UNIVERSITY OF CALIFORNIA SAN DIEGO

Effect of Perm1 Overexpression on OxPhos, Mitochondrial Dynamics, and Apoptotic Signaling Pathway Proteins Following Myocardial Ischemia-Reperfusion

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

TzuTung Lin

Committee in charge:

Professor Yoshitake Cho, Chair
Professor Kimberly Cooper, Co-Chair
Professor Jon C. Armour

2024

The Thesis of TzuTung Lin is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2024

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LIST OF ABBREVIATIONS

IR	Ischemia-reperfusion
Perm1	PGC-1/ERR-induced regulator in muscle 1
PGC-1	Peroxisome proliferator-activated receptor γ coactivator-1
ERR	Estrogen-related receptor
ETC	Electron transport chain
TCA	Tricarboxylic acid
ROS	Reactive oxygen species
SDH	Succinate dehydrogenase
OxPhos	Oxidative phosphorylation protein complex
Ckmt2	Mitochondrial creatine kinase 2
Drp1	Dynamin-related protein 1
MICOS	Mitochondrial contact site and cristae organizing system
MAPK	Mitogen activated protein kinase
JNK	c-Jun N-terminal kinase
p-JNK	Phosphorylated c-Jun N-terminal kinase
p-p38	Phosphorylated p38
Akt	Protein kinase B
p-Akt	Phosphorylated protein kinase B
IPC	Ischemic preconditioning

ACKNOWLEDGEMENTS

I would like to thank Dr. Yoshitake Cho for his mentorship and guidance for the past 3 and a half years.

I would like to thank members of the Cho lab for their guidance and support.

I would like to acknowledge the Brown lab and the Ross lab for providing technical support for experimentation.

Chapter 2 of this thesis also contains unpublished data co-authored with Yoshitake Cho and Shizuko Tachibana of the Cho lab at UCSD. The thesis author was the primary author of this chapter.

ABSTRACT OF THE THESIS

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Master of Science in Biology

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Proper contractile function of the heart requires the balance between energy demand and supply to be constantly maintained. This balance is disrupted by ischemia-reperfusion and leads to cardiac functional defects. Perm1 is a novel protein mainly expressed in skeletal and cardiac muscle and interacts with mitochondrial biogenesis encoding genes. Overexpression of Perm1 reduces cardiomyocyte damage and death following hypoxia-reoxygenation. However, the mechanism of Perm1 function remains unclear. We utilized Perm1 overexpression in transgenic

mice to evaluate the alterations in protein expression of various mitochondrial and cellular proteins in response to ischemia-reperfusion. We demonstrated that Perm1 overexpressed hearts did not affect cardiac mass and function at baseline and reduced infarct size upon global ischemia-reperfusion. Further experimentation is needed to verify my results and investigate Perm1 association with other processes of mitochondrial bioenergetics, mitochondrial dynamics, and apoptotic signaling.

CHAPTER I: Background on Cardiac Bioenergetics and Perm1

The heart is one of the most essential organs of the human body as heart (cardiac) muscles are constantly at work to pump blood throughout the body. Thus, the heart has a high energy demand [2,3]. Mitochondria serve as the primary source of energy production for the heart, making up nearly one-third of the volume of adult heart muscle cells (cardiomyocytes) [2]. The energy demand of the heart is supported by multiple sources of fuel, with fatty acids serving as the primary source [2, 3], followed by glucose oxidation, lactate oxidation, and glycolysis [3]. Cardiac function sets the requirement for energy production in the mitochondria to maintain constant ATP content in the myocardium [1,2]. Moreover, the heart is metabolically flexible in its ability to adjust fuel preference to adapt to physiological stress [2,3]. Energy metabolic processes, such as fuel oxidation rate, oxidation rate of reducing agents FADH2 and NADH, flux through electron transport chain (ETC), and oxygen consumption are also linked with one another and regulated by regulatory enzymes, inhibitory or stimulatory metabolites, and metabolic proteins [1]. When there is a defect in the balance between energy production and energy demand, mitochondrial dysfunction can occur, resulting in cardiac dysfunction.

