UNIVERSITY OF CALIFORNIA SAN DIEGO

MCL-1 Facilitates Mitochondrial Fission and Clearance

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by

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ABSTRACT OF THE THESIS

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by

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Master of Science in Biology

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Myeloid Cell Leukemia-1 (MCL-1) is an anti-apoptotic member of the BCL-2 family of proteins that is known to antagonize intrinsic apoptotic cell death. Our lab has previously shown that the cardiac-specific ablation of MCL-1 in mice leads to severe cardiomyopathy and mitochondrial dysfunction. Surprisingly, these defects were accompanied by necrotic rather than apoptotic cell death. This suggested that MCL-1 has additional functions at the mitochondria. This study demonstrates the mechanisms by which MCL-1 sustains mitochondrial homeostasis. We

found that overexpression of MCL-1 promotes mitochondrial perinuclear clustering. However, MCL-1 does not promote mitochondrial perinuclear clustering when Drp1 is knocked-down. Mutation of MCL-1's BH3-domain abrogates MCL-1 mediated mitochondrial clustering. Replacing MCL-1's BH-domains with the corresponding BH-domains from BCL-2 does not perturb this function. Additionally, overexpression of MCL-1 promotes mitochondrial clearance in response to FCCP and hypoxia. A screen of MCL-1 revealed three putative LC3-interacting region (LIR) motifs. Mitophagy was assessed when each of these three LIR motifs was mutated but no change was detected. However, mutation of MCL-1's three LIR motifs lead to a small but significant reduction in MCL-1 mediated mitophagy. Furthermore, we demonstrated that MCL-1 can promote mitophagy independently of its anti-apoptotic function and that MCL-1-mediated mitophagy and mitochondrial aggregation occur independently of one another. Instead, we found that MCL-1 interacts with known mitophagy receptor BNIP3 during hypoxia and FCCP treatment. Thus, our data suggest that MCL-1 promotes Drp1-dependent mitochondrial fission and aggregation as well as mitochondrial clearance.

Chapter 1: Introduction

BCL-2 Family of Proteins and MCL-1

The BCL-2 family of proteins control the intrinsic apoptotic pathway by regulating outer mitochondrial membrane (OMM) permeabilization. These proteins share homology in up to four domains (BH1-4) and are subdivided into three different classes that reflect their primary function in the apoptotic signaling cascade: pro-apoptotic proteins that permeabilize the OMM and subsequently cause apoptogenic protein release from the mitochondria; BH3-only proteins which are a subcategory of pro-apoptotic members that affect the OMM permeabilizing proteins; and anti-apoptotic proteins that promote cell-survival through inhibition of the pro-apoptotic family members (Giam *et al*., 2008; Singh *et al*., 2019).

Pro-apoptotic proteins BAX and BAK are activated in response to apoptotic stimuli and oligomerize on the OMM to form a pore through which cytochrome *c* and other apoptogenic proteins are released from the mitochondrial intermembrane space (Dewson & Kluck, 2009; Liu *et al*., 1996; Vaux, 2011). These apoptogenic proteins subsequently mediate activation of caspases, which ultimately execute the terminal step of apoptosis (Jiang & Wang, 2004). Anti-apoptotic members such as BCL-2, BCL-X_L and MCL-1 antagonize this process by directly binding and inhibiting pro-apoptotic proteins through their BH-domains (Kale, Osterlund, & Andrews, 2018). The BH3-only proteins, such as BNIP3, BIM, BID, PUMA, and NOXA, function as sensors of various stressors that promote apoptosis. Although they are functionally pro-apoptotic, these proteins cannot cause OMM permeabilization autonomously; BAX and BAK are required (Zong *et al*., 2001). While the exact mechanism through which BH3-only proteins promote apoptosis is still debated, it is currently thought that BH3-only proteins act by opposing the activity of anti-

apoptotic BCL-2 proteins and by directly interacting with and activating BAX/BAK (Giam *et al*., 2008).

Although the anti-apoptotic members of the BCL-2 protein family have functional overlap, MCL-1 is unique in that it has a short-half life, a non-homologous N-terminus, and lacks a BH4 domain (Day *et al*., 2005). There are two PEST sequences in the N-terminus of MCL-1 that have been shown to contribute to its rapid proteolytic degradation (Nijhawan *et al*., 2003). Furthermore, MCL-1 localizes to two different mitochondrial sub-compartments, where it exerts distinct functions. On the OMM, MCL-1 faces the cytosol and functions similarly to the other anti-apoptotic BCL-2 proteins by antagonizing apoptosis. The function of MCL-1 in the mitochondrial matrix is less known but is thought to involve regulation of bioenergetics (Perciavalle *et al*., 2012).

Mitochondrial Dynamics

Mitochondria are dynamic organelles that undergo constant cycles of fission and fusion in order to adapt to changes in the cellular environment. Mitochondrial fission is the process through which a single mitochondrion is divided into two smaller daughter mitochondria. This process is controlled by the fission regulator, Drp1. Drp1 is a large, cytosolic GTPase that is recruited to the OMM where it interacts with Fis1 and MFF to induce fission. Once at the mitochondria, Drp1 facilitates fission by creating a mechanical force through its GTPase activity to pinch off the membrane remaining between the two budding daughter mitochondria (Youle & Karbowski, 2005). This process is opposed by mitochondrial fusion, which is facilitated by the large GTPases MFN1, MFN2, and OPA1. MFN1 and MFN2 are localized in the OMM where they act as docking sites for adjacent mitochondria. Once the outer mitochondrial membranes are fused, OPA1 inside the mitochondria facilitates fusion of the inner mitochondrial membranes (Olichon *et al*., 2002; Scott & Youle, 2010). Mitochondrial fusion is thought to promote and preserve healthy

mitochondrial function while fission is generally considered to be a negative cellular stress-response. However, recent studies have suggested that mitochondrial fission can also be an adaptive response during stress (Coronado *et al*., 2018; Youle & Van Der Bliek, 2012).

Mitochondrial dynamics also have a role in regulating mitochondrial autophagy (mitophagy). Asymmetrical mitochondrial fission has been shown to facilitate mitophagy through the isolation of damaged mitochondrial content. Mitochondrial components are divided into healthy and damaged mitochondria via Drp1-mediated fission, where the damaged mitochondrion is then selectively targeted for degradation through autophagy (Twig *et al*., 2008). Studies have shown that inhibition of Drp1-mediated mitochondrial fission leads to an accumulation of damaged mitochondria (Ikeda *et al*., 2015).

Receptor-Mediated Mitophagy

General autophagy is known as a bulk-degradation process; however, autophagy can also be selective. Mitochondria can be selectively targeted for autophagy via the PINK1/parkin pathway or through receptor-mediated mitophagy. For instance, BNIP3 is anchored in the outer mitochondrial membrane where it can function as a mitophagy receptor by tethering mitochondria to forming autophagosomes. These proteins function as receptors for autophagy by directly interacting with LC3 on the phagophore membrane. The interaction between mitophagy receptors and LC3 is mediated via LC3-interacting region (LIR) motifs in the receptor. The consensus LIR motifs have been identified as a tryptophan, tyrosine, or phenylalanine followed by any two amino acids and then either a leucine, isoleucine, or valine (W/Y/F-X-X-L/I/V; Alemu *et al*., 2012). The autophagosome is then linked to the cargo which allows for efficient engulfment of the entire organelle (Johansen & Lamark, 2011). The mature autophagosome, containing the cargo, fuses

with a lysosome to form an autophagolysosome. In this compartment, the lysosomal enzymes degrade the autophagosome membrane and its content (Levine & Kroemer, 2008).

Rationale

MCL-1 has been well characterized as anti-apoptotic protein, but our laboratory recently found that MCL-1 may have an alternative role in maintaining mitochondrial integrity. Cardiac-specific ablation of MCL-1 lead to severe cardiomyopathy in mice. However, the loss of MCL-1 in myocytes did not lead to excessive activation of apoptosis. Instead, we observed a decline in mitochondrial function, structural deficiencies, and further signs of necrotic cell death (Thomas *et al*., 2013). Overall, these findings confirmed that the presence MCL-1 is crucial for mitochondrial homeostasis and function in myocytes, and that MCL-1 has an additional unknown function in maintaining mitochondrial function than just preventing apoptosis.

In my research, I have used in vitro models to examine the hypothesis that MCL-1 regulates mitochondrial morphology and turnover in cells. I have addressed the following questions to test the hypothesis:

1. How does overexpression of MCL-1 affect mitochondrial morphology, and what is the underlying mechanisms?
2. Does MCL-1 facilitate mitophagy, and if so, how does it promote the clearance of damaged mitochondria?

Chapter 2: Materials and Methods

Cell Culture

Mouse embryonic fibroblasts (MEFs) were maintained at 37 °C in 5% CO_2 and cultured in MEF media containing Dulbecco's Modified Eagle Medium (DMEM, Gibco), 10% fetal bovine serum (Gibco), 100 U/mL penicillin (Gemini), and 100 μg/mL streptomycin (Gemini). Normoxic and hypoxic conditions were established by placing cells in MEF media supplemented with 40mM HEPES (pH 7.4, Gibco) and then placing dishes in either a 5% CO_2 environment or a hypoxic pouch (BD Biosciences, BD GasPak EZ pouch). Glucose deprivation was accomplished by culturing cells in DMEM lacking D-glucose and sodium pyruvate (Gibco).

cDNA Constructs

The mouse HA-MCL-1 construct was generously provided by Joseph T. Opferman (Perciavalle et al., 2012). The HA-MCL-1-BH3-mutant (G198E,D199A), HA-MCL-1-LIR1 (W242A,I245A), and HA-MCL-1-LIR123 (W242A,I245A,F227A,V230A,F229A,V302A) mutants as well as a chimera of MCL-1 in which the BH1, BH2, and BH3 domains (aa 234-253, 285-300, & 190-204) were replaced with the corresponding BH domains from BCL-2 (133-152, 184-199, & 190-204) were synthesized by GenScript. PCR-based site-directed mutagenesis was performed to generate the HA-MCL-1-LIR2 (F227A,V230A) and HA-MCL-1-LIR3 (F299A,V302A) mutant constructs. The Myc-BNIP3 construct has previously been described (Kubli et al., 2008).

