL-1 and BNIP3 were assessed by Western blot analysis. MCL-1 and BNIP3 interact at baseline and this interaction is increased in response to 2 hours of hypoxia (Figure 5.10). However, as the time course

of hypoxia progresses, the interaction between MCL-1 and BNIP3 decreases (Figure 5.10).

Since I had previously shown that MCL-1 promotes mitophagy in response to FCCP, I wanted to determine if MCL-1 and BNIP3 also interact in response to FCCP treatment. MEFs were transfected with controls, MCL-1, and BNIP3 and treated with DMSO or 25 μ M FCCP for 2, 4, 6, 8, or 12 hours. An antibody against Myc was used to immunoprecipitate BNIP3, and MCL-1 and BNIP3 were assessed by Western blot analysis. Interestingly, the interaction between MCL-1 and BNIP3 in response to FCCP treatment also increased after 2 hours and then decreased as the time course progressed (Figure 5.11).

5.2.4 MCL-1 and BNIP3 Increase in the Border Zone and Remote Zone After MI

Given that my *in vitro* data suggested that MCL-1 interacts with and may facilitate clearance through the known mitophagy receptor BNIP3, I wanted to assess whether this also occurred in response to stress *in vivo*. I used myocardial infarction (MI) to induce mitochondrial damage and mitophagy (Kubli et al., 2013). To determine the levels of endogenous MCL-1_{OM} and BNIP3 in response to MI, I subjected mice to myocardial infarction by ligating the left anterior descending artery (LAD) and then assessed protein levels in a time course of 0, 4, 8, and 24 hours. I found that both MCL-1 and BNIP3 protein levels were significantly increased by 24 hours post-infarction in the border zone (Figure 5.12A and B). I saw a similar trend in the remote zone, where MCL-1 and BNIP3 levels gradually increased after the infarction (Figure 5.13A and B).

These results suggest that both MCL-1 and BNIP3 play an important role in stress response *in vivo*.

5.3 Discussion

My data suggests a novel function for MCL-1 in facilitating mitochondrial clearance. This role has not been previously explored because endogenous MCL-1 is unable to promote mitochondrial turnover in MEFs. This is likely due to the fact that MCL-1 is rapidly degraded in response to severe stress. However, I found that MCL-1 overexpression led to enhanced mitophagy in response to stress induced by both the potent mitochondrial uncoupler FCCP and the more physiological stimuli hypoxia. My data indicates that MCL-1-mediated mitochondrial clearance is occurring independently of PARKIN and suggests the MCL-1 is not acting as a mitophagy receptor. While individually mutating MCL-1's LIR motifs showed no significant differences in MCL-1's ability to induce mitophagy, this could be accounted for by functional compensation. However, while mutating all of the LIR motifs led to a slight decrease in mitophagy, it did not affect the ability of MCL-1 to interact with LC3 II in response to stress. This could be because the point mutations may interfere with MCL-1's functions. To more directly verify that MCL-1's LIR motifs are not required for induction of mitophagy, additional experiments comparing the ability of these mutants to facilitate clearance in response to FCCP and hypoxia via Western blot must be done.

Based on my results, it is more likely that MCL-1 is promoting mitophagy through a known mitophagy receptor, such as BNIP3. My data clearly demonstrates that MCL-1 and BNIP3 interact in response to stress induced by both hypoxia and FCCP. However,

the nature of this interaction needs to be further elucidated given that MCL-1 and BNIP3 are also involved in regulating apoptosis (Chen et al., 2016). It is still unknown what causes BNIP3 to switch from a mitophagy activator to a pro-death protein (Gustafsson, 2011), but there is likely a distinct temporal response. Thus, MCL-1 and BNIP3 may interact to remove aberrant mitochondria in response to acute cellular stress, but chronic stress may cause BNIP3 to switch to a pro-apoptotic protein and lead to a reduction in MCL-1. It will be important to clarify the relationship between MCL-1 and BNIP3 in regulating mitophagy and cell death moving forward.

MI is a relevant stress model for my studies because it results in both hypoxia and mitochondrial damage (Gomes et al., 2013; Siasos et al., 2018). My data demonstrates that both MCL-1 and BNIP3 significantly increase following MI, suggesting the possibility that they interact to facilitate mitophagy. Interestingly, BNIP3's significant increase at 24 hours is a distinct temporal response from when general autophagy is activated, which normally occurs only 4 hours after MI in the border zone (Kubli et al., 2013). Given this, it may be hard to decipher whether MCL-1's relationship with BNIP3 is involved in facilitating mitochondrial clearance or if it is instead part of its anti-apoptotic role. Further studies are necessary to clarify the relationship between MCL-1 and BNIP3 in response to MI and determine if they play protective roles together or separately.

Parts of Chapter 5 are currently being prepared for submission for publication. Moyzis, Alexandra; Lally, Navraj; Leon, Leonardo; Shires, Sarah; Orogo, Amabel; Liang, Wenjing; Najor, Rita, Gustafsson, Åsa. "Mcl-1 on the outer mitochondrial membrane

promotes Drp1-mediated mitochondrial fission and affects exercise capacity in the heart.” The dissertation author was the primary investigator and author of this paper.

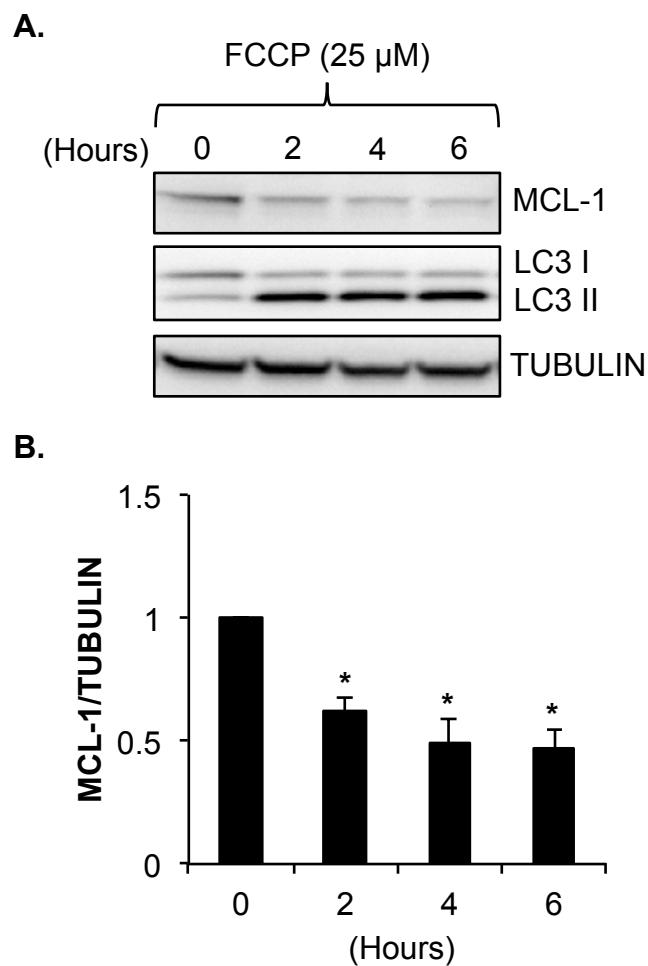


Figure 5.1. Endogenous MCL-1 is degraded in response to FCCP treatment.

A. Representative Western blot showing levels of MCL-1 and LC3 in MEFs in response to treatment with DMSO or 2, 4, or 6 hours of 25 μ M FCCP. **B.** Quantitation of MCL-1 levels (* p <0.05, n =3).

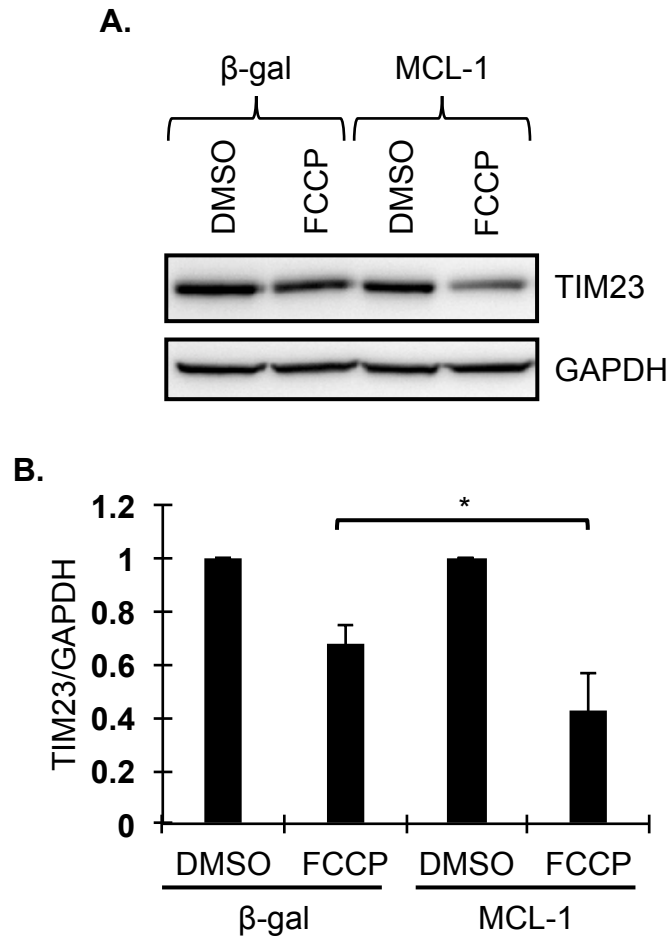


Figure 5.2. MCL-1 overexpression promotes mitochondrial clearance in response to FCCP.

A. Western blot analysis of the mitochondrial marker TIM23 after MEFs were infected with Ad-β-gal or Ad-HA-MCL-1 and treated with DMSO or 25 μM FCCP for 24 hours. GAPDH was used as a loading control. **B.** Quantitation of TIM23 levels (* $p < 0.05$, $n = 3$).

LC3-Interacting Region (LIR) Motifs: **W/F/Y-X-X-L/I/V**

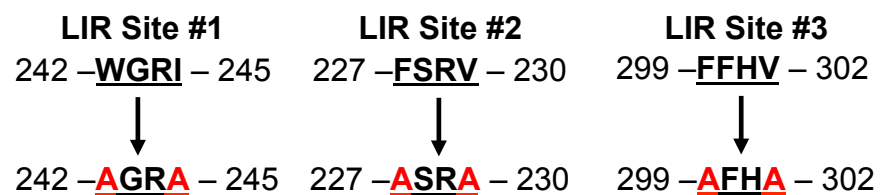


Figure 5.3. Mutation in MCL-1's potential conserved LC3-Interacting Region (LIR) motifs.

Figure 5.4. Individual mutation of MCL-1's LIR2 and LIR3 motifs does not affect MCL-1-mediated mitophagy.

A. MEFs were transfected with GFP-LC3 and either HA, HA-MCL-1, HA-MCL-1-LIR2, or HA-MCL-1-LIR3. Cells were treated with DMSO or 25 μ M FCCP for 2 hours and then stained for the TOM20 as a mitochondrial marker (red). Colocalization between mitochondria and GFP-LC3 positive vesicles was assessed by immunofluorescence microscopy. Scale bar: 10 μ m **B.** Quantitation of the number of GFP-LC3 puncta colocalized with mitochondria (n.s., not significant; n=3).