When the balance between the energy reservoir supplied by the mitochondria and the energy demand of the cardiac muscle (myocardium) is disturbed, myocardial energetic stress occurs [2]. Disruption can be due to a lack of oxygen (hypoxia) caused by a lack of blood flow (ischemia) to the heart, resulting in reduced oxidative phosphorylation (OxPhos). Energy deficit is commonly observed in cardiac muscle pathologies (cardiomyopathies) such as heart failure, due to the reduced ability of the mitochondria to support contractile function [2]. Failing hearts also lose metabolic flexibility [3], further contributing to the energy deficit. Although fatty acids remain the primary fuel source, the mitochondria’s abiity to utilize the electron transport chain

(ETC) for oxidative phosphorylation is significantly reduced, leading the mitochondria to increasingly depend on glucose utilization and glycolysis for ATP production [3]. Not only is glycolysis unable to produce enough ATP to support the heart's energy demand, but increased glycolysis coupled with lack of oxidation of glycolytic intermediates also causes accumulation of these intermediates [3]. Accumulation of glycolytic intermediates contributes to mitochondrial overload of calcium ion (Ca^{2+}) that can activate cell death (apoptosis) signaling pathways and activation of signaling pathways involved in cardiac remodeling [3]. These processes further contribute to mitochondrial and cardiac dysfunction. Cardiac function relies heavily on the mitochondria, and mitochondrial function, in turn, relies heavily on oxidative phosphorylation. Thus, energy deficiency leads to mitochondrial dysfunction that can result in cell damage and death, eventually leading to cardiac dysfunction. In other words, the ability of energy production through oxidative phosphorylation in the mitochondria to meet the cardiac energy demand is key to stable heart function.

A heart attack (myocardial infarction), producible by coronary artery occlusion, is one cardiomyopathy that can lead to heart failure. During myocardial infarction, the heart is subjected to ischemia, causing hypoxia in cardiomyocytes. While restoration of blood flow (reperfusion) is necessary for oxygen and nutrient supply [4], it aggravates existing ischemic injury, ultimately leading to cell death. Ichemia-reperfusion (I/R) injury can be separated into primary injury induced by ischemia and secondary injury induced by reperfusion. Ischemia-induced hypoxia causes dysfunction of the ETC and forces the mitochondria to rely on glycolysis and anaerobic metabolism to produce ATP [5], as previously mentioned. Anaerobic metabolism leads to decreased production of ATP and antioxidants that are important for reducing reactive oxygen species (ROS) [5], a byproduct of oxidative phosphorylation that can damage DNA and

cellular components. Cellular sodium-potassium ATPase pumps and calcium ATPase pumps also start to fail due to a lack of ATP activation, leading to an accumulation of electrolytes such as sodium (Na^+), calcium (Ca^{2+}), and hydrogen (H^+) in the cell [5]. Electrolyte retention leads to cell swelling and impaired enzymatic activity [5]. These processes induced by ischemia set the stage for further damage during reperfusion.

During reperfusion, the sudden increase in oxygen availability and switch to oxidative metabolism result in the rapid generation of ROS, which accumulates due to the low levels of antioxidative agents [5]. Together with accumulated Ca^{2+} overload, excessive concentrations of ROS can trigger the opening of mitochondrial permeability transition pores (mPTP) on the outer mitochondrial membrane (OMM), stimulating the cytochrome c release into the cytosol [2,5]. Cytochrome c, an electron carrier in ETC, can also trigger the caspase cascade when released into the cytosol, leading to the activation of apoptotic pathways, eventually resulting in cell death [2,5]. Due to ischemia-reperfusion injury, outcomes have not been optimal in myocardial infarction cases in the clinical field [5].

Current therapeutic strategies against ischemia-reperfusion injury include pre-ischemia conditioning, post-ischemia conditioning, and pharmacological interventions. These therapies aim to reduce ROS-induced cell damage by extending ischemia, inhibiting oxidative phosphorylation, or inducing autophagy [17]. Numerous experiments continue to be performed in search of more effective therapeutic strategies and preventative measures, and many scientists have directed their attention to genes relating to mitochondrial biogenesis and post-transcriptional modifications. Transcriptional regulators that play a crucial role in regulating cardiac mitochondrial function and biogenesis include peroxisome proliferator-activated receptor (PPAR) γ coactivator-1α (PGC-1α) and estrogen-related receptor α (ERRα). The two