Transient Transfections and Adenoviral Infections

MEFs were transiently transfected with cDNA using FuGene 6 Transfection Reagent (Promega) according to the manufacturer's protocol. Drp1 knockdown experiments were performed by transfecting MEFs with 50nM MISSION Universal Negative Control #1 siRNA

(Sigma, SIC001-1NMOL) or Drp1 siRNA (Sigma, SASI_Mm01 00125369/DNM1L) using Lipofectamine RNAiMAX transfection reagent (Invitrogen) for 48 hours, according to the manufacturer's protocol. For experiments involving infections, MEFs were infected with adenoviruses in DMEM supplemented with 2% heat-inactivated serum. After 3 hours, the infection media was replaced with MEF media described above. All experiments were performed 24 hours post-infection.

Assessment of Mitochondrial Morphology and Mitochondrial Autophagy

Cells cultured on MaTek dishes were fixed with 4% paraformaldehyde for 20 minutes, permeabilized in 0.2% Triton X-100, and blocked with 5% normal goat serum (Vector). Cells were incubated at 4 °C overnight with primary antibodies against TOM20 (Santa Cruz), COX-IV (Invitrogen), or HA (Sigma) in 5% normal goat serum. Cells were washed with PBS and subsequently incubated with secondary antibodies at 37°C for 1 hour and counter-stained with Hoescht 33342 (1mg/mL). Images were captured using a Carl Zeiss AxioObserver Z1 inverted microscope equipped with a 63x objective, motorized Z-stage, and ApoTome for optical sectioning or a Nikon ECLIPSE Ti2 inverted microscope equipped with 10x and 60x objectives and a Nikon DS-Qi2 monochrome microscope camera. Mitochondrial morphology was assessed in individual cells and the mitochondrial networks were classified as either intact or clustered. A minimum of 200 cells from at least 5 different fields per experiment were analyzed. Autophagosome and mitochondrial colocalization was assessed by counting the number GFP-LC3 puncta in the green channel that overlapped with mitochondria in the red channel and showed a clear yellow color when the channels were merged.

Western Blot Analysis and Immunoprecipitation Experiments

MEFs were lysed in ice-cold lysis buffer comprised of 50mM Tris-HCl (pH 7.4), 150mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, and Complete protease inhibitor cocktail (Roche). Lysates were cleared via centrifugation at 13,000 rpm for 20 minutes and then added to 1X NuPAGE LDS Sample Buffer (Novex) plus 50mM dithiothreitol (DTT). Proteins were separated on NuPAGE Bis-Tris gels (Life Technologies) and transferred to nitrocellulose membranes. Proteins were detected using antibodies against MCL-1 (Rockland), BNIP3 (Sigma), TIM23 (BD Biosciences), Actin (GeneTex), Tubulin (Sigma), and GAPDH (GeneTex). Proteins were visualized using a Bio-Rad ChemiDoc XRS+.

For immunoprecipitation experiments, cell lysates were pre-cleared with Protein A agarose beads (Santa Cruz) for 1 h and then incubated with anti-Myc (Sigma) overnight. The antibody bound protein was captured with Protein A agarose for 2 hours. Beads were pelleted at 3500 rpm, washed 3 times with 1X PBS and resuspended in 2X SDS sample buffer and 50mM DTT. Proteins were separated on NuPAGE Bis-Tris gels, transferred to nitrocellulose membranes, and detected using antibodies against MCL-1 and BNIP3. Proteins were visualized on a Bio-Rad ChemiDoc XRS+.

Cell Death Assay

Cells infected with adenoviruses encoding β-galactose (β-gal), HA-MCL-1, or HA-MCL-1-BH3-mutant were treated with dimethyl sulfoxide (DMSO) or 1μM staurosporine for 24 hours. Cells with Drp1 knocked down that were infected with β-gal or HA-MCL-1 were incubated in DMEM with or without glucose for 24 hours. Viability was assessed by incubating cells with YO-PRO-1 Iodide (Invitrogen) and Hoescht 33342 for 20 minutes. Cells were visualized with the 10x objective of the Nikon ECLIPSE Ti2 inverted microscope. Images were taken from at least 5

different fields and a minimum of 1000 cells were counted. Percent cell death was reported as the number of cells permeable to YO-PRO-1 Iodide divided by the total number of Hoescht 33342 positive cells.

Statistical Analysis

All values were expressed as mean ± standard error of mean. All values are from a minimum of three independent experiments. Statistical analyses were performed using Student's T-Test. Multiple comparisons between experimental groups were performed using ANOVA with the Tukey multiple-comparisons post-test. A p-value of less than 0.05 was considered significant.

Chapter 3: MCL-1 Promotes Drp1-Dependent Mitochondrial Fission and Clustering

Introduction

We have previously demonstrated that MCl-1 is necessary for the maintenance of healthy mitochondria. We showed that ablation of MCL-1 in mouse cardiomyocytes lead to severe mitochondrial dysfunction and cell death. However, the mitochondrial defects led to necrotic rather than apoptotic cell death (Thomas *et al*., 2013). This suggests that MCL-1 has an alternative role in maintaining healthy mitochondria that is independent of its function as an anti-apoptotic protein. Here, I investigated whether MCL-1 had an effect on mitochondrial morphology in cells.

A. Overexpression of MCL-1 Results in Drp-1 Mediated Perinuclear Clustering of Mitochondria

To investigate if MCL-1 had an effect on mitochondrial morphology, we overexpressed MCL-1 in MEFs and assessed the effect on the mitochondrial network. MEFs were infected with adenoviruses encoding β-gal or MCL-1. After 24 h, cells were fixed and stained with an antibody against TOM20 to label mitochondria. We monitored alterations in the mitochondrial network by immunofluorescent microscopy. We observed distinct perinuclear clustering of mitochondria in a significant number of cells overexpressing MCL-1 (Figure 1A). Examination of cells at higher magnification revealed the presence of punctate-like mitochondria within these perinuclear clusters, suggesting that mitochondrial fragmentation is linked to the perinuclear clustering.

Drp1 is a large GTPase that regulates mitochondrial fission, a process where a mitochondrion is divided into two smaller mitochondria. This process is characterized by the presence of a fragmented mitochondrial network consisting of small, round, punctate-like mitochondria (Tilokani *et al*., 2018). The presence of such punctate-like mitochondria within the clustered mitochondria observed upon MCL-1 overexpression suggested a role for Drp1 in MCL-

1 mediated mitochondrial clustering. To investigate if Drp1 was involved in MCL-1 mediated mitochondrial perinuclear clustering, we knocked down Drp1 using siRNA and assessed the effect that overexpressing MCL-1 had on the mitochondrial morphology of MEFs. MEFs were transfected with either control or Drp1 siRNA and subsequently infected with either Ad-β-gal or Ad-MCL-1. Knockdown of Drp1 was confirmed by western blot (data not shown). Fluorescence microscopy confirmed that MCL-1 overexpression induced the perinuclear clustering of mitochondria (Figure 2A). Interestingly, the number of cells exhibiting this mitochondrial morphology was significantly reduced when Drp1 was knocked-down (Figure 2B). These findings suggest that MCL-1-induced perinuclear clustering of mitochondria involves Drp1-mediated fission.

B. MCL-1-Mediated Perinuclear Clustering Requires a Functional BH3-Domain

MCL-1 is known to antagonize intrinsic apoptotic cell death by interacting with pro-apoptotic BCL-2 proteins through its BH3-domain (Giam *et al*., 2008). To investigate whether MCL-1's BH3-domain was required for the mitochondrial perinuclear clustering, we generated a mutant of MCL-1 with two point mutations in its BH3-domain. Specifically, we mutated glycine 198 to glutamic acid and aspartic acid 199 to alanine (HA-MCL-1-BH3 mutant; Figure 3A). These mutations have previously been shown to abrogate the anti-apoptotic function of MCL-1 (Clohessy *et al*., 2006). To confirm the disruption in anti-apoptotic activity, we assessed cell viability in MEFs overexpressing either MCL-1 or the MCL-1-BH3-mutant in response to an apoptotic stimulus. MEFs were infected with either Ad-β-gal, Ad-MCL-1, or Ad-MCL-1-BH3-mutant and subsequently treated with DMSO or 1μM staurosporine for 24 hours. As expected, we found that overexpression of MCL-1 led to a significant decrease in cell death in response to staurosporine

treatment. Furthermore, we found that overexpression of MCL-1-BH3-mutant led to reduced protection against staurosporine-induced cell death (Figure 3B).

Next, we assessed the effect of the MCL-1-BH3-mutant on mitochondrial morphology in MEFs. MEFs were infected with either β-gal, MCL-1, MCL-1-BH3-mutant and fixed cells were stained with anti-TOM20 to label mitochondria. Surprisingly, immunofluorescent microscopy revealed that overexpression of the MCL-1-BH3-mutant failed to induce mitochondrial perinuclear clustering (Figure 4A & 4B). This suggests that a functional BH3-domain is needed for MCL-1 mediated mitochondrial clustering.

To investigate if the change in the mitochondrial network observed upon MCL-1 overexpression was unique to MCL-1's BH3-domain, we generated a MCL-1-BCL-2 chimera where MCL-1's BH1, BH2, and BH3 domains were replaced with the corresponding BH-domains of BCL-2 (MCL-1-BCL-2-Chimera). MEFs were transfected with HA, HA-MCL-1, or HA-MCL-1-BCL-2-Chimera and stained with anti-TOM20 to label mitochondria and HA to label cells transfected with MCL-1. We found no differences in the number of cells with mitochondrial perinuclear clustering between the wild-type and the chimera (Figure 5A & 5B). These findings suggest that although the BH3-domain is important for mitochondrial aggregation, the BH domains are not specific for MCL-1.