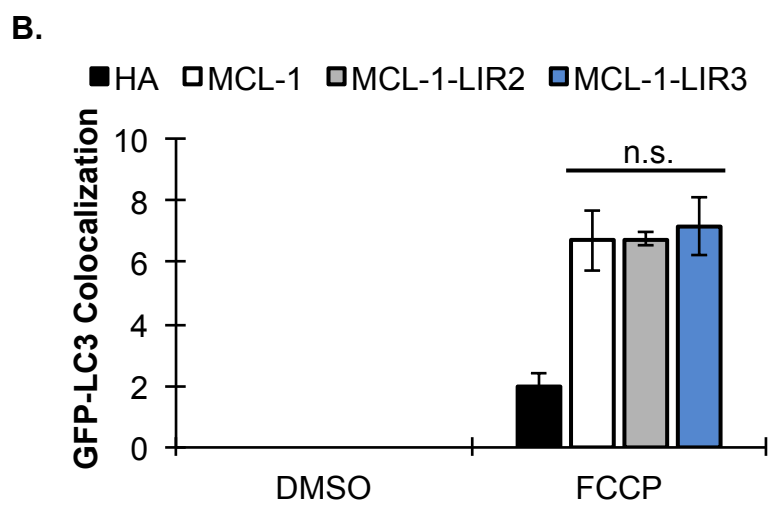
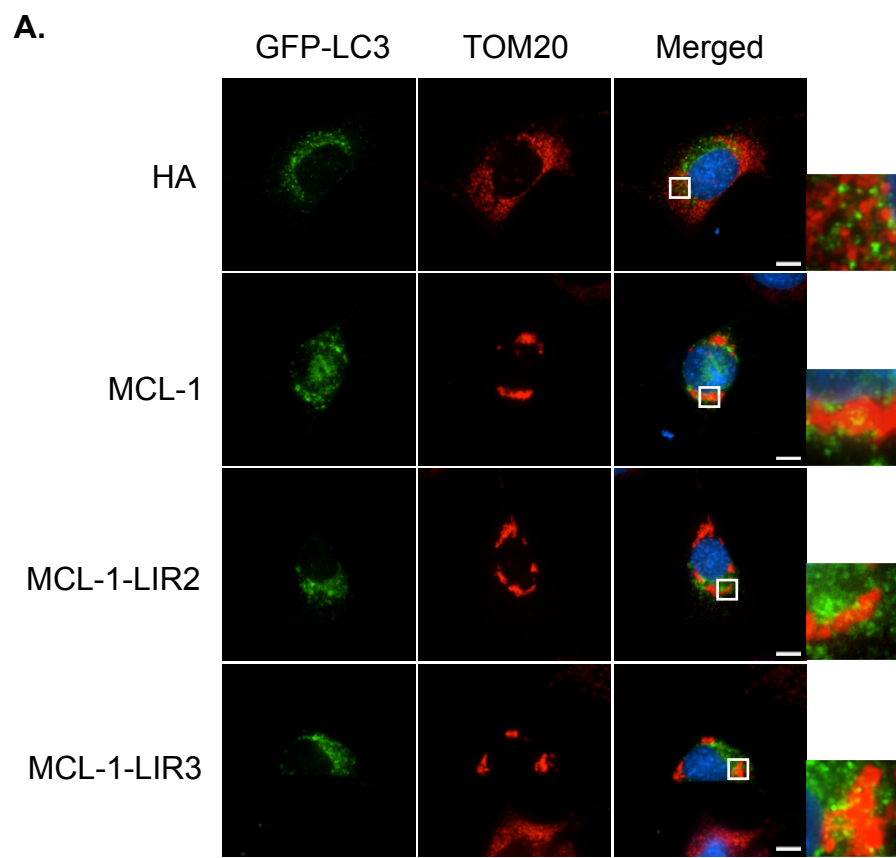
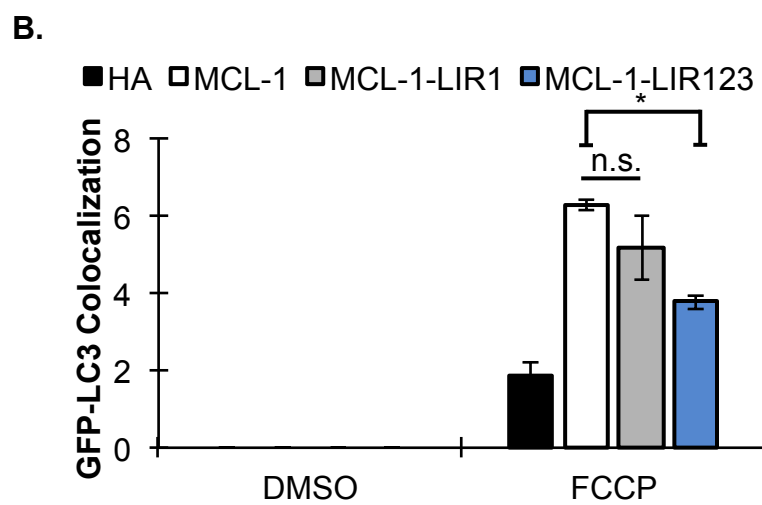
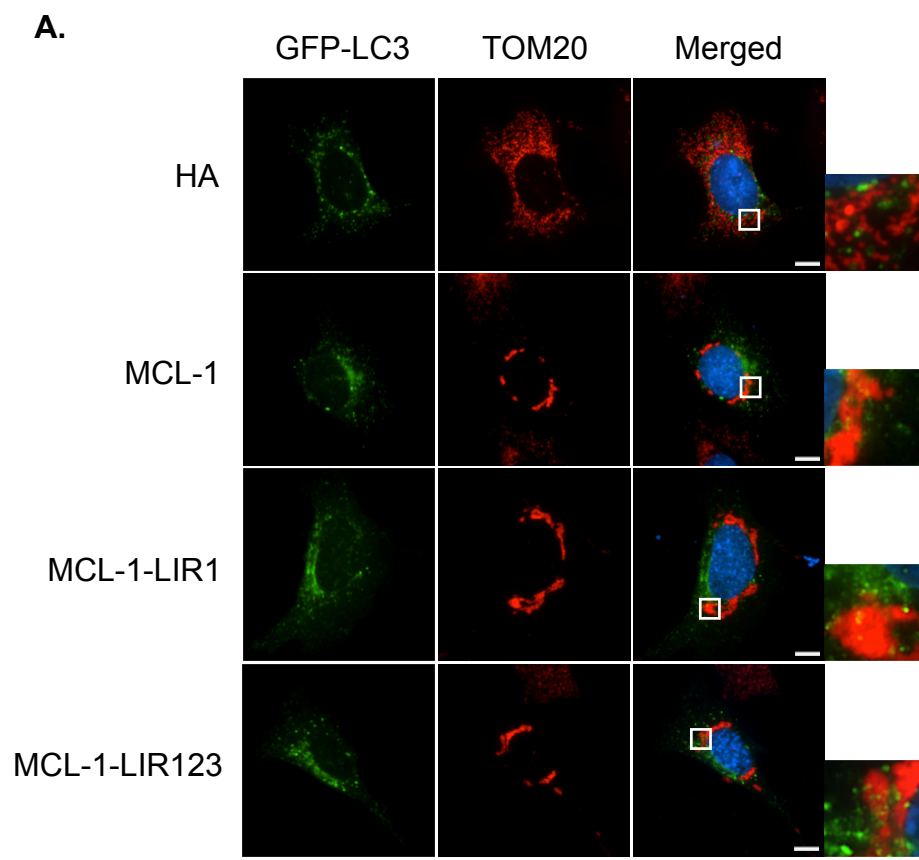


Figure 5.5. Mutating all of MCL-1's LIR motifs leads to reduced mitophagy.

A. MEFs were transfected with GFP-LC3 and either HA, HA-MCL-1, HA-MCL-1-LIR1, or HA-MCL-1-LIR123. Cells were treated with DMSO or 25 μ M FCCP for 2 hours and then stained for the TOM20 as a mitochondrial marker (red). Colocalization between mitochondria and GFP-LC3 positive vesicles was assessed by immunofluorescence microscopy. Scale bar: 10 μ m **B.** Quantitation of the number of GFP-LC3 puncta colocalized with mitochondria (* p <0.05; n.s., not significant; n =3).



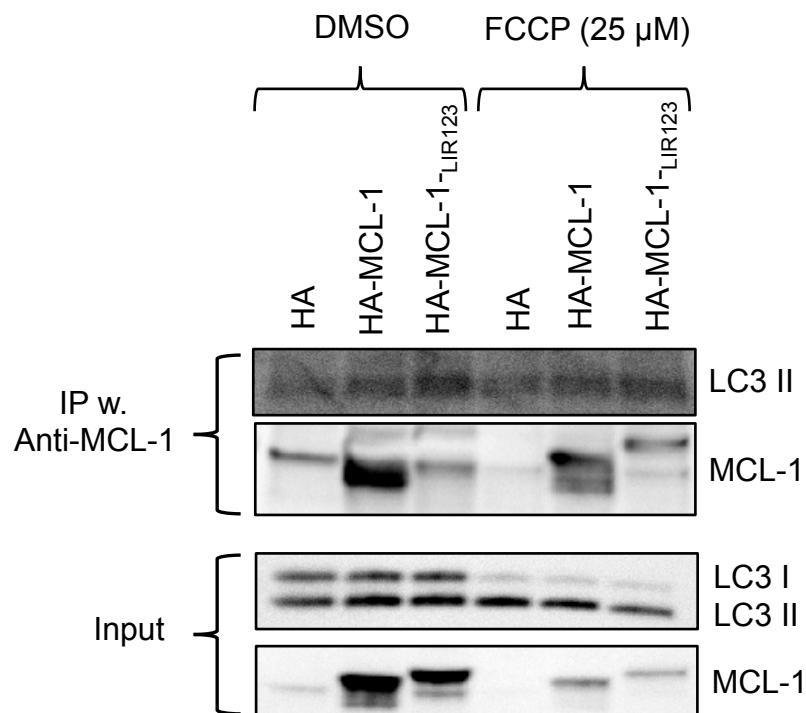


Figure 5.6. Mutation of all three of MCL-1's LIR motifs does not reduce MCL-1's interaction with endogenous LC3.

MEFS were transfected with HA, HA-MCL-1, or HA-MCL-1-LIR123 and treated with DMSO or 25 μ M FCCP for 1 hour. MCL-1 was immunoprecipitated and interaction between MCL-1 and LC3 II was assessed via Western blot analysis.

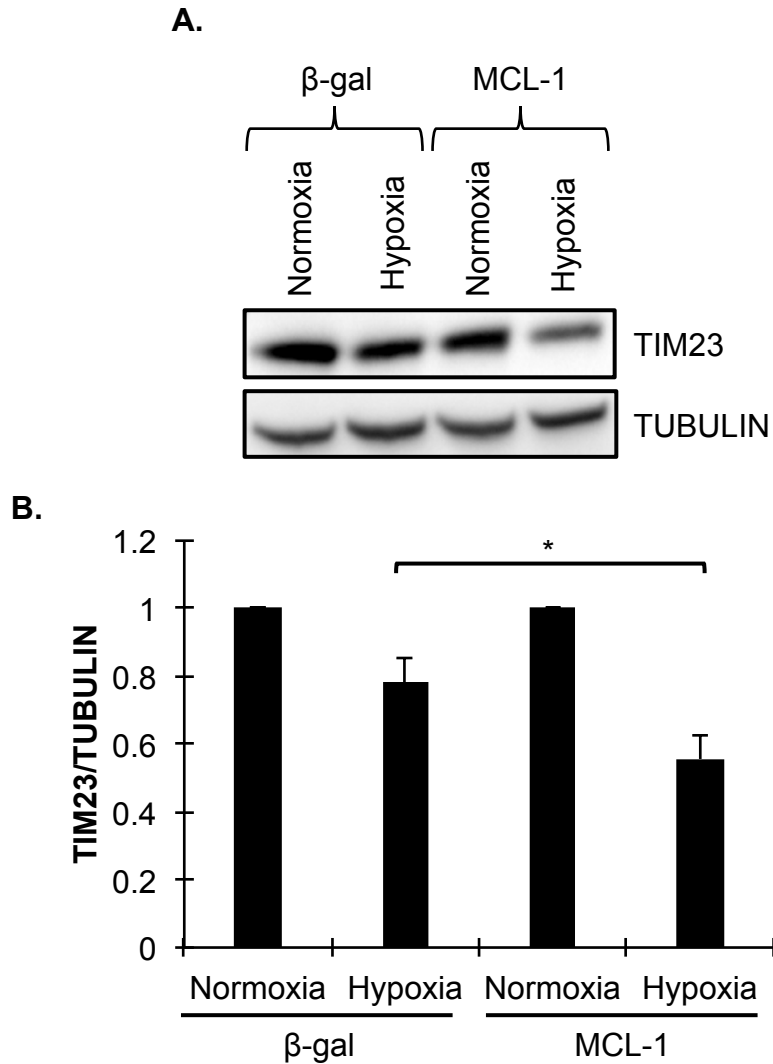


Figure 5.7. MCL-1 promotes mitochondrial clearance in response to hypoxia.

A. Western blot analysis of the mitochondrial marker TIM23 after MEFs were infected with Ad- β -gal or Ad-HA-MCL-1 and subjected to normoxia or hypoxia for 24 hours. TUBULIN was used as a loading control. **B.** Quantitation of TIM23 levels (* p <0.05, n =4).