C. MCL-1-Mediated Protection from Cell Death is Abrogated when Drp1 is Knocked-Down

Drp1-mediated mitochondrial fission has been reported to be both protective and detrimental in cells (Coronado et al., 2018; Youle & Karbowski, 2005). To investigate the functional role of the mitochondrial fission and perinuclear clustering observed with MCL-1 overexpression, we assessed cellular viability in response to glucose deprivation. Glucose deprivation is known to induce mitochondrial fragmentation (Rambold *et al*., 2011). MEFs

infected with Ad-β-gal or Ad-MCL-1 after being transfected with either control or Drp1 siRNA were assessed for viability after glucose deprivation. We found that MCL-1 overexpression led to a significant reduction in glucose deprivation-induced cell death. However, the MCL-1-mediated protection from cell death was abrogated in cells with Drp1 knockdown. We observed a significant increase in cell death after Drp1 was knocked down in cells overexpressing MCL-1 (Figure 6).

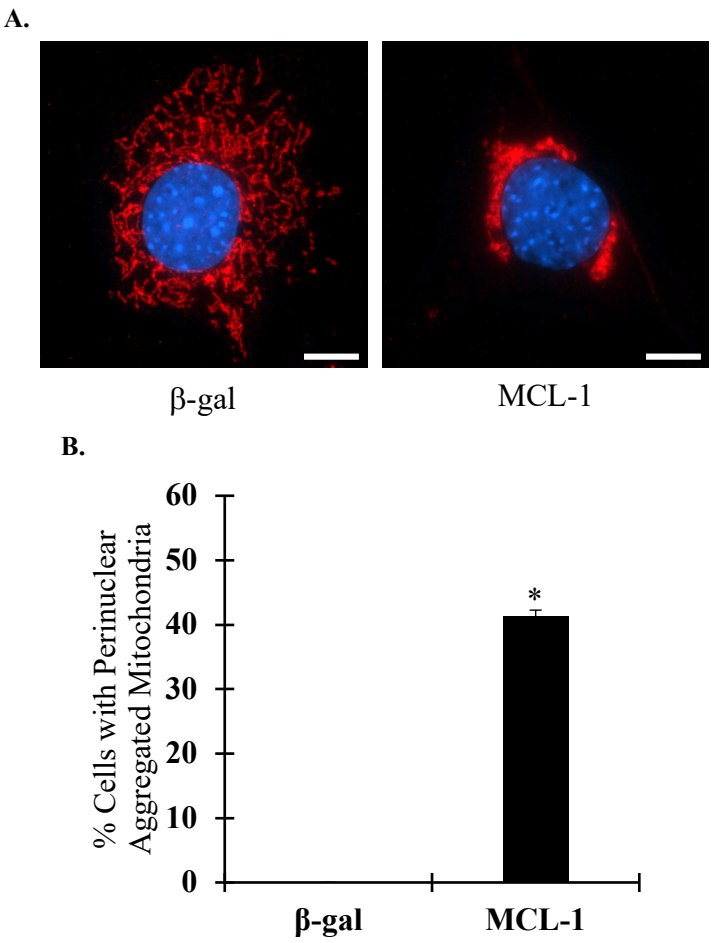


Figure 1. Overexpression of MCL-1 induces the perinuclear clustering of mitochondria in MEFs. **A.** MEFs were infected with Ad-β-gal or Ad-HA-MCL-1 and stained for TOM20 (red) as a mitochondrial-marker. Scale bar: 10μm. **B.** Quantification of the percentage of cells exhibiting clustered mitochondria (*$p<0.05$; n=3).

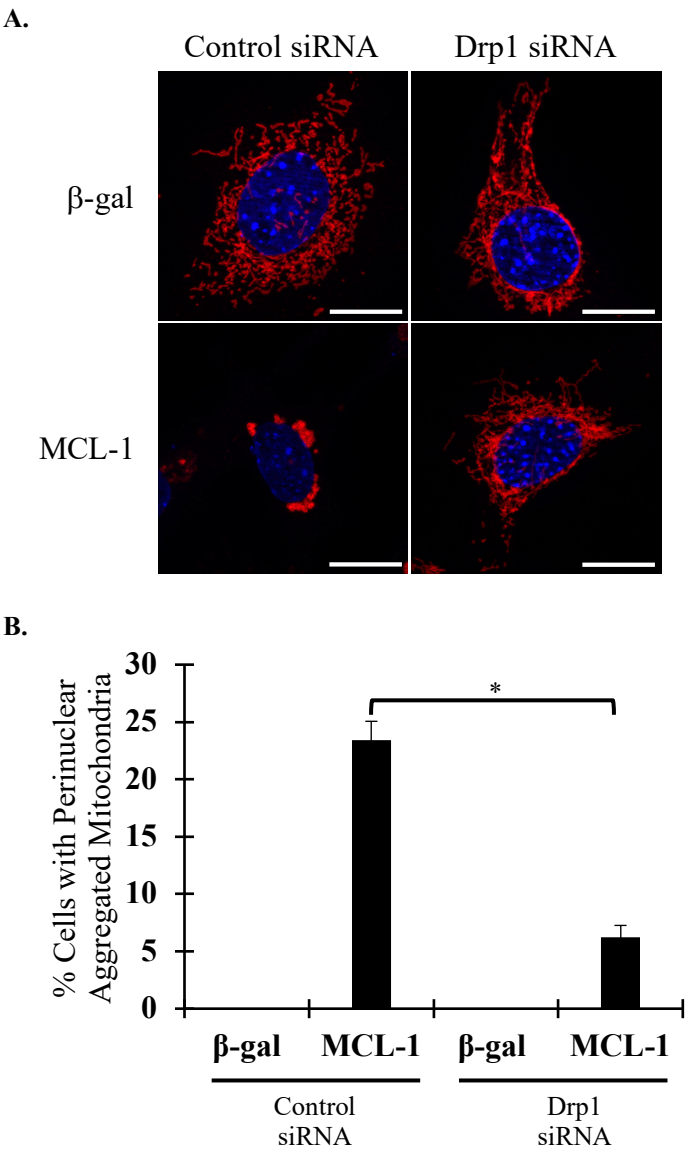


Figure 2. MCL-1 induced perinuclear clustering of mitochondria is Drp1-dependent. **A.** MEFs were transfected with 50nM control or Drp1 siRNA and subsequently infected with either Ad-β-gal or Ad-HA-MCL-1. TOM20 was stained for as a mitochondrial marker Scale bar: 20μm. **B.** Quantification of the percentage of cells exhibiting clustered mitochondria (*$p<0.05$; n=3).

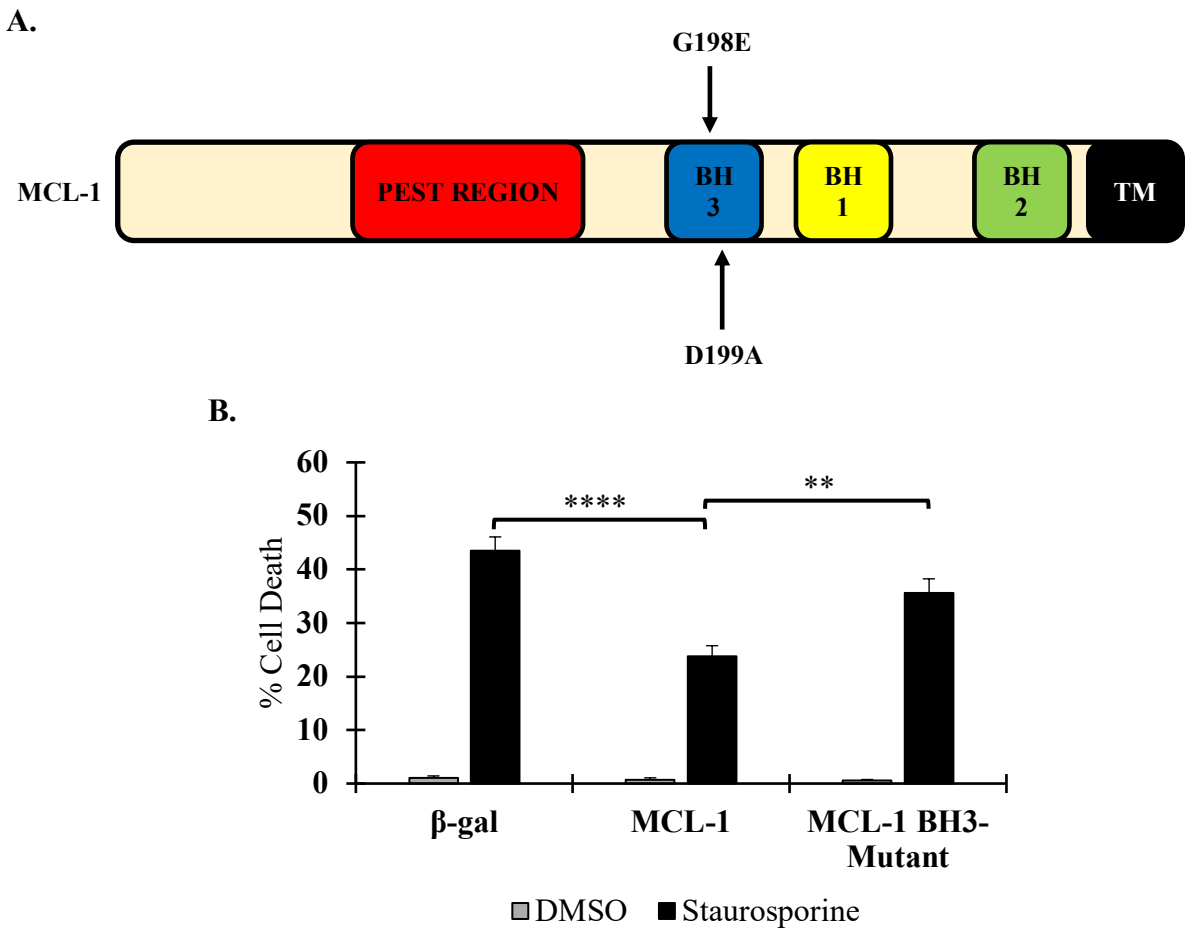
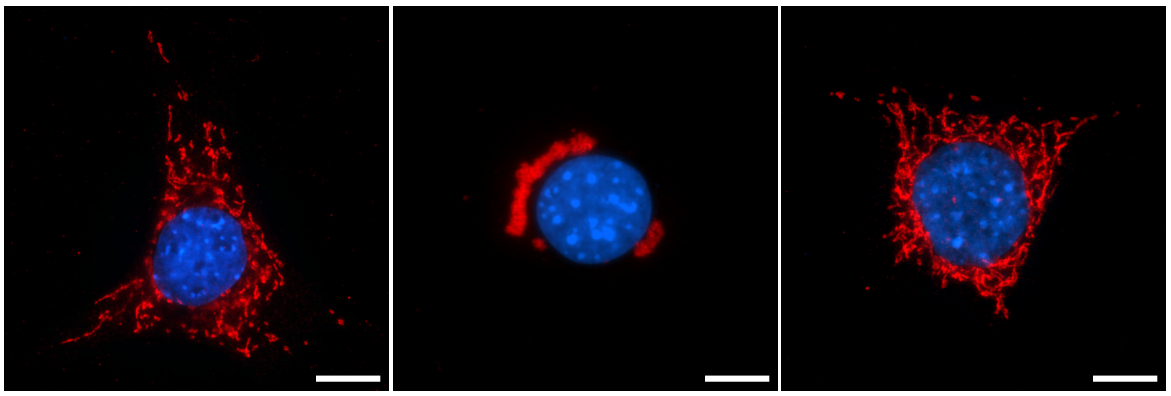


Figure 3. Mutation of MCL-1's BH3 domain disrupts its ability to protect from cell death in response to staurosporine. **A.** Schematic representation of the MCL-1 BH3-mutant generated through the mutation of two conserved residues (Gl98E; D199A) in MCL-1's BH3-domain (MCL-1-BH3-Mutant). **B.** Quantification of cell death after treatment with staurosporine. MEFs were infected with either Ad-β-gal, Ad-HA-MCL-1, or Ad-HA-MCL-1-BH3mutant and subsequently treated with 1 μM staurosporine for 24 hours (**p<0.01; ****p<0.0001; n=3).

A.



β-gal MCL-1 MCL-1 BH3-mutant

B.

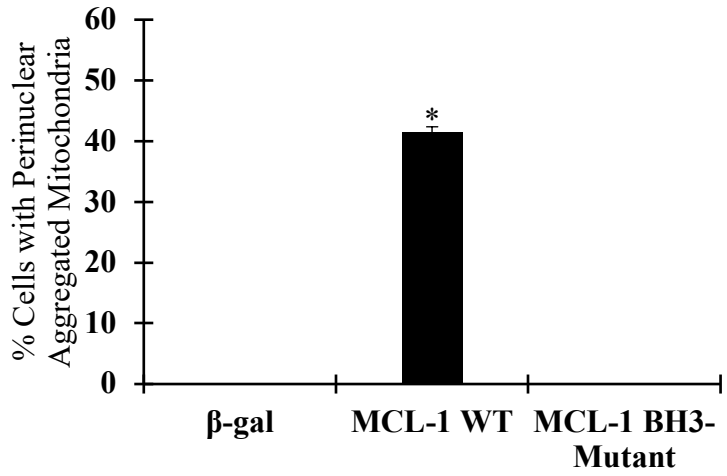


Figure 4. Mutation of MCL-1's BH3 domain abrogates the perinuclear clustering of mitochondria. **A.** MEFs were infected with Ad-β-gal, Ad-HA-MCL-1, or Ad-HA-MCL-1-BH3-mutant and stained for TOM20 (red) as a mitochondrial-marker. Scale bar: 10μm **B.** Quantification of the percentage of cells exhibiting perinuclear clustered mitochondria (*$p<0.05$; n=3).

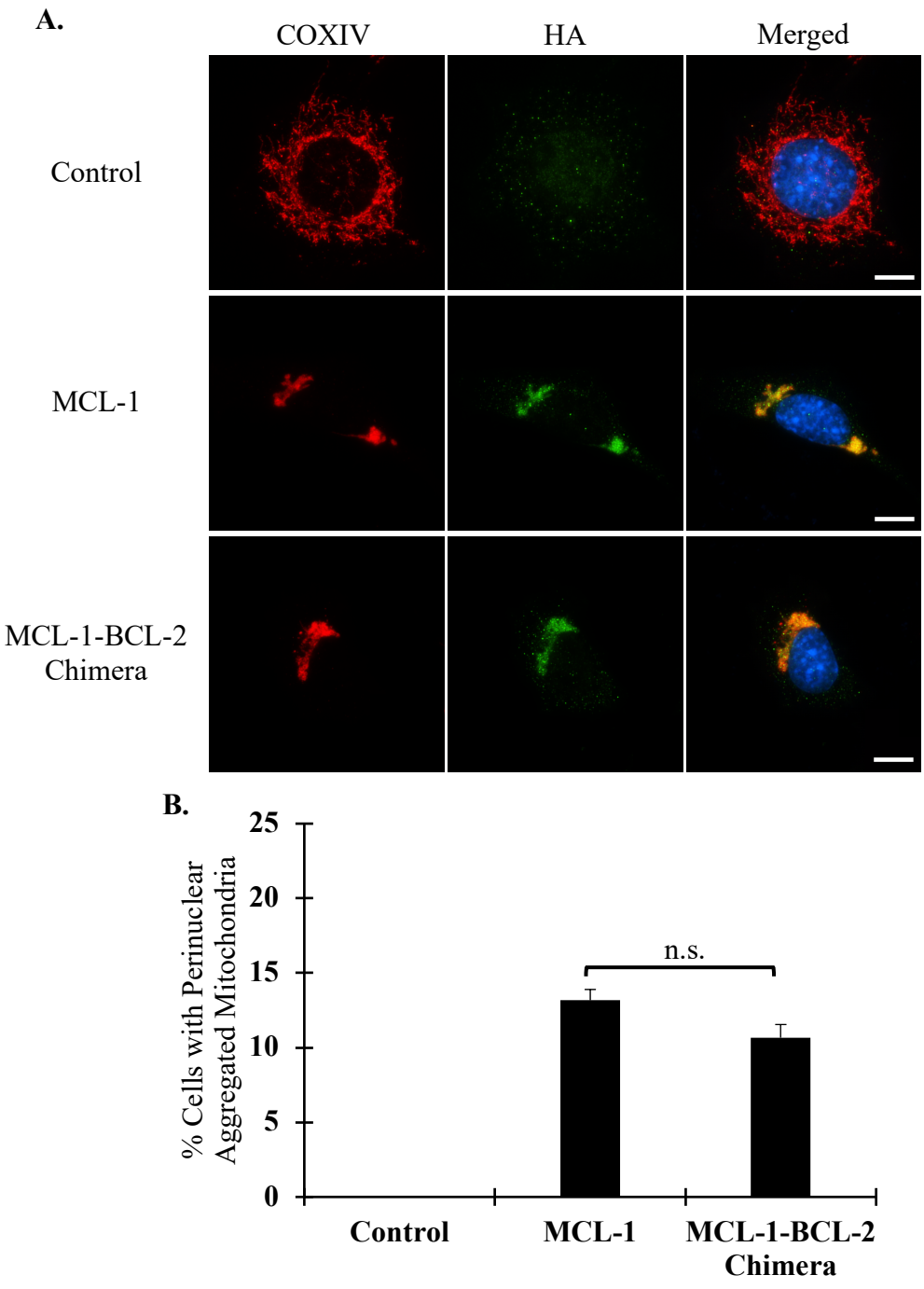


Figure 5. MCL-1 induced perinuclear clustering of mitochondria requires a functional BH3 domain. **A.** An MCL-1 BCL-2 BH-domain chimera was made by replacing all BH-domains in MCL-1 with the corresponding BH-domains of BCL-2 (HA-MCL-1-BCL-2 Chimera). MEFs were transfected with HA, HA-MCL-1, or HA-MCL-1-BCL-2 Chimera and stained for HA (green) as a marker for overexpressed MCL-1 and COX-IV (red) as a mitochondrial marker. Scale bar: 10μm **B.** Quantification of the percentage of cells exhibiting perinuclear clustered mitochondria (n.s., not significant; n=3).

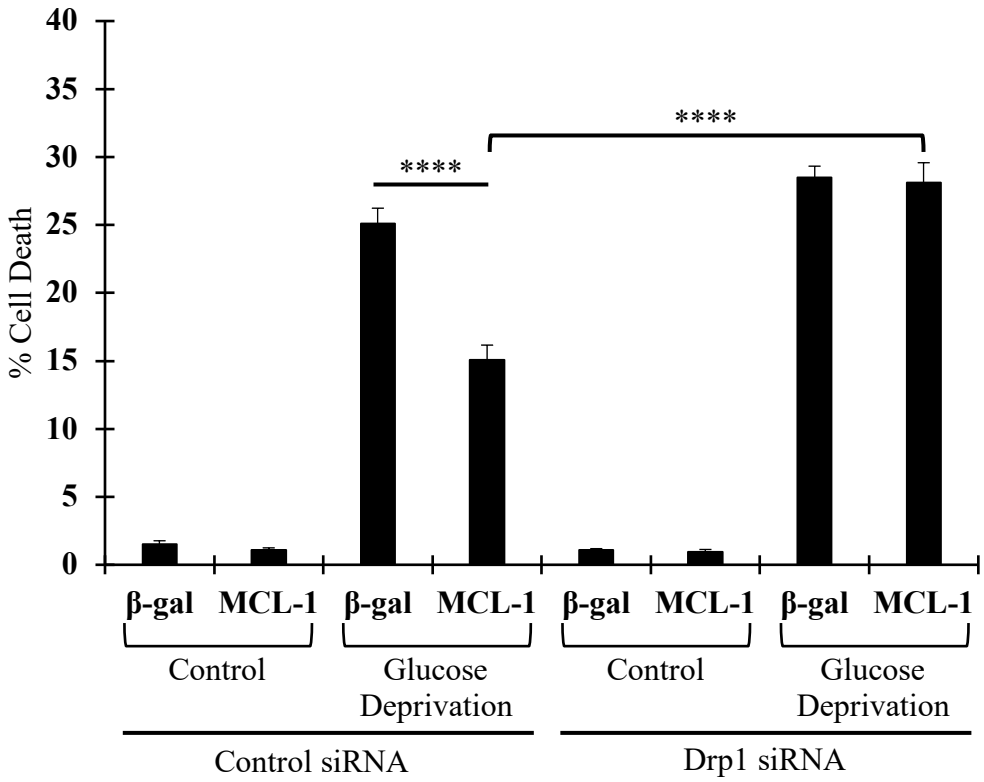


Figure 6. MCL-1's ability to protect from cell death is lost when Drp1 is knocked down. MEFs were transfected with 50nM control or Drp1 siRNA and subsequently infected with either Ad-β-gal or Ad-HA-MCL-1. Cells were subjected to glucose deprivation or control conditions for 24 hours and cell death was assessed (****$p<0.0001$; n=3).