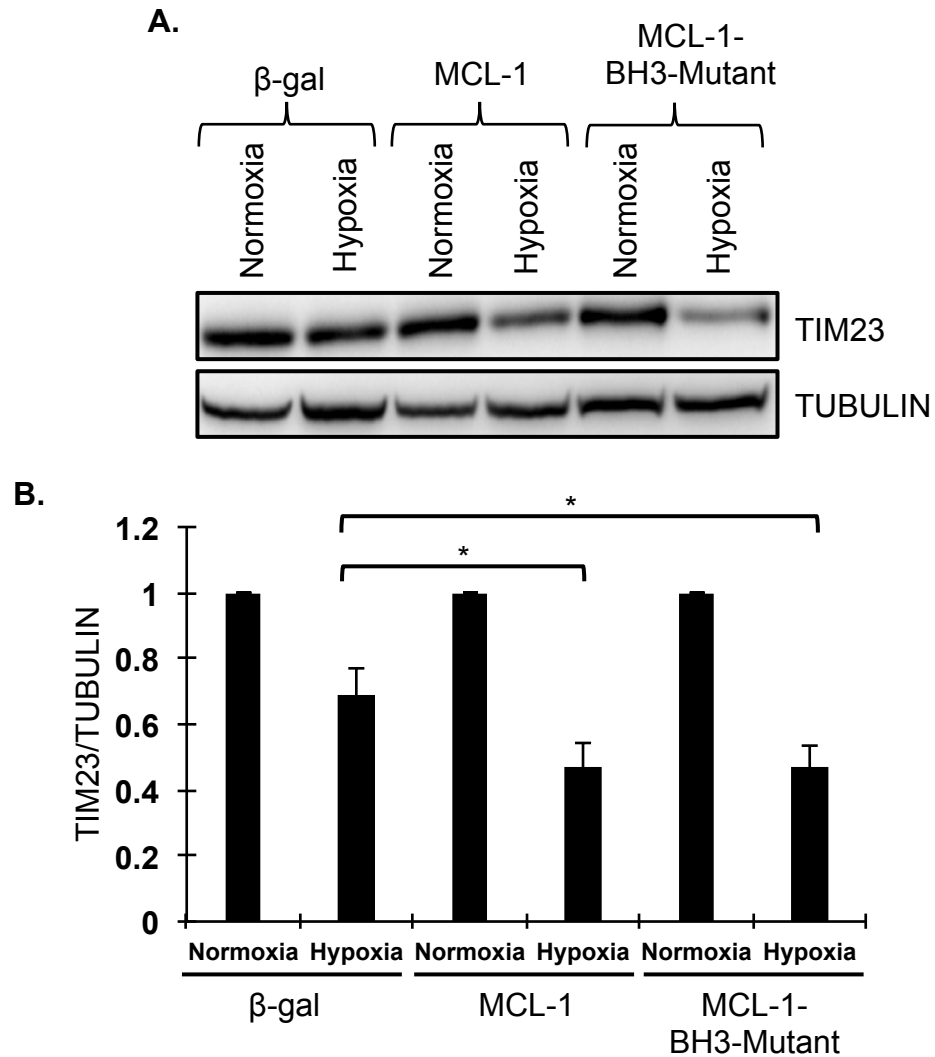


Figure 5.8. Mutation of MCL-1's BH3 domain does not affect MCL-1-mediated mitochondrial clearance.

A. Western blot analysis of the mitochondrial marker TIM23 after MEFs were infected with Ad-β-gal, Ad-HA-MCL-1, or Ad-HA-MCL-1-BH3-mutant and subjected to normoxia or hypoxia for 24 hours. TUBULIN was used as a loading control. **B.** Quantitation of TIM23 levels (* $p < 0.05$, $n = 4$).

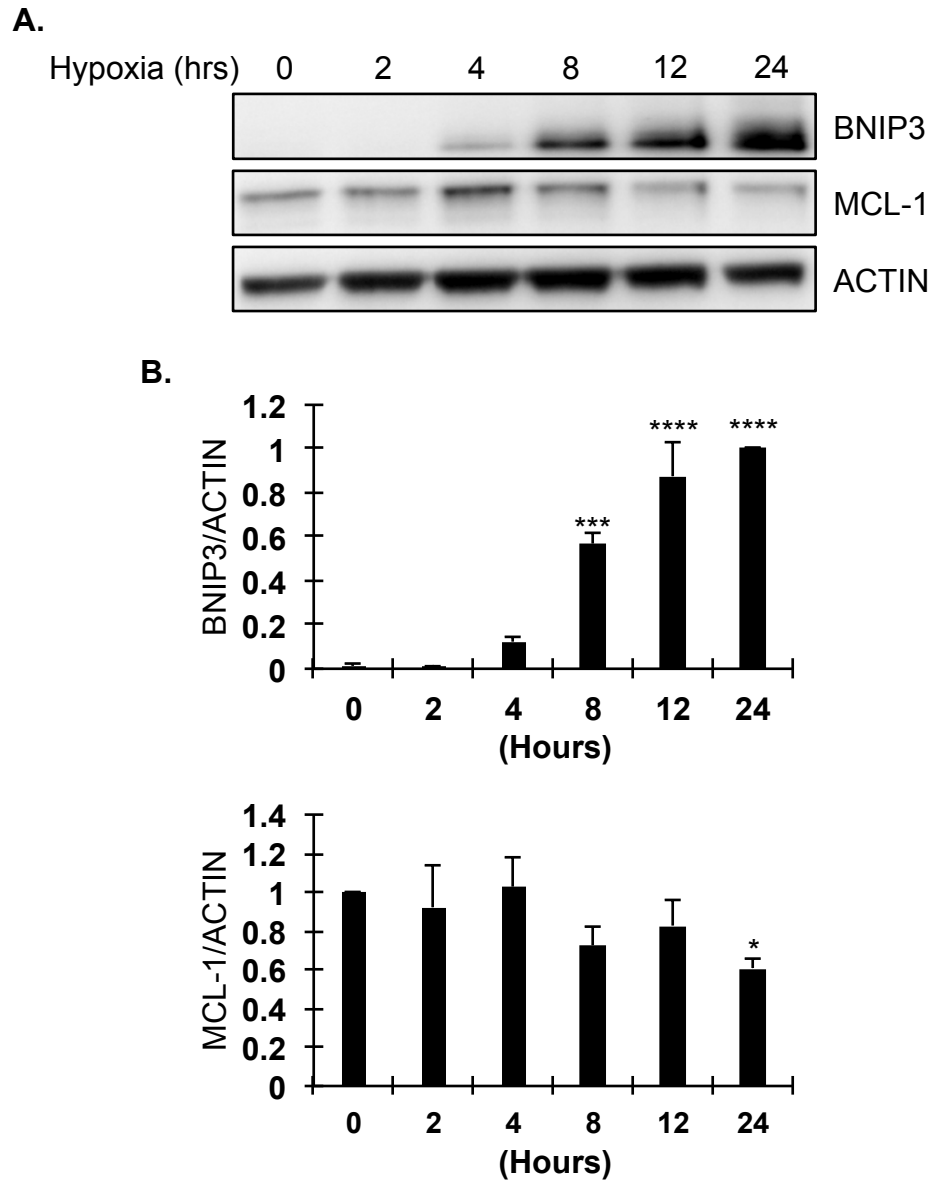


Figure 5.9. BNIP3 levels significantly increase in response to hypoxia.

A. Western blot analysis of BNIP3 and MCL-1 after MEFs were subjected to hypoxic conditions for 0, 2, 4, 8, 12, and 24 hours. ACTIN was used as a loading control. **B.** Quantitation of BNIP3 and MCL-1 levels (* $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$; $n = 4$).

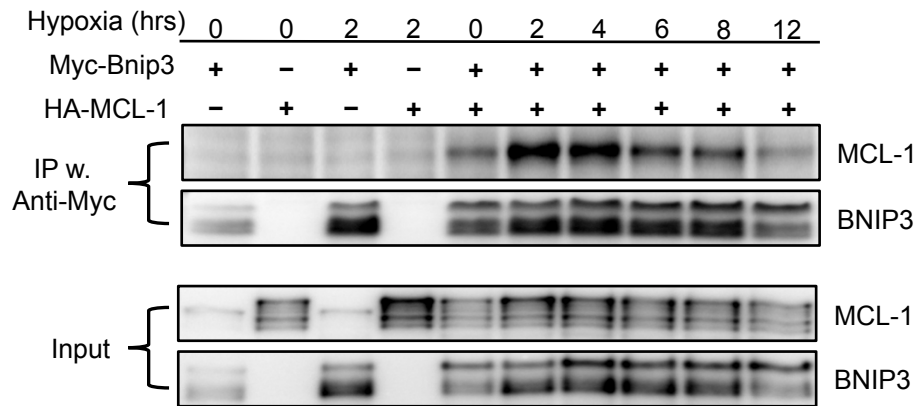


Figure 5.10. Co-immunoprecipitation of HA-MCL-1 with Myc-BNIP3 in response to hypoxia.

HA-MCL-1 and Myc-BNIP3 were co-overexpressed in MEFs. Cells were subjected to normoxia or hypoxia for 2, 4, 6, 8, or 12 hours and immunoprecipitation was performed using an anti-Myc antibody. Representative Western blot from 3 independent experiments.

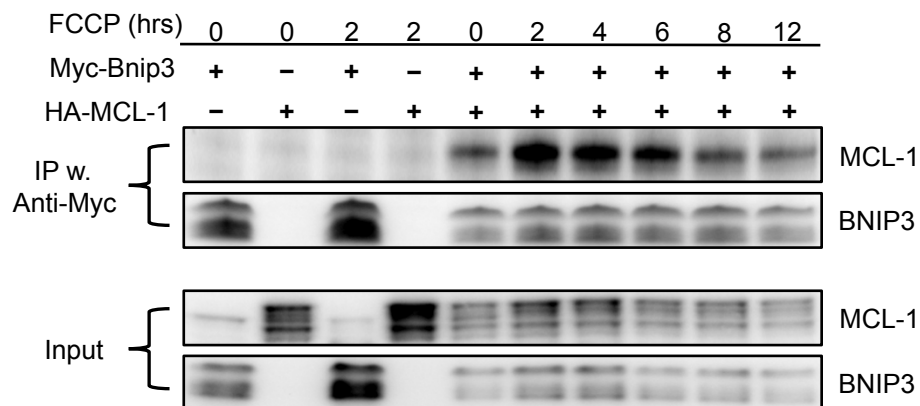


Figure 5.11. Co-immunoprecipitation of HA-MCL-1 with Myc-BNIP3 in response to FCCP treatment.

HA-MCL-1 and Myc-BNIP3 were co-overexpressed in MEFs. Cells were treated with DMSO or 25 μ M FCCP for 2, 4, 6, 8, or 12 hours and immunoprecipitation was performed using an anti-Myc antibody. Representative Western blot from 3 independent experiments.

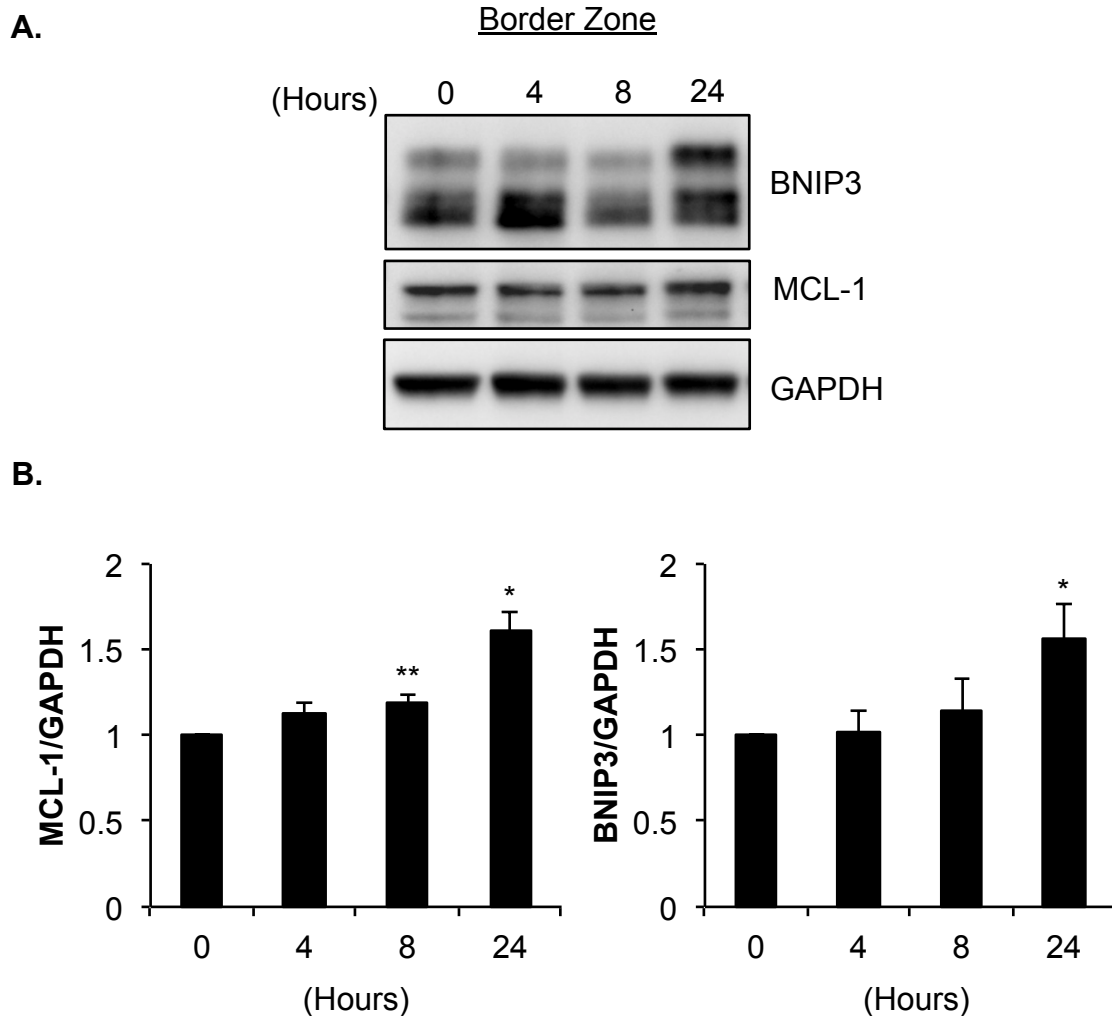


Figure 5.12. Endogenous MCL-1 and BNIP3 levels increase in the border zone after myocardial infarction (MI).