Chapter 4: MCL-1 Promotes Mitophagy in Response to FCCP and Hypoxia

Introduction

Mitochondrial fission has previously been shown to facilitate mitophagy (Twig *et al*., 2008). Specifically, the BH3-only protein BNIP3 has been shown to facilitate mitophagy by inducing the translocation of Drp1 to the mitochondria and causing fragmentation. Inhibition of Drp1 lead to a reduction in BNIP3-mediated mitophagy (Lee *et al*., 2011). We have demonstrated that overexpression of MCL-1 induces Drp1-dependent mitochondrial fission and clustering. Here, I investigated whether MCL-1 also promotes the clearance of damaged mitochondria.

A. MCL-1 May Function as a Mitophagy Receptor

To investigate if MCL-1 had an effect on mitochondrial clearance, we investigated whether overexpression of MCL-1 would facilitate mitochondrial clearance in response to FCCP. FCCP is a mitochondrial uncoupler and well-known inducer of Parkin-mediated mitophagy (Narendra *et al*., 2008). To assess whether MCL-1 had an effect on mitophagy, MEFs infected with adenoviruses encoding β-gal or MCL-1 were treated with DMSO or 25μM FCCP for 24 hours. We assessed mitochondrial clearance by Western blot analysis of the mitochondrial protein TIM23 to assess mitochondrial content. We observed a significant reduction in TIM23 levels during FCCP treatment when MCL-1 was over-expressed (Figure 7A & 7B), suggesting that MCL-1 can facilitate mitophagy in MEFs.

Autophagy receptors such as BNIP3 and NIX promote the selective clearance of mitochondria by recruiting and directly interacting with LC3 (Hanna et al., 2012; Novak et al., 2010). Autophagy receptors have conserved LC3 interacting region (LIR) motifs through which they can directly bind LC3 on autophagosomes. LIR motifs have been shown to consist of either 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; Figure 8; Johansen & Lamark, 2011). We conducted a screen of MCL-1's amino acid sequence and identified three conserved, putative LIR motifs (242-WGRI-245; 227-FSRV-230; 299-FFHV-302). To investigate MCL-1's function as a potential mitophagy receptor, we generated four different MCL-1 mutants with point-mutations in the putative LIR motifs. Specifically, we 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 a mutant with all six of the aforementioned point mutations (MCL-1-LIR123; Figure 8). We used immunofluorescence microscopy to assess MCL-1-mediated mitophagy by evaluating mitochondrial colocalization with LC3-positive autophagosomes in MEFs overexpressing MCL-1. MEFs were transfected with GFP-LC3 and either HA, MCL-1, MCL-1-LIR2, or MCL-1-LIR3 and in a separate experiment transfected with GFP-LC3 and either HA, MCL-1, MCL-1-LIR1, MCL-1-LIR123. MEFs were subsequently treated with DMSO or 25μM FCCP for 2 hours, fixed, and stained with an antibody against the mitochondrial protein TOM20. We confirmed that overexpression of MCL-1 led to a significant increase in the co-localization between GFP-LC3-positive autophagosomes and mitochondria, an indicator of mitophagy. However, we found that individually mutating either of MCL-1's putative LIR motifs had no effect on MCL-1-mediated mitophagy (Figure 9 & Figure 10). Interestingly, overexpressing MCL-1-LIR123 lead to a small but significant reduction in the number of GFP-LC3 puncta colocalized with mitochondria (Figure 10). Overall, these findings suggest that MCL-1 might not directly interact with LC3 on the autophagosomes via a LIR motif.

B. MCL-1 Promotes Mitochondrial Clearance in Response to Hypoxia

Next, we investigated if MCL-1 facilitated mitochondrial clearance by interacting with a mitophagy receptor in the outer mitochondrial membrane. Mitophagy receptors BNIP3 and NIX have been shown to promote mitophagy in response to hypoxia (Hanna *et al*., 2012; Novak *et al*., 2010). Because BNIP3 is known to be upregulated during hypoxia in cells, we investigated if MCL-1 also promoted mitophagy in response hypoxia (Bellot *et al*., 2009). MEFs were infected with Ad-β-gal or Ad-MCL-1 before they were subjected to normoxia or hypoxia for 24 hours. We assessed mitochondrial clearance by Western blot analysis of mitochondrial TIM23. We observed a significant reduction in TIM23 levels during hypoxia in cells overexpressing MCL-1 compared to β-gal (Figure 11A & 11B). This suggests that MCL-1 can enhance hypoxia-mediated mitophagy.

D. MCL-1 Mediated Mitophagy Occurs Independently of its Anti-Apoptotic Activity

We previously found that MCL-1-induced perinuclear clustering of mitochondria required a functional BH3-domain. To investigate if MCL-1 induced mitophagy also required an intact BH3-domain, we evaluated mitochondrial clearance in MEFs that were overexpressing the MCL-1-BH3-mutant. MEFs were infected with Ad-β-gal, Ad-MCL-1, or Ad-MCL-1-BH3-mutant and treated with DMSO or 25μM FCCP for 24 hours. We assessed mitochondrial clearance by Western blot analysis of TIM23, which was used as a marker for mitochondrial content. We observed a significant reduction in TIM23 levels during FCCP treatment when MCL-1-BH3-Mutant was overexpressed (Figure 12A & 12B). This suggests that MCL-1 facilitates mitochondrial clearance independently of its anti-apoptotic function.

E. MCL-1 Interacts with the Mitophagy Receptor BNIP3

BNIP3 is upregulated in response to hypoxia (Drake *et al*., 2017). Because we found that MCL-1 also facilitates mitophagy during hypoxia, we investigated whether MCL-1 may be interacting with BNIP3 to facilitate this process. To confirm that BNIP3 was upregulated in MEFs during hypoxia, we subjected MEFs to hypoxia and assessed BNIP3 levels. MEFs were subjected to either control or hypoxic conditions for 2, 4, 8, 12, or 24 hours and assessed for BNIP3 and MCL-1 levels by Western blot analysis. We observed that BNIP3 levels were significantly increased 8, 12, and 24 hours after hypoxia, and that MCL-1 levels were significantly reduced after 24 hours of hypoxia (Figure 13A & Figure 13B).

Next, we assessed whether MCL-1 interacted with BNIP3 during hypoxia. MEFs were transfected with controls, MCL-1 and BNIP3 and subsequently subjected to control or hypoxic conditions for 2, 4, 6, 8, or 12 hours. BNIP3 was immunoprecipitated using an antibody against Myc, and MCL-1 and BNIP3 were assessed by Western blot analysis. Interestingly, we found that MCL-1 and BNIP3 co-immunoprecipitate at baseline, and that the amount of MCL-1 that co-immunoprecipitates with BNIP3 increases after 2 hours of hypoxia. As the duration of hypoxia increases, the interaction between MCL-1 and BNIP3 is decreased (Figure 14).

We found that MCL-1 also promotes mitophagy in response to FCCP. To investigate if MCL-1 facilitated mitophagy through BNIP3, we investigated whether there was an interaction between MCL-1 and BNIP3 in response to FCCP. We transfected MEFs with vector controls, MCL-1 and BNIP3 and then treated cells with DMSO or 25μM FCCP for 2, 4, 6, 8, or 12 hours. BNIP3 was immunoprecipitated using an antibody against Myc, and MCL-1 and BNIP3 were assessed by Western blot analysis. Interestingly, the interactions between MCL-1 and BNIP3 were similar during both hypoxia and FCCP treatment where the amount of MCL-1 that co-

immunoprecipitates with BNIP3 increases after two hours of FCCP but the amount decreases as the treatment progresses (Figure 15).

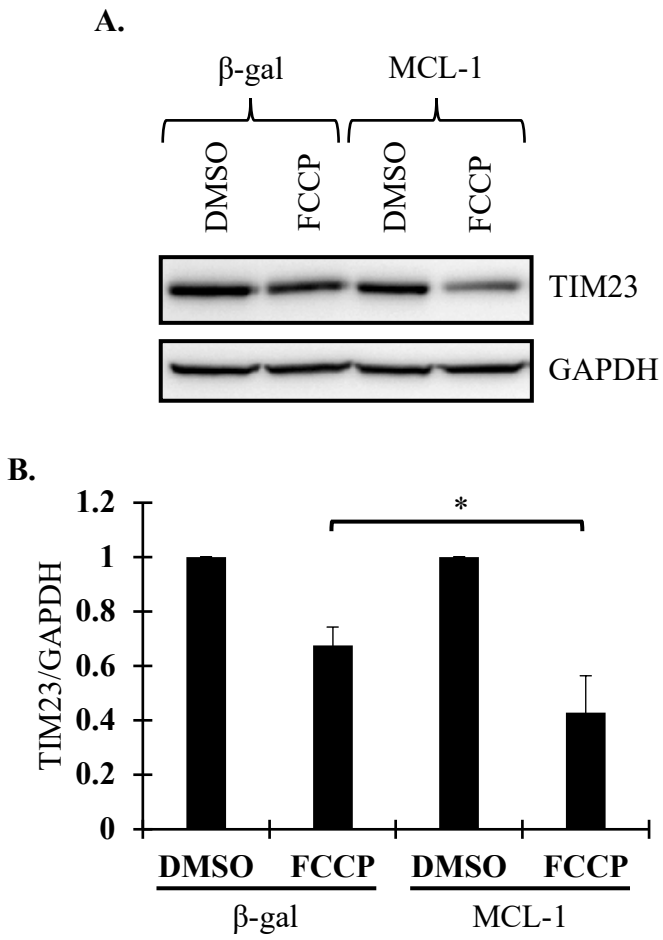


Figure 7. MCL-1 promotes mitochondrial clearance in response to FCCP. **A.** Western blot analysis of the mitochondrial marker TIM23 after MEFs were infected with Ad-β-gal or Ad-HA-MCL-1 and subsequently treated with DMSO or 25μM FCCP for 24 hours. GAPDH was used a loading control. **B.** Quantification of TIM23 levels (*$p<0.05$; n=3).

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

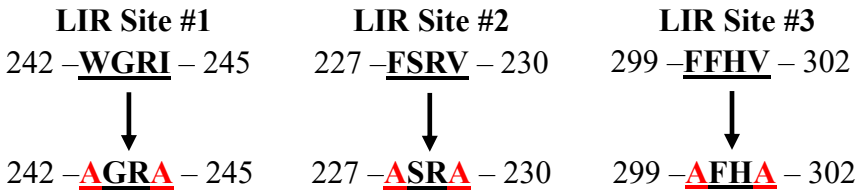


Figure 8. Mutation in MCL-1's conserved, putative LC3-Interacting Region (LIR) motifs.

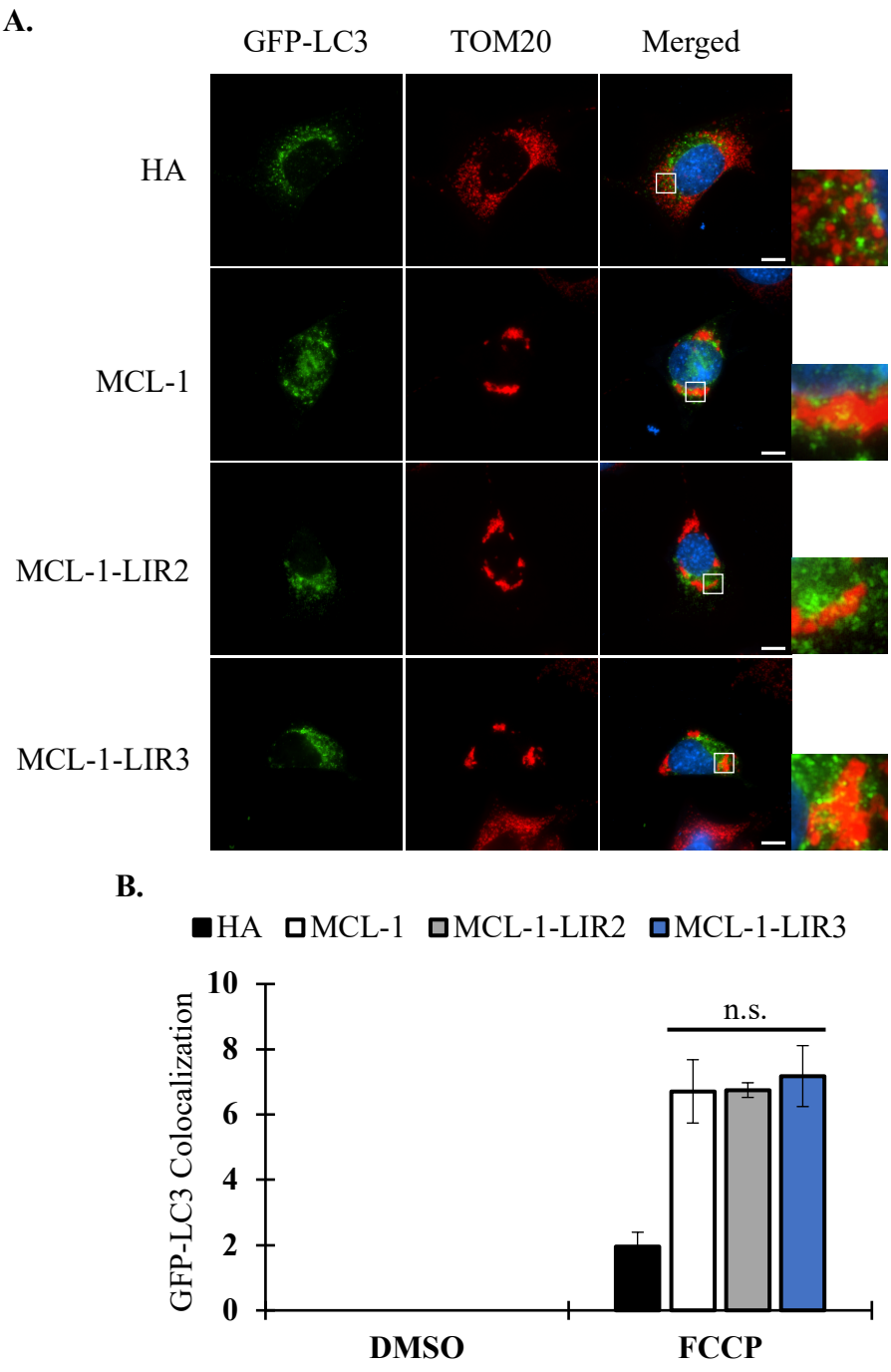


Figure 9. Individually mutating the LIR2 and LIR3 motifs of MCL-1 does not affect MCL-1 mediated mitophagy. **A.** MEFs were transfected with GFP-LC3 and either HA, HA-MCL-1, HA-MCL-1-LIR2, or HA-MCL-1-LIR3. Cells were treated with DMSO or 25μM FCCP for 2 hours and then stained for TOM20 (red). Colocalization between mitochondria and GFP-LC3 positive vesicles was assessed by immunofluorescence microscopy. Scale bar: 10μm **B.** Quantification of the number of GFP-LC3 puncta colocalized with mitochondria (n.s., not significant; n=3).

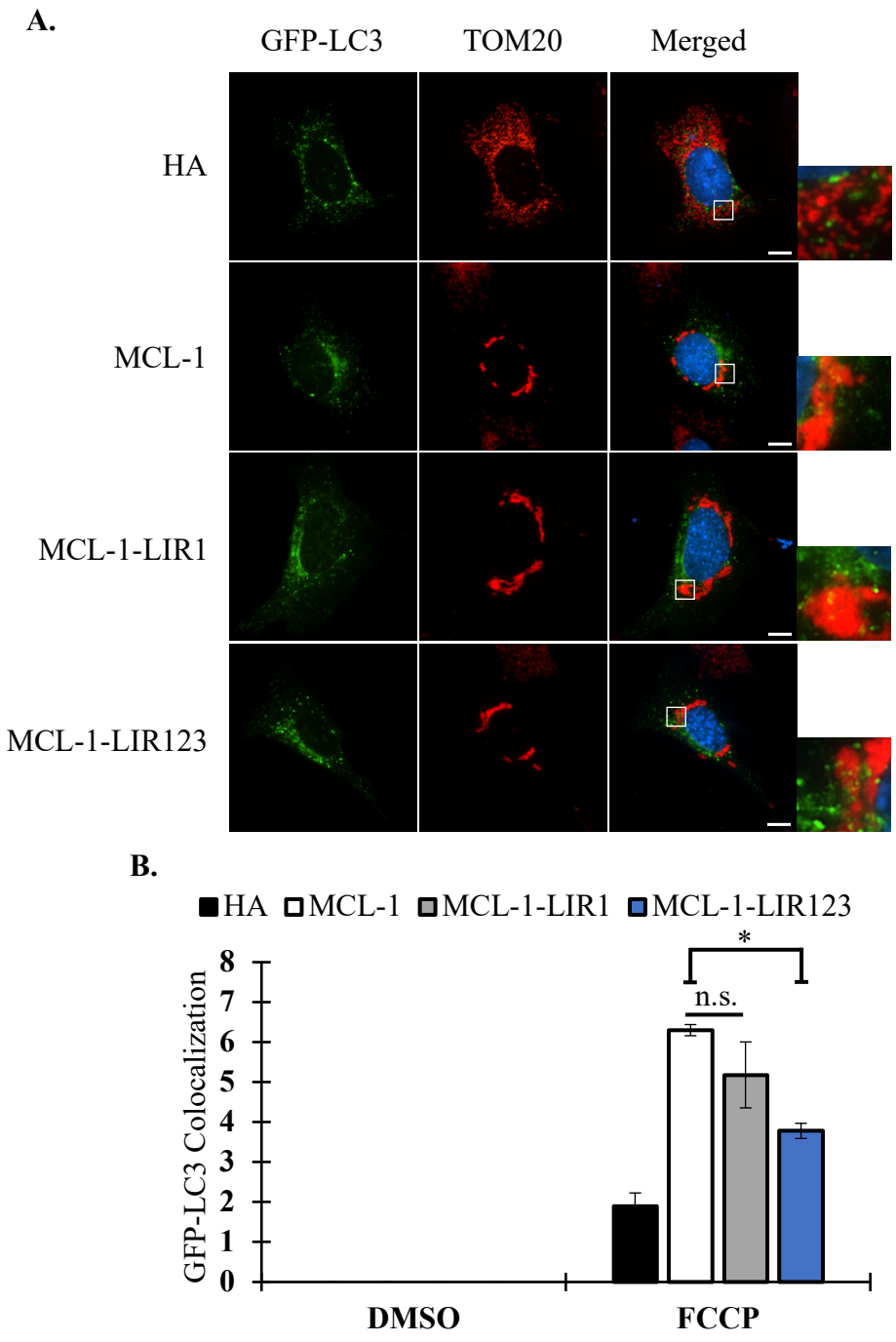


Figure 10. Mutating all three LIR motifs of MCL-1 leads to a reduction in mitophagy. **A.** MEFs were transfected with GFP-LC3 and either HA, MCL-1, MCL-1-LIR1, or MCL-1-LIR123. Cells were treated with DMSO or FCCP for 2 hours and then stained for TOM20 (red). Colocalization between mitochondria and GFP-LC3 positive vesicles was assessed by immunofluorescence microscopy. Scale bar: 10μm **B.** Quantification of the number of GFP-LC3 puncta colocalized with mitochondria (*$p<0.05$; n.s., not significant; n=3).