A. Representative Western blot showing MCL-1 and BNIP3 levels in the border zone 0, 4, 8, and 24 hours after permanent ligation of the LAD. **B.** Quantitation of MCL-1 and BNIP3 levels in the border zone region (n=7-8, *p<0.05, **p<0.01 vs. 0 hour).

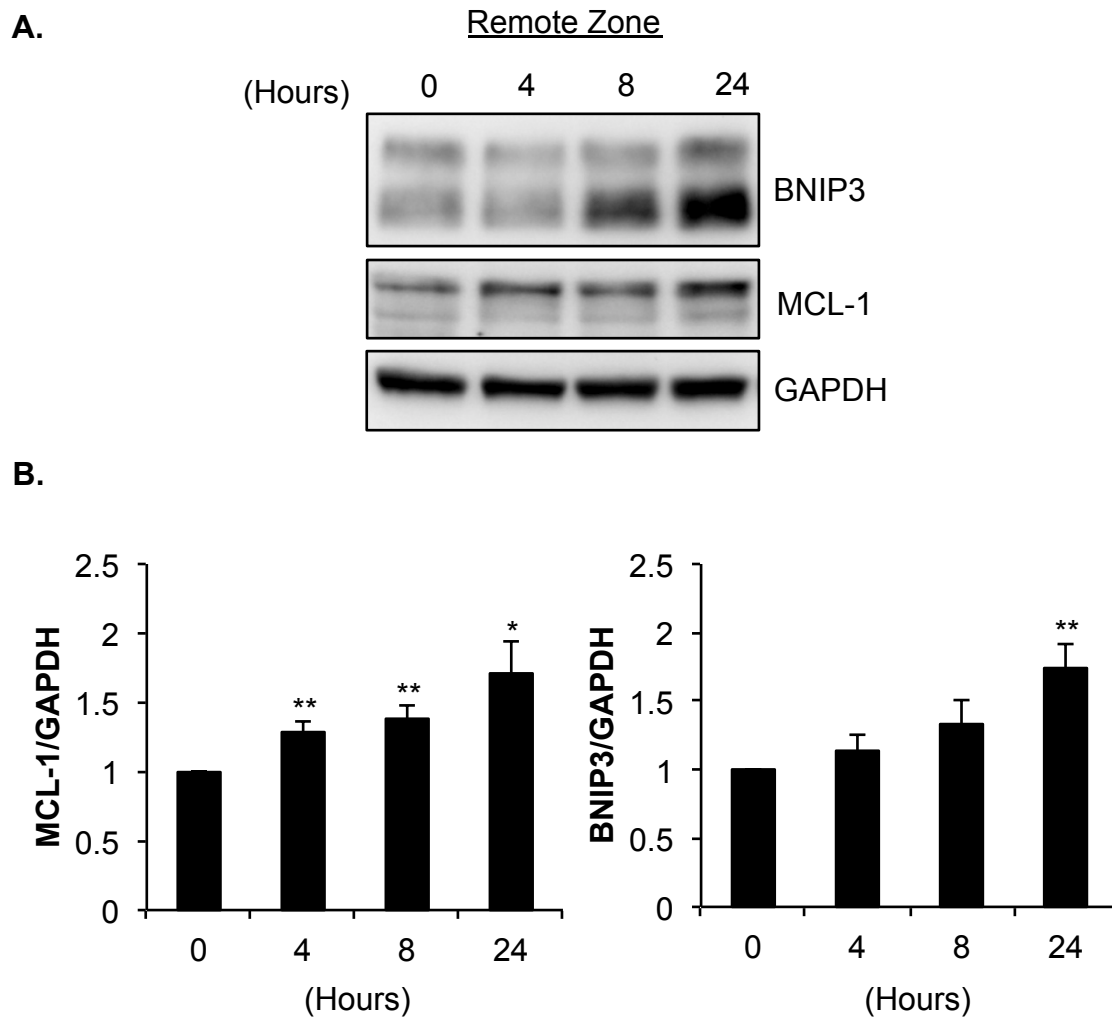


Figure 5.13. Endogenous MCL-1 and BNIP3 levels increase in the remote zone after myocardial infarction (MI).

A. Representative Western blot showing MCL-1 and BNIP3 levels in the remote zone 0, 4, 8, and 24 hours after permanent ligation of the LAD. **B.** Quantitation of MCL-1 and BNIP3 levels in the remote zone region (n=7-8, *p<0.05, **p<0.01 vs. 0 hour).

CHAPTER 6: THE EFFECT OF MCL-1 OVEREXPRESSION ON AUTOPHAGY

6.1 Introduction

Although my results suggest that MCL-1 facilitates mitochondrial clearance in response to stress, anti-apoptotic BCL-2 proteins can also inhibit general autophagy by binding to the autophagy protein BECLIN1 through their BH3 domains (Maiuri et al., 2007; Pattingre et al., 2005). In order for autophagy to proceed, this interaction needs to be disrupted for BECLIN1 to be released. While this has been predominantly characterized with BCL-2, both BCLX_L and MCL-1 have also been reported to inhibit autophagy (Maiuri et al., 2007; Tai et al., 2013). MCL-1 can act as a negative regulator of autophagy in cortical neurons (Germain et al., 2011), but whether MCL-1 inhibits autophagy in the heart still needs to be investigated. Thus, I wanted to confirm that MCL-1 inhibits general autophagy *in vitro* and assess the effect of overexpressing MCL-1 on autophagy in the heart *in vivo*. I also wanted to determine whether MCL-1's ability to inhibit autophagy varied with different types of stressors. I was particularly interested in comparing stress that induces general autophagy versus stress that elicits mitochondrial damage and mitophagy.

6.2 Results

6.2.1 MCL-1 Inhibits Rapamycin-Induced Autophagy

To confirm reports that MCL-1 inhibits general autophagy, I assessed the effect of MCL-1 overexpression in response to rapamycin treatment. Rapamycin inhibits mTOR, which is a negative regulator of autophagy (Li et al., 2014). I infected MEFs

with either Ad- β -gal or Ad-MCL-1 and treated them with DMSO or 100 nM Rapamycin for 1 and 3 hours. To assess autophagy, I monitored LC3 II levels via Western blot analysis. Conversion of cytosolic LC3 I to the active autophagosome-bound LC3 II correlates with the increase in autophagosome formation (Kabeya et al., 2000). In the Ad- β -gal-infected cells I observed a significant increase in LC3 II levels indicative of enhanced autophagy after both 1 and 3 hours (Figure 6.1A and B). However, the rapamycin failed to significantly increase LC3 II at either 1 or 3 hours in cells overexpressing MCL-1, suggesting that autophagosome formation is not occurring (Figure 6.1A and B). These findings indicate that MCL-1 overexpression is able to suppress initiation of autophagy in MEFs in response to mTOR inhibition.

6.2.2 MCL-1 does not Inhibit Autophagy in Response to FCCP Treatment

Unlike the general mechanism of action employed by rapamycin, FCCP induces autophagy by causing the mitochondria to depolarize. Given that MCL-1 overexpression promoted clearance in response to FCCP, I investigated its effect on autophagosome formation and induction of autophagy under these conditions. I infected MEFs with either Ad- β -gal or Ad-MCL-1 and treated them with DMSO or 25 μ M FCCP for 6, 12, and 24 hours. I assessed levels of LC3 II via Western blot analysis. I found that autophagosome formation significantly increased after 6 and 12 hours of FCCP treatment in cells overexpressing β -gal (Figure 6.2A and B). Similarly, I observed that LC3 II levels also significantly increased at 6 and 12 hours in cells overexpressing MCL-1 (Figure 6.2A and B). There was no significant difference between the increase in LC3 II levels observed in cells infected with β -gal or MCL-1, suggesting that MCL-1

overexpression does not inhibit autophagosome formation in response to stress induced by FCCP. These results indicate that MCL-1 does not negatively regulate autophagy in response to all stressors and suggests that it varies based on distinct mechanisms of action.

6.2.3 MCL-1_{OM} TG Mice do not Show Any Alterations in Autophagy Proteins or Autophagic Flux Under Baseline Conditions

Based on my *in vitro* findings, I also investigated the role of MCL-1 overexpression on autophagy *in vivo*. First, I determined whether MCL-1_{OM} overexpression in the heart had an effect on autophagic activity at baseline. To assess autophagy, I monitored levels of the autophagy markers LC3 and p62. In addition to LC3, the adaptor protein p62 is also used to evaluate autophagic activity because it is normally degraded by the autophagosome-lysosomal pathway and impaired or reduced autophagy leads to its accumulation in cells (Komatsu et al., 2007). I found no significant alterations in the levels of the autophagy proteins LC3 and p62 in the MCL-1_{OM} TG mice under baseline conditions (Figure 6.3A and B). This suggests that there are no inherent effects of MCL-1 overexpression on the initiation and progression of the autophagy pathway. To further confirm that MCL-1_{OM} has little effect on autophagy at baseline, I assessed autophagic flux in these hearts. Autophagic flux is a measure of autophagic degradation activity and reveals how quickly turnover of autophagosomes is occurring (Loos et al., 2014). I injected WT and MCL-1_{OM} TG mice with chloroquine, which is a compound that causes increased LC3 II levels by decreasing autophagosome-lysosome fusion (Mauthe et al., 2018). I found that WT and MCL-1_{OM}

TG hearts showed a similar increase in LC3 II levels after chloroquine injections, indicating that autophagic flux is intact in hearts overexpressing MCL-1_{OM} (Figure 6.4A and B).

6.2.4 MCL-1_{OM} TG Mice Exhibit Reduced Autophagy in Response to Fasting

Next, I wanted to assess the effect of MCL-1_{OM} overexpression on autophagy in response to various stressors. I subjected mice to fasting, which is a traditional and potent physiological inducer of autophagy (Bagherniya et al., 2018). Previous studies have demonstrated that fasting activates autophagy in a variety of tissues, including the heart (Bagherniya et al., 2018). First, I evaluated endogenous MCL-1 levels in response to fasting. Interestingly, I found that endogenous MCL-1_{OM} was significantly reduced in response to fasting at 12, 16, and 24 hours in WT mice (Figure 6.5A and B). I also saw corresponding and progressive autophagosome formation as measured by increased LC3 II levels (Figure 6.5A and B). This increase ultimately became significant by 24 hours (Figure 6.5B). Next, I wanted to determine the effect of MCL-1_{OM} overexpression in response to fasting. Using WT and MCL-1_{OM} TG mice, I performed a fasting time course where the mice were fasted for 0, 16, and 24 hours. Similar to endogenous MCL-1_{OM} WT mouse hearts, I found that MCL-1_{OM} protein from the transgene was significantly reduced in response to fasting at both 16 and 24 hours (Figure 6.6A and B). However, even with the decrease, the transgene was still maintained at levels that are noticeably higher than endogenous MCL-1_{OM}. Next, I assessed LC3 II levels to determine the effect of this on autophagy. Interestingly, while the WT mice showed the expected significant increase in LC3II levels at 24 hours, the MCL-1_{OM} TG hearts failed

to increase LC3II at the same time point (Figure 6.7A and B). This indicates that MCL-1_{OM} needs to be degraded to allow fasting-induced autophagy to proceed. Overall, these findings suggest that MCL-1 in the outer membrane has little effect on autophagy at baseline. However, MCL-1_{OM} must be eliminated during fasting in order for autophagy to proceed, indicating that under these energy-depriving conditions, MCL-1 becomes an inhibitor of autophagy.

6.2.5 MCL-1_{OM} does not Inhibit Autophagy or Mitophagy in Response to MI

Next, I wanted to assess the role of MCL-1_{OM} overexpression in response to a stimulus associated with oxidative stress and cellular damage rather than energetic stress. A myocardial infarction (MI) causes acute cardiac injury associated with oxidative stress and mitochondrial damage and leads to increased autophagy and mitophagy (Kiyuna et al., 2018). To induce MI in mice, the left anterior descending artery was permanently ligated. After MI, the heart can be divided into three distinct regions: the infarct region, the border zone, and the remote zone (Kung et al., 2018). As was previously reported, I monitored activation of autophagy and mitophagy in the border zone 4 hours post-MI (Kubli et al., 2013). Intriguingly, in response to MI, MCL-1_{OM} overexpression does not inhibit general autophagy, as MCL-1_{OM} transgenic mice exhibit increased LC3 II levels comparable to WT mice (Figure 6.8A and B). I also assessed mitophagy in the border zone by evaluating LC3 II levels specifically at the mitochondria. Again, I found that overexpression of MCL-1_{OM} did not inhibit induction of mitophagy (Figure 6.9A and B). This confirms that MCL-1 overexpression has distinct effects on autophagy depending on the type of stress *in vivo* as well. MCL-1_{OM}

overexpression does not inhibit autophagy or mitophagy in response to cellular stress and mitochondrial damage caused by MI.

6.3 Discussion

The present study demonstrates that the effect of MCL-1 overexpression on autophagy varies with different stressors both *in vitro* and *in vivo*. My data indicates that overexpression of MCL-1 does not alter autophagic machinery under baseline conditions. However, consistent with previous reports that MCL-1 and other BCL-2 family proteins can inhibit general autophagy, I found that MCL-1 overexpression inhibits autophagy in response to rapamycin treatment *in vitro* and fasting *in vivo*. To my knowledge, this is the first time that MCL-1 has been shown to suppress autophagy *in vivo* in the heart. Based on prior studies, it is most likely that MCL-1 is inhibiting autophagy by sequestering BECLIN1 (Germain et al., 2011). As rapamycin is an mTOR inhibitor, MCL-1 could be acting downstream to suppress autophagy through interaction with BECLIN1. Additionally, since fasting also leads to mTOR inhibition, this could be a conserved mechanism. However, additional investigation is necessary to assess if overexpression of MCL-1 leads to increased interaction with BECLIN1 in order to inhibit autophagy or if it is occurring through an alternative mechanism.

In contrast to rapamycin treatment and fasting, autophagy was not inhibited by MCL-1_{OM} overexpression in response to FCCP treatment and MI. FCCP causes rapid and extensive mitochondrial damage and I previously found that MCL-1 overexpression facilitated clearance in response to FCCP treatment. This may account for why MCL-1 does not inhibit autophagy in this case. Additionally, given that the ability to upregulate

autophagy has been established as an important survival mechanism for cardiomyocytes (Gustafsson and Gottlieb, 2008), it would be detrimental for it to be compromised in response to MI. MI is also a stressor that elicits mitochondrial damage, and since MCL-1 is an important regulator of cell survival at the mitochondria, potential links between autophagy and apoptosis need to be further explored.

Taken together, my findings suggest that MCL-1's ability to inhibit autophagy varies in response to models of energetic stress versus cellular damage. Energetic stress occurs more slowly, allowing time for MCL-1 to be degraded so that autophagy can proceed. MCL-1's ability to inhibit autophagy could be a mechanism to protect cells from bulk degradation. However, in instances of cellular stress and damage that occur more rapidly, MCL-1 could be inhibited without being degraded so that autophagy can proceed. Under these circumstances there is no need to protect cells from bulk degradation to generate energy because repair is more important. While the exact mechanisms accounting for this paradigm are still under investigation, my data indicates a more complex role for MCL-1 in regulating autophagy. To further clarify which stressors cause MCL-1 to inhibit autophagy and which do not, additional stimuli must also be assessed. Exercise, which induces a general energetic autophagy response, would be ideal to confirm the results from the fasting study. Additionally, other heart failure models would be beneficial to explore to determine if MCL-1_{OM} overexpression may play a protective role.

Parts of Chapter 6 are coauthored/unpublished material. Moyzis, Alexandra; Lally, Navraj; Najor, Rita; Gustafsson, Åsa. Chapter 1, Introduction. Chapter 2,

Experimental Methods and Materials. Chapter 6, The Effect of Mcl-1 Overexpression on Autophagy. Chapter 7, Discussion. The dissertation author was the primary investigator and author of this paper.

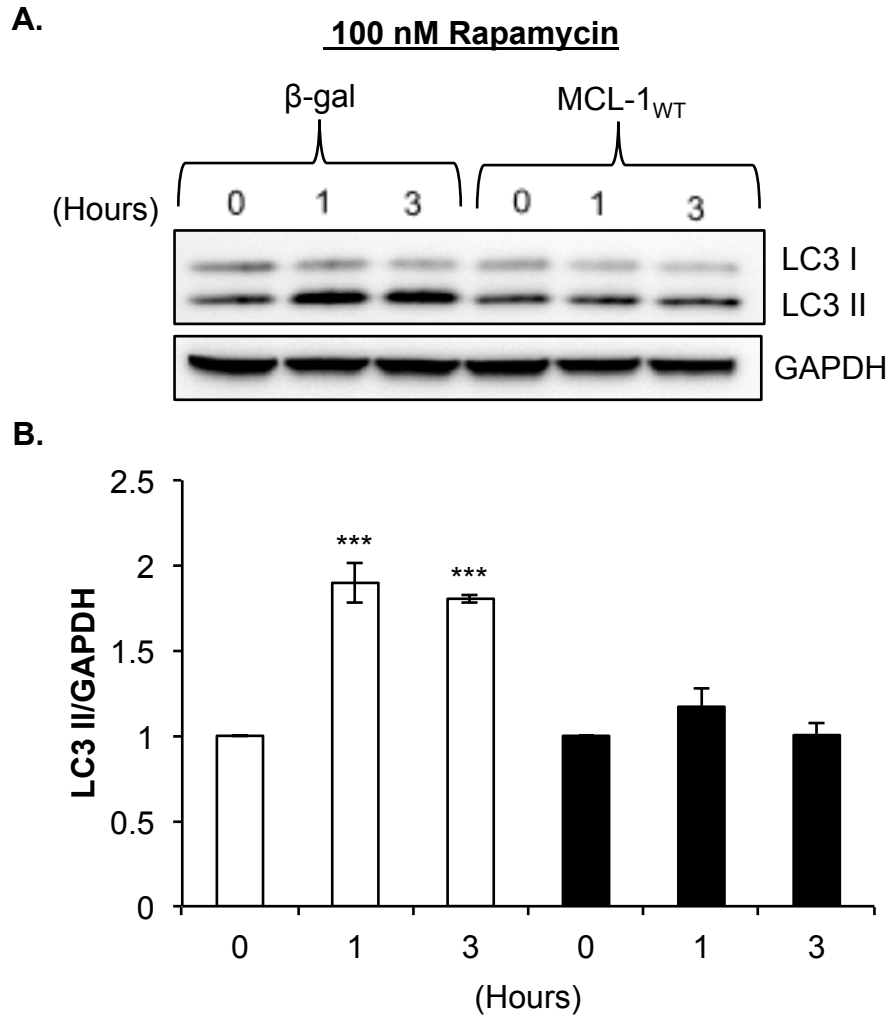


Figure 6.1. MCL-1 overexpression inhibits rapamycin-induced autophagy.

A. Representative Western blot showing LC3 levels in MEFs in response to 0, 1, or 3 hours of treatment with 100 nM rapamycin **B.** Quantitation of LC3 II levels (**p<0.001 compared to 0 h; n=3).

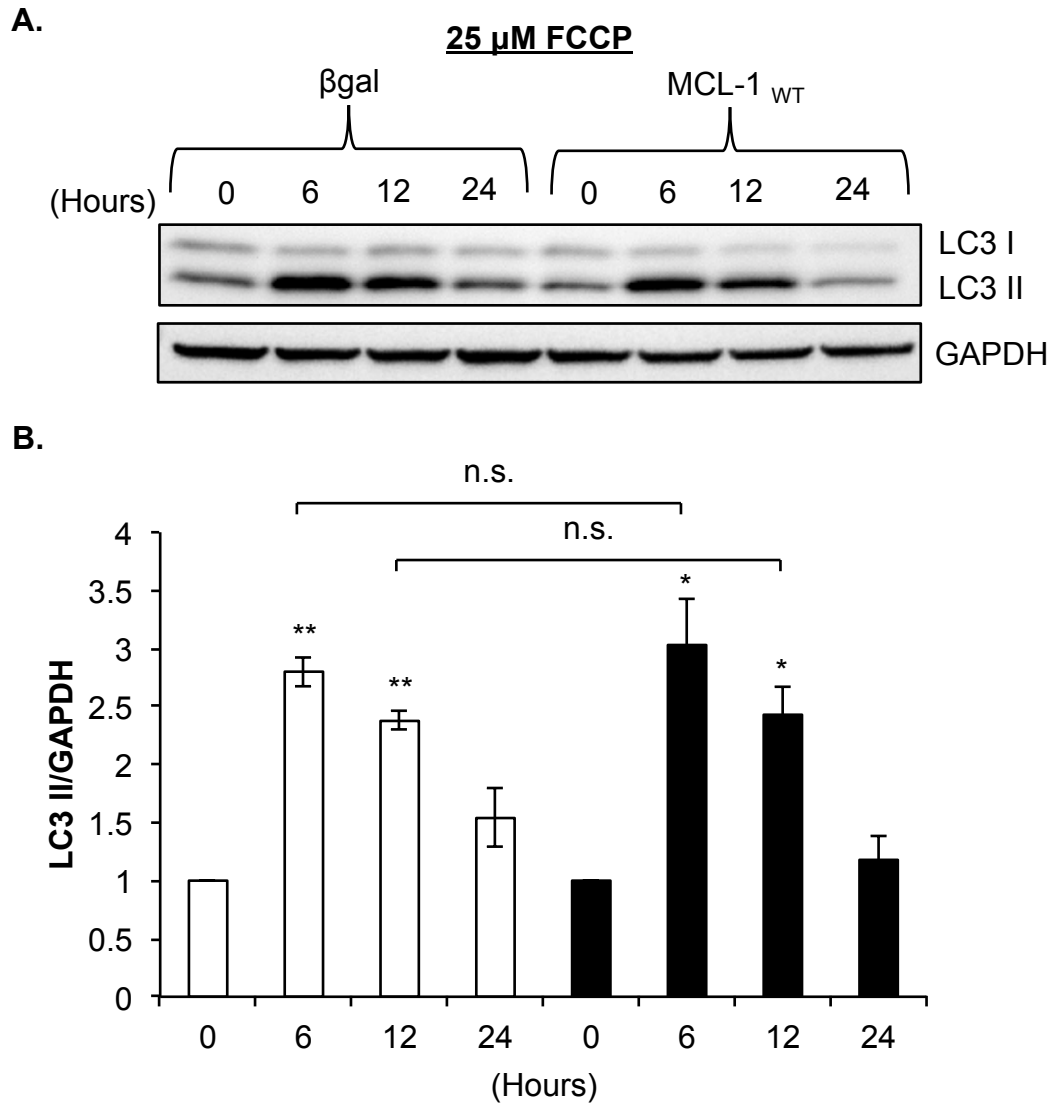


Figure 6.2. MCL-1 overexpression does not inhibit autophagy in response to FCCP treatment.

A. Representative Western blot showing LC3 levels in MEFs in response to 0, 6, 12 or 24 hours of treatment with 25 μ M FCCP. **B.** Quantitation of LC3 II levels (* p <0.05, ** p <0.01; n =3).

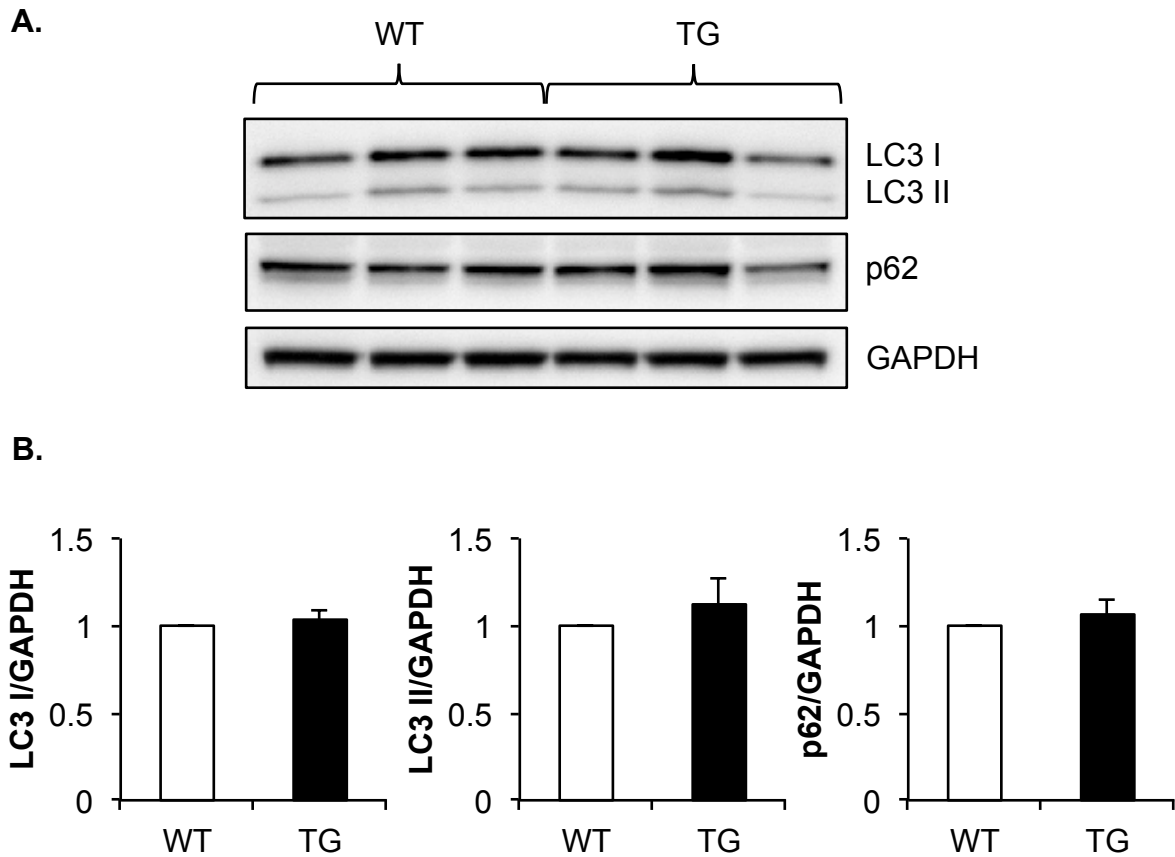
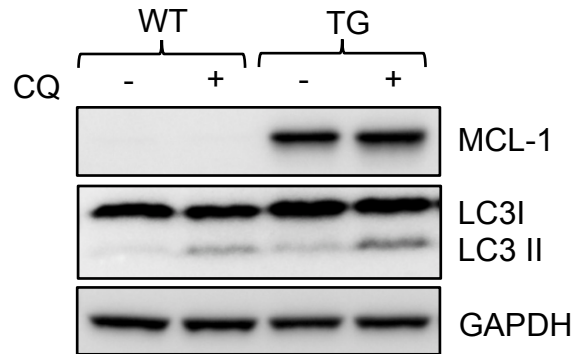


Figure 6.3. Autophagy proteins are not altered under baseline conditions in MCL-1_{OM} transgenic mice.