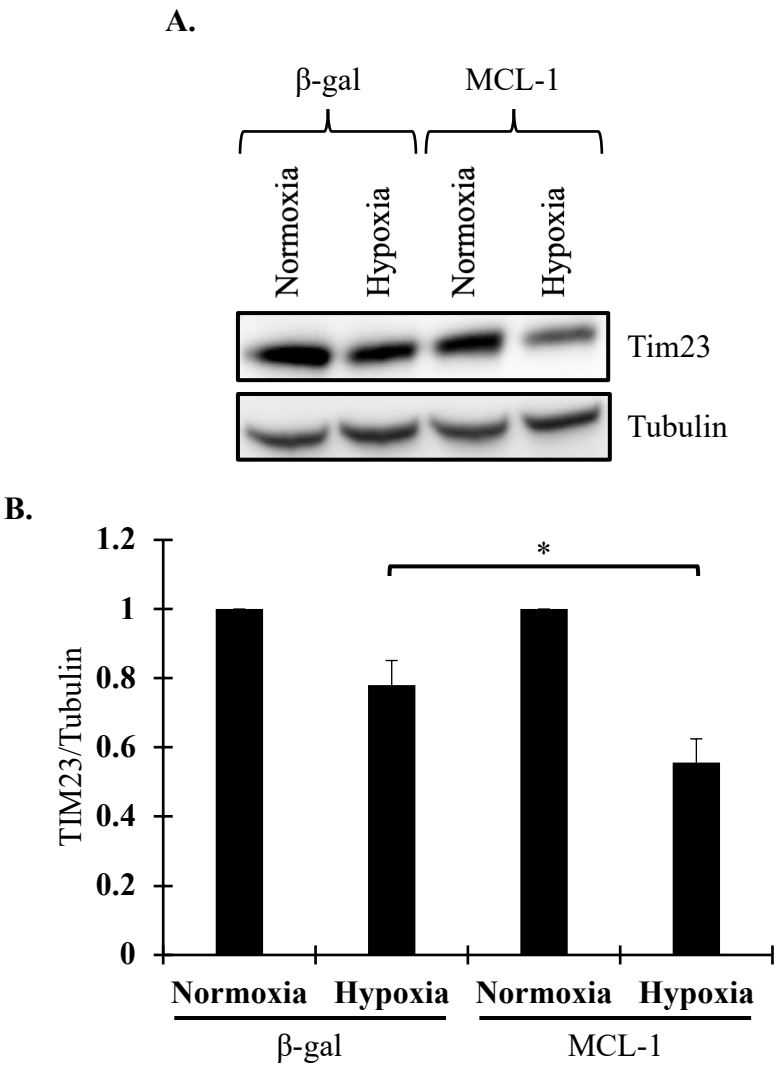


Figure 11. MCL-1 promotes mitochondrial clearance during hypoxia. **A.** Western blot analysis of TIM23 after MEFs were infected with Ad-β-gal or Ad-HA-MCL-1 and subsequently subjected to hypoxia or control conditions (normoxia) for 24 hours. Tubulin was used a loading control. **B.** Quantification of TIM23 levels (*$p<0.05$; n=4).

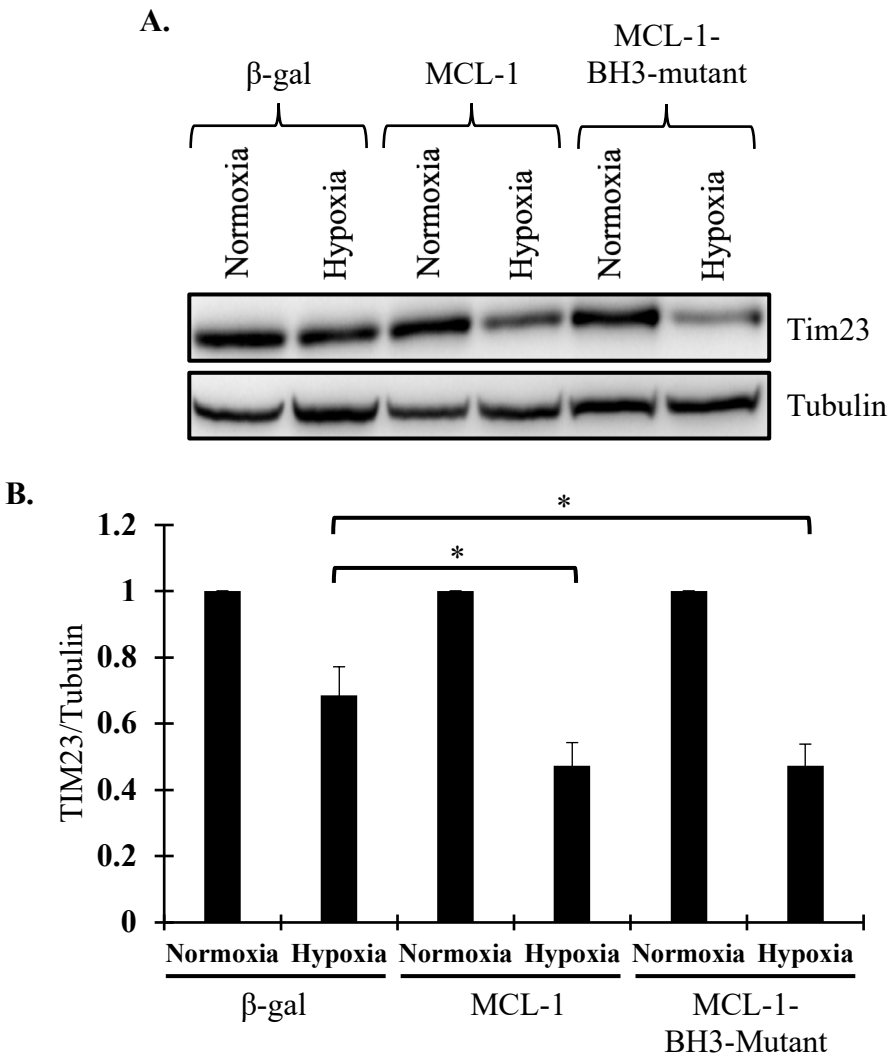


Figure 12. Mutating MCL-1's BH3-domain does not affect MCL-1 mediated mitochondrial clearance. **A.** Western blot analysis of TIM23 after MEFs were infected with Ad-β-gal, Ad-HA-MCL-1, or Ad-HA-MCL-1-BH3-mutant and subsequently subjected to hypoxia or control conditions (normoxia) for 24 hours. Tubulin was used a loading control. **B.** Quantification of TIM23 levels (*$p<0.05$; n=4).

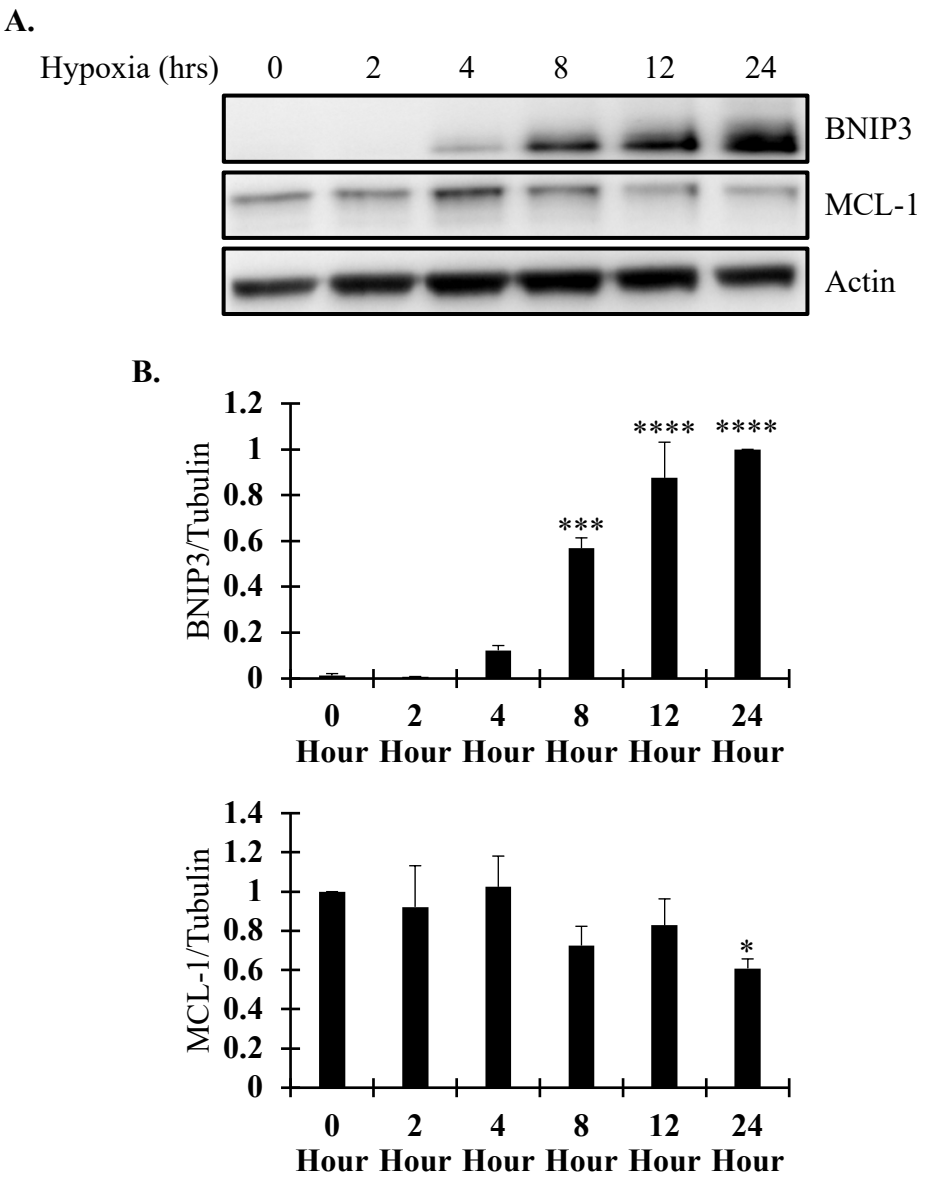


Figure 13. BNIP3 levels significantly increase during hypoxia. **A.** Western blot analysis of BNIP3 and MCL-1 after MEFs were subjected to hypoxic conditions for 2, 4, 8, 12, and 24 hours. Actin was used as a loading control. **B.** Quantification of BNIP3 and MCL-1 levels (***$p<0.001$; ****$p<0.0001$; n=4).

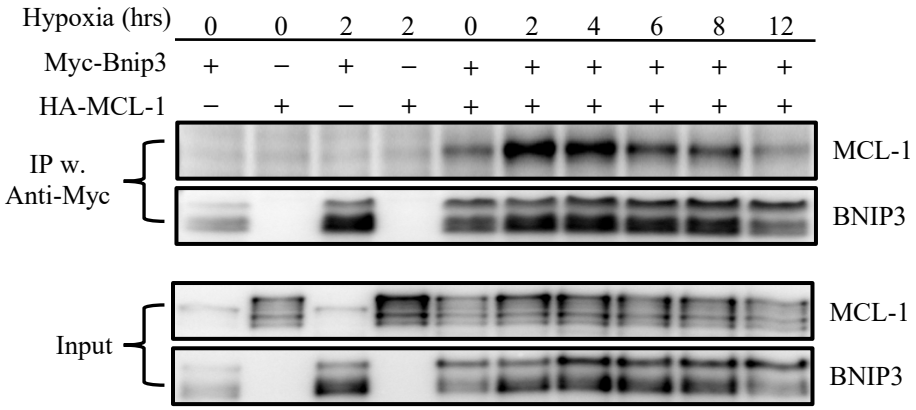


Figure 14. Coimmunoprecipitation of HA-MCL-1 with Myc-BNIP3 during hypoxia. HA-MCL-1 and Myc-BNIP3 were co-overexpressed in MEFs. Cells were subjected to either control conditions (0 hr) or hypoxia for 2, 4, 6, 8, or 12 hours and immunoprecipitation was performed using anti-Myc antibody. Representative western blot from 3 independent experiments.

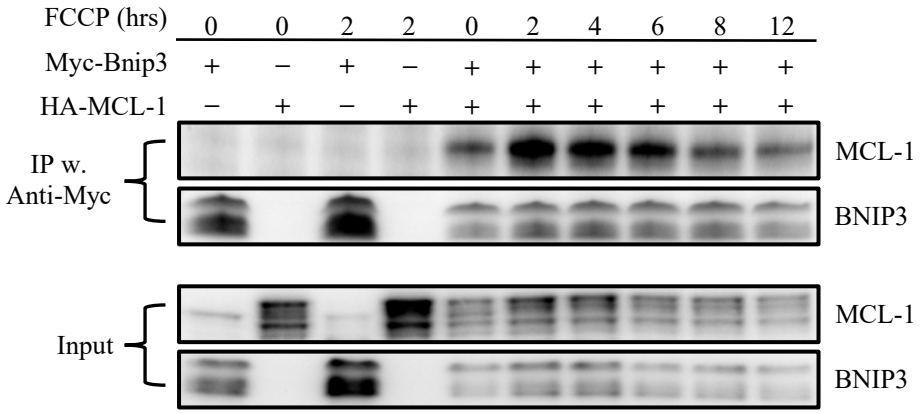


Figure 15. Coimmunoprecipitation of HA-MCL-1 with Myc-BNIP3 during FCCP treatment. HA-MCL-1 and Myc-BNIP3 were co-overexpressed in MEFs. Cells were treated with DMSO or 25μM FCCP for 2, 4, 6, 8, or 12 hours and immunoprecipitation was performed using anti-Myc antibody. Representative western blot from 3 independent experiments.

Chapter 5: Summary

The data presented describe possible mechanisms for MCL-1's critical function at the mitochondria. MCL-1 promotes Drp1-mediated mitochondrial perinuclear clustering as a protective mechanism. Mutation of MCL-1's BH3 domain inhibits its anti-apoptotic function and abrogates MCL-1's ability to facilitate mitochondrial clustering. However, replacing MCL-1's BH-domains with those of BCL-2 does not alter this function. Furthermore, MCL-1 promotes the clearance of damaged mitochondria in response to hypoxia and FCCP. We found that MCL-1 contains 3 putative LIR motifs, and mutation of these motifs had little effect on MCL-1 mediated mitophagy; suggesting that MCL-1 is not a mitophagy receptor. Interestingly, the pro-mitophagy function of MCL-1 is independent of its function as an anti-apoptotic protein. Mutation of MCL-1's BH3-domain caused no changes to MCL-1 facilitated mitochondrial clearance. Furthermore, our data suggest that MCL-1 interacts with BNIP3 to promote mitophagy in cells.

Chapter 6: Discussion and Conclusion

Our results provide important insight into new functions of MCl-1 in regulating mitochondrial dynamics and turnover. First, we demonstrated that MCL-1 facilitates morphological changes in the mitochondrial network by promoting Drp1-mediated mitochondrial fission and subsequent perinuclear clustering. Second, we determined that MCL-1-mediated mitochondrial fission is protective and might be part of its anti-apoptotic activity. Lastly, MCL-1 facilitates mitophagy which might occur through its interaction with the mitophagy receptor BNIP3. Thus, our findings demonstrate an important role for MCL-1 in mitochondrial maintenance.

Mitochondrial fission is controlled by the large GTPase Drp1, and Drp1-mediated fission has been linked to apoptosis. Several studies of the intrinsic apoptotic cell death pathway have shown that mitochondrial dynamics are an important piece of the signaling cascade. For instance, inhibition of Drp1-mediated mitochondrial fission has been shown to delay caspase activation and cell death (Youle & Karbowski, 2005). Interestingly, our data demonstrate that Drp1-mediated fission by MCL-1 is protective during nutrient deprivation and that abrogating fission disrupts the protective effect of MCL-1. Thus, the mitochondrial fission and perinuclear aggregation by MCL-1 is an adaptive response that leads to MCL-1 mediated protection from cell death. Our data suggests that MCL-1-mediated fission is protective, and that the fission required an intact BH3 domain. However, further studies are needed to dissect whether this is part of MCL-1's anti-apoptotic response in cells.

Another interesting and unexpected finding in our study was that mutation of MCL-1's BH3 domain abrogated MCL-1 mediated perinuclear clustering of mitochondria. The anti-apoptotic members of the BCL-2 family of proteins are known to antagonize apoptosis by

interacting with pro-apoptotic proteins through their shared BH-domains (Giam *et al*., 2008). The inability of the MCL-1-BH3-Mutant to induce mitochondrial aggregation suggests that MCL-1's anti-apoptotic activity might be coupled to its effects on mitochondrial dynamics.

Another key finding in our study is that MCL-1 promotes mitochondrial clearance in response to FCCP and hypoxia. However, the mechanism through which MCl-1 facilitates mitophagy is still under investigation. FCCP rapidly depolarizes the mitochondrial membrane which is largely known to be the initiator for PINK1/Parkin-mediated mitophagy (Moyzis *et al*., 2015). However, MCL-1-mediated mitophagy is independent of Parkin as MEFs lack endogenous Parkin. Our data also suggest that MCL-1 is most likely not functioning as a mitophagy receptor. Mitophagy receptors contain LIRs that are needed to tether mitochondrial to the autophagosomes and mutating the LIRs leads to disruption of their function (Hanna *et al*., 2012). However, we found that individually mutating MCL-1's three putative LIR motifs had no effect on MCL-1's ability to induce mitophagy. Although mutating all three putative LIR motifs led to a decrease in mitophagy, it is possible that the point mutations are interfering with other MCL-1 functions. Additional experiments are needed to confirm that the MCL-1-LIR mutants are still as effective as MCL-1 WT in clearing mitochondria in response to hypoxia and FCCP treatment. This suggest that there may be another mechanism through which MCL-1 promotes mitophagy.

It is more likely that MCL-1 is promoting mitophagy through a mitophagy receptor, such as BNIP3. MCL-1 and BNIP3 have been shown regulate apoptosis during hypoxia through one another and our results clearly demonstrate that MCL-1 and BNIP3 interact during hypoxia and that this interaction increases after 2 hours (Chen *et al*., 2016). The same transient increase in their interaction was observed in response to FCCP treatment. Hence, it is possible that MCL-1 and BNIP3 interact during acute cellular stress to remove aberrant mitochondria. During chronic stress,

MCL-1 levels are reduced and BNIP3 switches to a pro-apoptotic protein. Further studies are needed to confirm the relationship between MCL-1 and BNIP3 in regulating mitophagy and cell death.

Additional research is clearly needed to fully elucidate MCL-1's complete function at the mitochondria, however, our data has confirmed that MCL-1 is involved in regulating mitochondrial dynamics and clearance. We have previously shown that loss of MCL-1 in the heart is lethal, and loss of the functions of MCL-1 that were demonstrated in this study may be contributing factors. Furthermore, MCL-1 has been widely studied in regard to cancer as its expression has been shown to be high in different malignant tumors (Xiang *et al.*, 2018). Chemotherapeutics targeting MCL-1 have been studied, however, it is clear that MCL-1 contributes much more than just its anti-apoptotic activity to maintain cellular homeostasis. Our studies suggest that therapies targeting MCL-1 must be tightly regulated because these other pathways that MCL-1 is involved with may be unintentionally disrupted.

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