A. Representative Western blot showing that there are no alterations in the levels of the autophagy proteins LC3 and p62 in the hearts of WT vs. TG mice. **B.** Quantitation of LC3 I, LC3 II, and p62 levels in WT vs. TG mice (n=5).

A.



B.

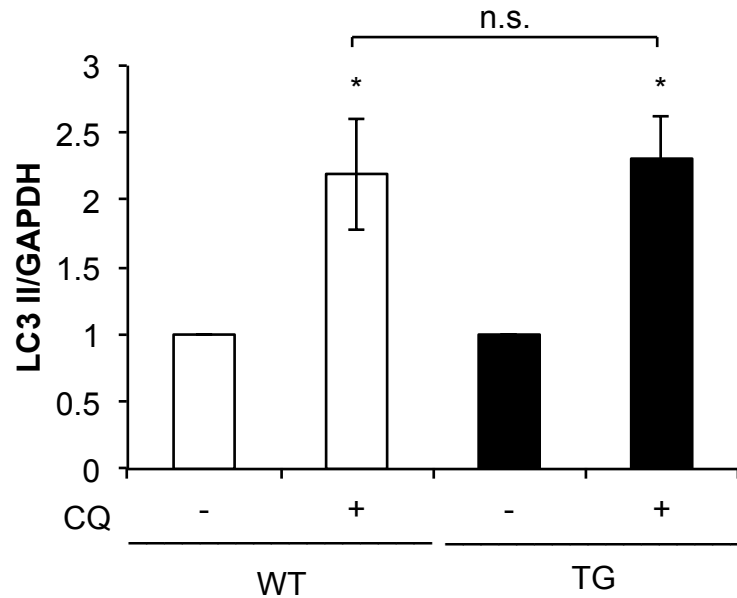


Figure 6.4. Assessing autophagic flux in WT vs. MCL-1_{OM} transgenic mice.

A. Representative Western blot shows increased LC3 II levels in hearts of both WT and MCL-1_{OM} TG mice after injection with 80 mg/kg chloroquine (CQ) for 2 hours. **B.** Quantitation of LC3 II levels (n=4-5, *p<0.05 compared to - CQ control; n.s., not significant).

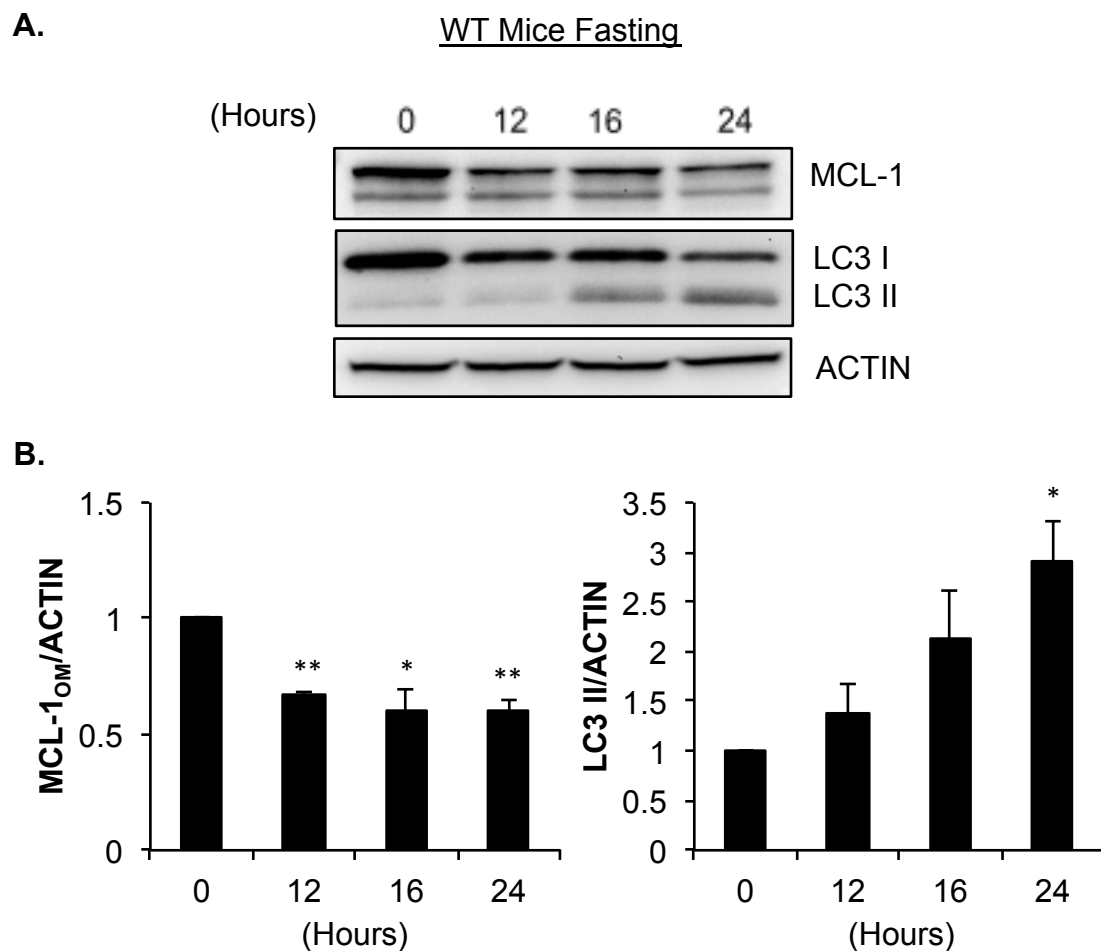


Figure 6.5. Endogenous MCL-1 is decreased in response to fasting in WT mice.

A. Representative Western blot showing MCL-1 and LC3 levels in the hearts of WT mice in response to a fasting time course (0, 12, 16, and 24 hours). **B.** Quantitation of MCL-1_{OM} and LC3 II protein levels in response to fasting (n=3, *p<0.05, **p<0.01).

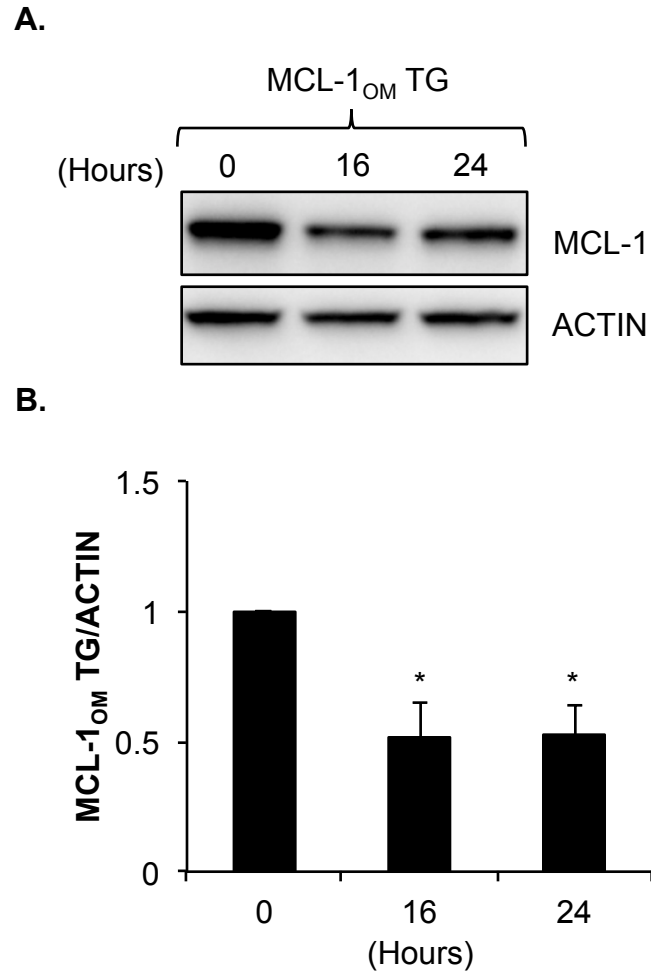


Figure 6.6. MCL-1_{OM} transgene expression is reduced in response to fasting.

A. Representative Western blot of MCL-1_{OM} transgene levels in the hearts of transgenic mice in response to 0, 16, and 24 hours of fasting **B.** Quantitation of MCL-1_{OM} transgene levels (n=4, *p<0.05).

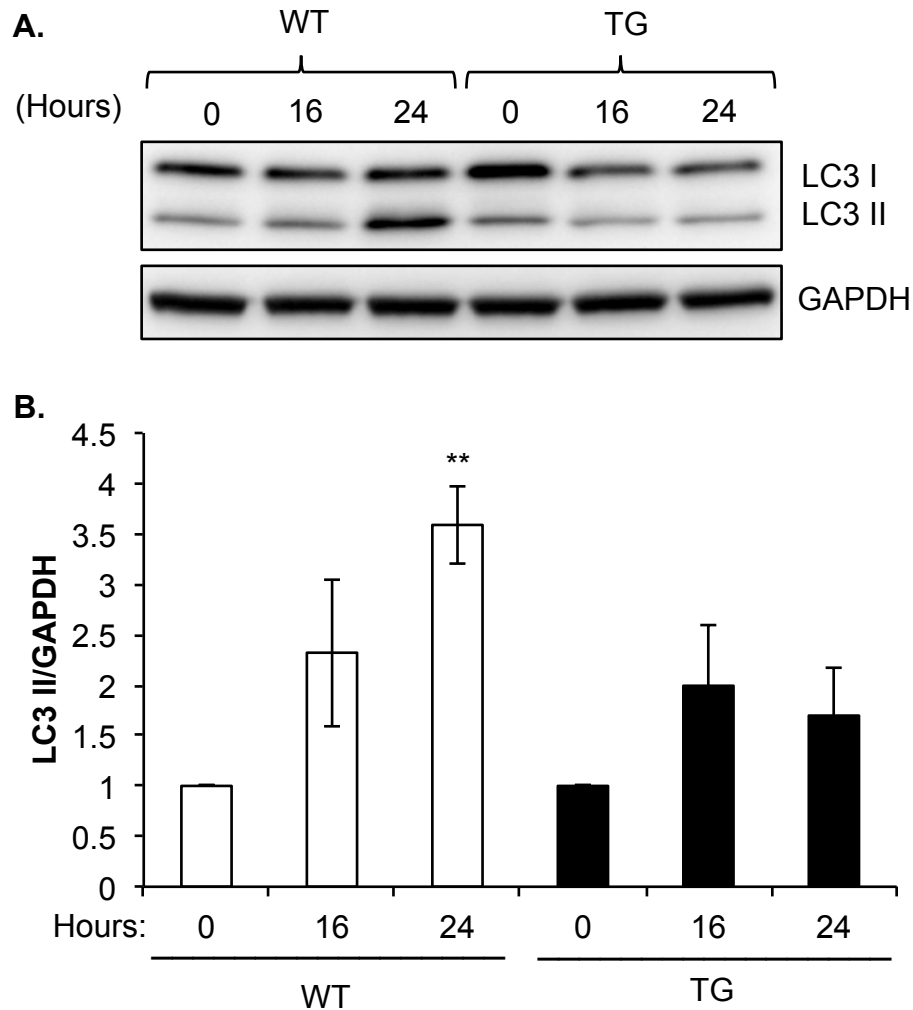


Figure 6.7. MCL-1_{OM} transgenic mice show reduced autophagy in response to fasting.

A. Representative Western blot showing LC3 levels in the hearts of WT and TG mice after 0, 16, and 24 hours of fasting. **B.** Quantitation of LC3 II levels in WT and TG mice reveals a significant increase in the WT, but not TG, hearts in response to 24 hours of fasting (n=4, **p<0.01).

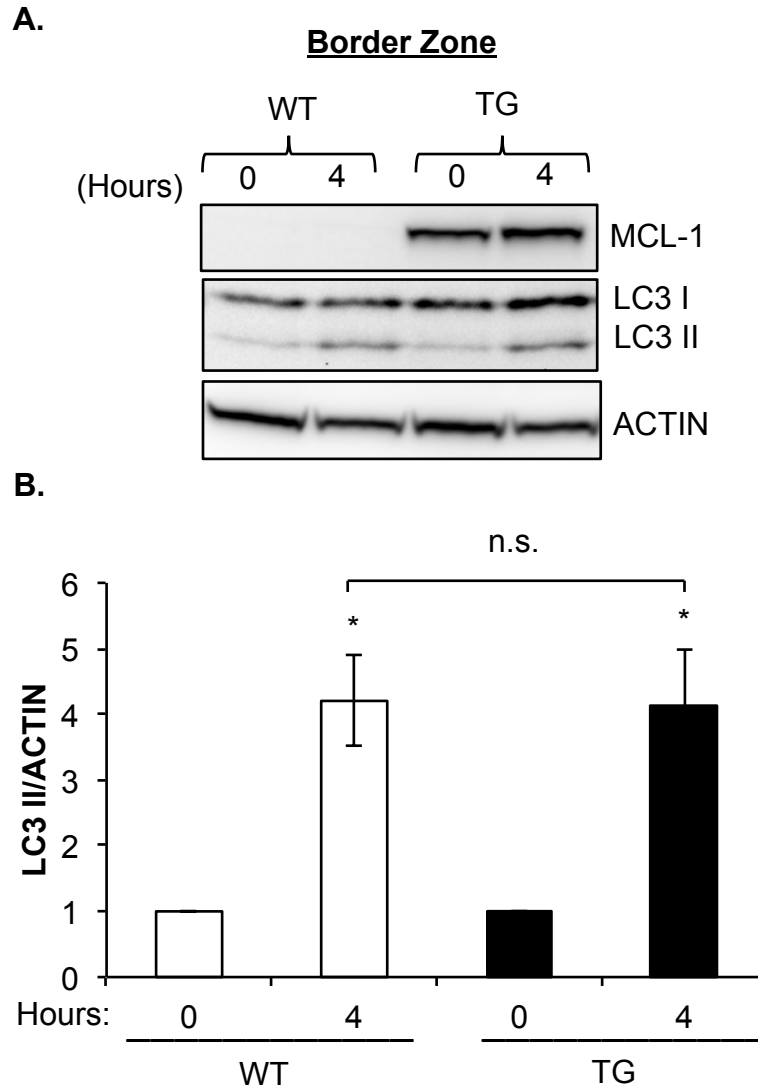


Figure 6.8. MCL-1_{OM} overexpression does not inhibit autophagy the border zone in response to myocardial infarction (MI).

A. Representative Western blot showing LC3 levels in the border zone in response to myocardial infarction, 4 hours after permanent ligation of the left anterior descending artery (LAD). **B.** Quantitation of LC3 II levels in WT and TG mice (n=5, *p<0.05 compared to 0 h control, n.s., not significant).

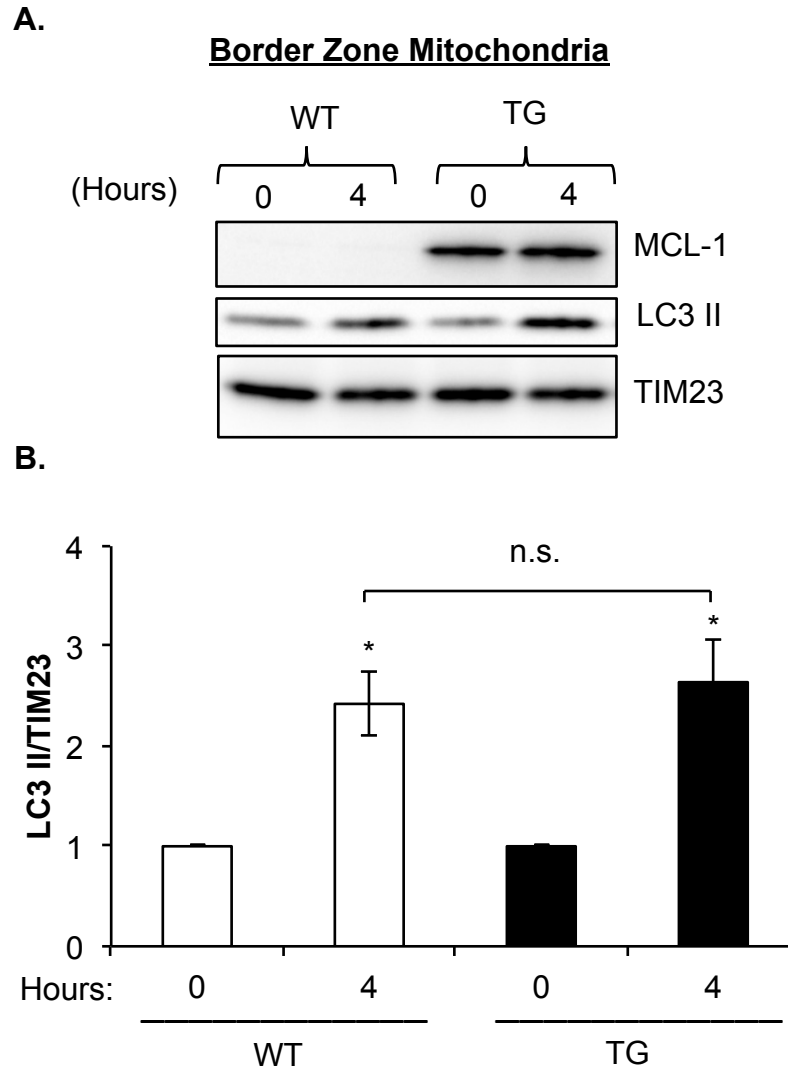


Figure 6.9. MCL-1_{OM} overexpression does not inhibit mitophagy in the border zone in response to myocardial infarction (MI).

A. Representative Western blot showing LC3 II levels in mitochondria isolated from the border zone of WT and TG mice 4 hours after myocardial infarction by permanent ligation of the left anterior descending artery (LAD). **B.** Quantitation of LC3 II levels at the mitochondria 4 hours after MI (n=5, *p<0.05 compared to 0 h control, n.s., not significant).

CHAPTER 7: DISCUSSION

7.1 Introduction

My dissertation research extends our understanding of the function of MCL-1 in the heart and provides further insight into why the presence of MCL-1 is required to maintain homeostasis. MCL-1 localizes to two distinct mitochondrial locations, and my studies have focused specifically on MCL-1 on the outer mitochondrial membrane (MCL-1_{OM}). This is the location responsible for MCL-1's anti-apoptotic activity, but my data reveals that it also has additional roles in regulating mitochondrial morphology and turnover. This provides important new insights into the complexity of MCL-1's involvement in a variety of processes. Taken together, my findings suggest that MCL-1_{OM} has many functions in the cell including anti-apoptosis, mitochondrial fission, mitophagy, and inhibiting autophagy (Figure 7.1). Specifically, my proposed model is that MCL-1_{OM} is involved in facilitating DRP1-mediated mitochondrial fission and regulating mitochondrial clearance through the mitophagy receptor BNIP3 (Figure 7.2).

7.2 MCL-1 Facilitates DRP1-mediated Fission *in vitro* and *in vivo*

My data indicates that MCL-1 regulates DRP1-mediated mitochondrial fission both *in vitro* and *in vivo*. Taken together, my findings provide strong evidence that MCL-1 is involved in recruiting DRP1 to the mitochondria. Endogenous MCL-1 and DRP1 interact rapidly in response to a variety of fission-promoting stimuli and MCL-1_{OM} overexpression alone leads to increased DRP1 levels at the mitochondria. Furthermore, it was previously reported that DRP1 is absent from the mitochondria in

MCL-1-deficient hearts (Thomas et al., 2013). MCL-1 may act as a DRP1 receptor directly or be part of a larger complex. For instance, MCL-1 could interact with one of the known DRP1 receptors FIS1, MFF, or MiD49/51. Further investigation of the mechanism by which MCL-1 is able to facilitate DRP1-mediated mitochondrial fission will be important moving forward.

Increased mitochondrial fission has been linked to apoptosis (Youle and van der Bliek, 2012). However, my data suggests that MCL-1 facilitates DRP1-mediated fission as a pro-survival response that confers protection against cell death induced by staurosporine. Additionally, DRP1 is also required for MCL-1-mediated protection against cell death in response to glucose deprivation. This suggests that MCL-1's pro-fission function is related to its pro-survival activity. Similar to my findings, it was reported that MCL-1's involvement in regulating mitochondrial dynamics with DRP1 is associated with the intrinsic apoptotic pathway and is protective against cell death (Morciano et al., 2016). In contrast, it has also been proposed that MCL-1's role in modulating mitochondrial dynamics in stem cells is distinct from its anti-apoptotic function, as MCL-1 modulated fission under conditions that did not induce cell death (Rasmussen et al., 2018). Therefore, while MCL-1 can facilitate fission in the absence of cell death, this function may become incorporated into its anti-apoptotic role upon induction of stress. Interestingly, while MCL-1 inhibitors have been shown to induce mitochondrial fission in a DRP1-dependent manner, it was also recently reported that DRP1 can facilitate BH3 mimetic-mediated cytochrome c release and apoptosis downstream of mitochondrial fission (Milani, 2019 et al). Additional research is required to more fully elucidate the roles of MCL-1 and DRP1 in regulating mitochondrial

dynamics and apoptosis.

Whereas my *in vitro* data indicates that MCL-1-mediated fission is adaptive, my *in vivo* data demonstrates that enhanced mitochondrial fission is not beneficial in response to exercise. While outcomes are variable based on the type and duration of exercise, it has been reported that mitochondrial fusion is favorable in response to exercise training in skeletal muscle (Arribat et al., 2019; Axelrod et al., 2019). My results indicate that even though MCL-1 is primarily regarded as an anti-apoptotic protein, enhanced levels of MCL-1 are not always adaptive because of its additional functions. Overall, my findings have important implications in the heart, as exercise is associated with many cardioprotective benefits (Nystoriak and Bhatnagar, 2018).

While MCL-1_{OM}-mediated mitochondrial fission was not advantageous in response to exercise *in vivo*, it may be adaptive in response to other stimuli. Interestingly, a recent study reported that promoting DRP1-mediated mitochondrial fission in midlife led to a significant increase in health and lifespan in *Drosophila* (Rana et al., 2017). The authors found that DRP1-mediated fission facilitated mitophagy, which was essential for the protective effects, and improved respiratory function and proteostasis (Rana et al., 2017). However, the induction of mitochondrial fission was only beneficial if induced in midlife, but not early life (Rana et al., 2017). This suggests that temporal regulation of mitochondrial fission may indicate whether it is protective or not. Based on these findings, it would be interesting to determine if increased DRP1-mediated fission in the MCL-1_{OM} TG mice could also have beneficial effects in response to aging. While my studies indicate an important physiologic role for MCL-1_{OM} in facilitating mitochondrial fission, further clarification of when this plays an adaptive role

is required.

7.3 MCL-1 Facilitates Mitochondrial Turnover via the Mitophagy Receptor BNIP3

My results demonstrate that in addition to promoting DRP1-mediated fission, MCL-1 overexpression can also facilitate mitochondrial clearance. This is a novel function for MCL-1. However, the physiological relevance of this still needs to be determined given that my data suggests endogenous MCL-1 does not induce clearance in response to stress induced by mitochondrial uncouplers. One potential explanation is that endogenous MCL-1 is rapidly degraded in response to FCCP treatment. Thus, overexpression of MCL-1 could allow for facilitation of mitochondrial clearance in response to stress even though it is not normally expressed at high enough levels to do this.

My data indicates that MCL-1's three potential LIR motifs are not required for MCL-1 to interact with LC3 and facilitate mitophagy. Instead, my findings suggest that MCL-1 most likely regulates mitochondrial clearance through the known mitophagy receptor BNIP3. While I have confirmed that MCL-1 and BNIP3 interact both at baseline and in response to stress, it is unknown how the two coordinate to facilitate mitochondrial clearance. Furthermore, MCL-1 and BNIP3 are also involved in regulating apoptosis so separating their functional roles in cell death from their ability to induce mitophagy is necessary. Interestingly, mutation of MCL-1's conserved BH3 domain did not affect its ability to induce mitochondrial clearance, suggesting that it is able to perform this function independently of its anti-apoptotic activity. This provides further evidence that MCL-1's ability to promote mitophagy is a novel function.

While my results suggest that MCL-1 is able to induce mitochondrial clearance independently of PARKIN, previous reports have also implicated MCL-1 in regulating PARKIN-mediated mitophagy. MCL-1 overexpression inhibited PARKIN translocation to depolarized mitochondria and subsequent mitophagy by interacting with both PINK1 and PARKIN (Hollville et al., 2014). Additionally, another study by the same group found that MCL-1 underwent rapid PARKIN- and PINK1-dependent polyubiquitination and degradation in response to mitochondrial depolarization that led to activation of BAX/BAK and subsequent apoptosis (Carroll et al., 2014). This suggests that in addition to facilitating mitophagy, PARKIN can also sensitize cells toward apoptosis depending on the degree of mitochondrial damage. However, both of these studies overexpressed PARKIN in cell types that lack detectable endogenous levels of PARKIN, so they do not address the more physiologic role of MCL-1 in these systems. Another recent study showed that PARKIN-knockout mice, which do not normally exhibit neurodegeneration or other Parkinson's disease phenotypes, show signs of dopaminergic loss and motor impairment when MCL-1 is reduced by 50% (Ekholm-Reed et al., 2019). The authors speculate that MCL-1 upregulation in PARKIN-knockout mice may be a compensatory mechanism during development (Ekholm-Reed et al., 2019). Taken together, these findings suggest that MCL-1 may suppress mitophagy under circumstances when PARKIN is overexpressed to avoid excessive clearance and apoptosis, but in PARKIN-deficient systems MCL-1 may functionally compensate for PARKIN.

Finally, my data suggests that MCL-1's ability to induce mitochondrial fission is separate from its role in facilitating mitophagy. However, there may be potential links

between MCL-1 mediated fission and mitochondrial clearance. As mitochondrial fission often precedes mitophagy, several studies have highlighted the necessity of DRP1-mediated fission in promoting mitochondrial clearance (Kageyama et al., 2014; Lee et al., 2011; Shirakabe et al., 2016; Twig et al., 2008). Interestingly, inhibition of DRP1 reduced BNIP3-mediated autophagy (Lee et al., 2011), raising the possibility that MCL-1 may be regulating both of these activities through BNIP3. It is possible that MCL-1 facilitates both processes to allow for more efficient removal of damaged mitochondria. However, whether MCL-1-induced fission and mitophagy are coupled processes may vary based on the type of stress. For instance, MCL-1-mediated mitochondrial fission and mitophagy may act together in response to some stressors, such as FCCP, whereas they may also be separable in adaptation to stimuli that cause fission but do not result in mitophagy. It will be important to clarify the interplay between MCL-1-mediated mitochondrial fission and mitophagy in order to gain a greater understanding of their functional roles.

7.4 MCL-1 Inhibits Autophagy *in vitro* and *in vivo*

My studies confirm previous reports that MCL-1 inhibits autophagy *in vitro* and indicate that MCL-1 can also act as negative regulator of autophagy in response to stress *in vivo* in the heart. MCL-1 was shown to inhibit autophagy in the brain (Germain et al., 2011), but my data extends these findings to the heart. However, my results suggest that MCL-1 overexpression does not alter autophagy or autophagic flux under baseline conditions. Additionally, instead of MCL-1 generally inhibiting autophagy in response to all stressors, my data signifies that this is specifically in the context of

energetic stress to avoid bulk degradation and not in response to stimuli that induce mitochondrial damage and require repair. These results were confirmed both *in vitro* and *in vivo*, as MCL-1 overexpression suppressed autophagy in response to treatment with the mTOR inhibitor rapamycin and fasting, but not in response to FCCP treatment and myocardial infarction (MI). Given that autophagy elicits cardioprotective benefits in response to heart failure (Bravo-San Pedro et al., 2017; Santulli, 2018), further elucidation of under what specific contexts MCL-1 is capable of inhibiting autophagy could have potential therapeutic relevance.

The other anti-apoptotic BCL-2 family members, BCL-2 and BCL-X_L, modulate autophagy in the heart through their interaction with BECLIN1 (He et al., 2013; Maejima et al., 2013). MCL-1 has also been shown to interact with BECLIN1 (Germain et al., 2011), but mechanistically, it is still unclear how exactly MCL-1 inhibits autophagy. It was previously reported that MCL-1 inhibits PARKIN-mediated mitophagy independently of BECLIN1 (Hollville et al., 2014). Similarly, another recent study implicated MCL-1 in suppressing autophagy upstream of BECLIN1 via AMBRA1 (Strappazzon et al., 2019). Interestingly, AMBRA1 contains a LIR domain and is involved in regulating both PARKIN-dependent and -independent mitophagy (Di Rita et al., 2018; Strappazzon et al., 2015). Additional avenues for investigation include assessing the interaction between MCL-1, BECLIN1, and AMBRA1 in response to various stressors to gain insight into how MCL-1 inhibits autophagy.

7.5 Therapeutic Relevance

The therapeutic potential of MCL-1 needs to be weighed carefully. My results add to the concern raised by previous MCL-1 knockout studies (Thomas et al., 2013; Wang et al., 2013) that anticancer therapeutics directed toward MCL-1 will lead to cardiotoxicity in the heart. Even if it is possible to specifically target MCL-1 on the outer mitochondrial membrane to inhibit its anti-apoptotic function, my data suggests that MCL-1 at this location is also involved in regulating other important processes, such as mitochondrial dynamics and mitophagy. Given that my results indicate that MCL-1-mediated mitochondrial fission plays an adaptive role in response to stress, inhibiting MCL-1 would likely be detrimental. Previous studies conducted in our lab revealed that partial MCL-1 ablation in the heart results in transient cardiac dysfunction that improves with time, suggesting that the heart can compensate for reduced MCL-1 levels. Similarly, it was recently reported that the combination of standard chemotherapeutics was tolerable in MCL-1 heterozygous mice and didn't lead to signs of overt damage in a broad range of tissues including heart, liver, kidney, and the haematopoietic compartment (Brinkmann et al., 2017). However, although this study attempted to use doses and schedules aimed to recapitulate conventional clinical protocols, the combined toxicities over longer periods of time need to be monitored and carefully assessed. Thus, it is possible that more temporary and reversible MCL-1 antagonists may still be a viable therapeutic approach.

Current studies are inadequate to determine whether MCL-1 will make an effective therapeutic target in the heart. While MCL-1 is essential in maintaining cardiac homeostasis, it has been less extensively studied in the context of heart failure. Interestingly, decreased MCL-1 expression was associated with susceptibility to

myocardial infarction (MI) in a recent integrative genomics study (Salisbury-Ruf et al., 2018). Our lab also showed that MCL-1 at the two different mitochondrial locations responded differently to stress induced by MI, suggesting distinct functional and temporal roles (Thomas et al., 2013). Given that MCL-1 is involved in regulating apoptosis, mitochondrial dynamics, autophagy, and mitophagy, all of which have important implications in heart failure, it could be a potentially interesting target. Increased mitochondrial fission has been shown to be detrimental in some instances (Catanzaro et al., 2019; Ong et al., 2010). Given the proposed link between fission and apoptosis, it is possible that increased fission is harmful in heart failure by leading to a rise in apoptotic cell death. However, alternatively, other studies suggest that fission is required to facilitate mitophagy, which is protective (Shirakabe et al., 2016; Wu et al., 2017). Thus, determining if overexpression of MCL-1 will elicit cardioprotective benefits in different models of heart failure will be important moving forward. Additional evaluation of the interplay between MCL-1's ability to induce mitochondrial fission and its modulation of autophagy will also be necessary to evaluate therapeutic potential.

7.6 Future Studies

My current findings recommend a number of future studies that would extend our understanding of the role of MCL-1_{OM} in the heart. Based on my data showing that MCL-1 facilitates DRP1-mediated fission, future efforts should address the underlying mechanism. This includes clarifying whether MCL-1 is responsible for recruiting DRP1 to the mitochondria and investigating whether MCL-1 regulates fission directly or through other known DRP1 receptors and/or novel binding partners. Additionally, the

interplay between MCL-1's ability to induce fission and turnover of damaged mitochondria requires further elucidation. Studies disrupting mitochondrial fission by inhibiting DRP1 or overexpressing fusion proteins and determining the effect on MCL-1-mediated clearance could help to solidify a more definitive link between these two processes.

Although my data indicates that MCL-1 is likely acting through BNIP3 to facilitate mitophagy, it would be worth investigating if MCL-1 interacts with any other mitophagy receptors as well. Additionally, given that mitochondrial clearance can occur independently of PARKIN via PINK1 (Lazarou et al., 2015; Villa et al., 2017), determining whether MCL-1-mediated clearance is PINK1-dependent would also be informative. To assess this, the clearance experiments would be repeated in PINK1^{-/-} MEFs to determine if MCL-1 overexpression is still able to induce mitophagy.

My results have also illuminated that the effect of MCL-1_{OM} overexpression on autophagy varies in response to energetic stress versus stimuli that induce cellular damage. Expanding these studies would help to confirm my previous findings and provide additional evidence for understanding MCL-1's effect on autophagy in the heart. Another energetic stress resulting in autophagy that could be investigated is exercise. Additionally, I/R could be used as an additional model of cellular damage.

While I have uncovered several novel functions for MCL-1_{OM}, it is still unknown whether ablation of MCL-1 at one or both mitochondrial locations is predominantly responsible for the rapid mortality and cardiomyopathy upon MCL-1 deletion (Thomas et al., 2013; Wang et al., 2013). Future studies crossing both the transgenic MCL-1_{OM} and MCL-1_{Matrix} mice with the MCL-1 knockout mice and assessing survival and cardiac

function would be beneficial. However, distinguishing the functional contribution of MCL-1_{OM}'s anti-apoptotic activity from its other novel roles in mitochondrial dynamics and mitophagy may prove to be challenging in this case.

7.7 Concluding Remarks

My work has demonstrated that MCL-1 on the outer mitochondrial membrane is involved in regulating DRP1-mediated mitochondrial fission and facilitating mitochondrial clearance through the mitophagy receptor BNIP3. My data also suggests that MCL-1 can inhibit autophagy in response to general energy stress, but does not do this in models that invoke cellular and mitochondrial damage. My findings have important implications for MCL-1's involvement in broader functional roles and may also help explain why other anti-apoptotic proteins are insufficient in compensating for its loss in the heart. Results from my experiments provide valuable new knowledge into the relationship between mitochondrial function, dynamics, and turnover in the heart and has the potential to identify new therapeutic targets to preserve mitochondrial function and prevent development of heart failure. These additional insights into how MCL-1 regulates mitochondrial survival and function will also guide future development of MCL-1 inhibitors and help to limit potential cardiotoxic side effects of MCL-1 inhibition in cancer patients.

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promotes Drp1-mediated mitochondrial fission and affects exercise capacity in the heart.” The dissertation author was the primary investigator and author of this paper.

Parts of Chapter 7 are coauthored/unpublished material. Moyzis, Alexandra; Lally, Navraj; Najor, Rita; Gustafsson, Åsa. Chapter 1, Introduction. Chapter 2, Experimental Methods and Materials. Chapter 6, The Effect of Mcl-1 Overexpression on Autophagy. Chapter 7, Discussion. The dissertation author was the primary investigator and author of this paper.

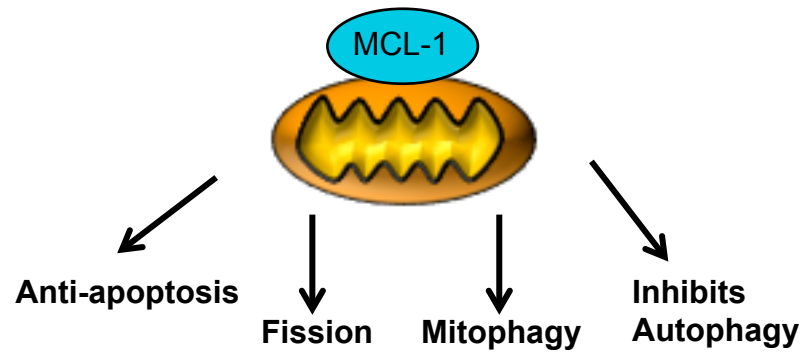


Figure 7.1. Functions of MCL-1_{OM} in the cell.

MCL-1_{OM} has many functions in the cell. These include anti-apoptosis, regulating mitochondrial fission, facilitating mitophagy, and inhibiting autophagy.

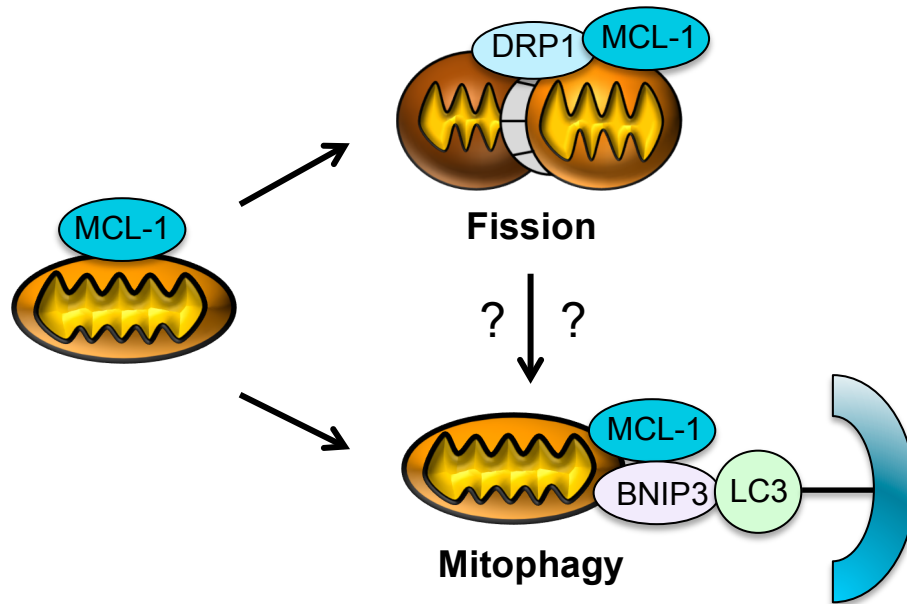


Figure 7.2. Proposed model for additional functions of MCL-1 on the outer mitochondrial membrane.

MCL-1_{OM} interacts with DRP1 to induce mitochondrial fission. MCL-1_{OM} also interacts with the mitophagy receptor BNIP3 to facilitate mitochondrial clearance. Whether these two processes are connected is still under investigation.

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