transcriptional regulators coactivate each other and target genes involved in glucose metabolism, apoptosis, and mitochondrial functions. These mitochondrial functions include mitochondrial fatty acid oxidation, the tricarboxylic acid (TCA) cycle, electron transport, oxidative phosphorylation, ATP synthesis, and lipid homeostasis [6,7]. PGC-1α interacts with nuclear respiratory factors (NRF) 1 and 2 and mitochondrial transcription factor A (TFAM) to regulate mitochondrial DNA (mtDNA) transcription and mitochondrial biogenesis [4,7]. ERRα targets genes that mediate the ratio of cellular phospho-creatine (PCr) and ATP[8]. It also plays a more direct role in regulating cardiac function by targeting genes that encode cardiac contractile proteins [6]. PGC-1α also protects against apoptosis by targeting the anti-apoptotic extracellular signal-related kinases (ERK) pathways [6]. Previous studies have indicated that a loss of PGC-1α showed reduced coupling of ATP synthesis to oxygen consumption [6], blunted ATP production, and increased susceptibility to the development of heart failure following exposure to physiological stress [10]. Animal models with deletion of ERRα demonstrated increased pathological hypertrophy, reduced capability to maintain the PCR/ATP ratio, and contractile defects when the heart is under physiological stress [8]. PGC-1α and ERRα are thus crucial components involved in maintaining cardiac function.

In addition, other members and coactivators of the PGC-1/ERR family also play essential roles in the regulating mitochondrial function. PPARδ, another transcription factor that coactivates with PGC-1α, regulates the expression of genes that encode mitochondrial proteins involved in fatty acid and glucose oxidation [6]. ERRγ, which acts similarly to ERRα, serves as the primary regulator of nuclear-encoded mitochondrial genes and coordinates the postnatal metabolic transition from glucose dependence to oxidative metabolism [9]. ERRγ-null mice demonstrated metabolic and cardiac abnormalities that resulted in death in the early postnatal

period [9]. These findings suggest that downregulation of PGC-1/ERR pathways, which is observed in heart pathology such as heart failure, may underlie the energy imbalance and mitochondrial dysfunction that consequentially leads to cardiac dysfunction [6,10]. PGC-1/ERR plays key roles in mitochondrial biogenesis and oxidative metabolism.

PGC-1/ERR interacts with a protein called Perm 1 (PGC-1/ERR-induced regulator in muscle 1), which was first found by Cho and colleagues [11]. Perm1 is mainly expressed in skeletal muscle and cardiac muscle [11]. The *Perm1* gene includes a nuclear localization signal and nuclear export signals that indicate Perm1 protein localization to the nucleus and an Ø*XX*LL-type motif that allows protein-protein interaction [11]. The gene also contains an ERRα response element (ERRE), allowing the binding of ERRα to the *Perm1* gene and induction of the protein by PGC-1α/ERRα [11,12]. The Perm1 protein acts downstream of the two transcriptional regulators and regulates specific PGC-1α/ERRα target genes [11,12]. As a component of the PGC-1/ERR pathway, Perm1 also plays an important role in mitochondrial biogenesis and oxidative metabolism.

Previous experiments have studied Perm1 extensively in skeletal muscle. In accordance with the nuclear localization and export signals found on the *Perm1* gene, the Perm1 protein is mainly localized in the nucleus and cytosol of skeletal myotubes [11]. Perm1 maintains basal and PGC-1α-induced mitochondrial oxidative capacity [11]. Increased Perm1 expression in skeletal muscle tissue showed increased mitochondrial DNA (mtDNA) content, increased expression of mtDNA genes that encode for the OxPhos complexes, consequential increased abundance and activity of OxPhos complexes, and increased expression of other mitochondrial enzymes involved in oxidative metabolism [12]. On the other hand, depletion of Perm1 showed reduced basal levels and PGC-1α-induced increases in the abundance of OxPhos complexes [11]. In

skeletal muscle, signaling and gene induction following exercise can induce positive adaptations such as increases in increases in the abundance of mtDNA, OxPhos complexes, and mitochondrial enzymes involved in oxidative metabolism [13]. Depletion of Perm1 showed attenuation of these exercise-induced adaptations [13]. These findings confirmed the role of Perm1 in skeletal muscle and also suggested expectations for a similar role for Perm1 in cardiac muscle.

While much has been found regarding the function of Perm1 in skeletal muscle, the full role of Perm1 in the heart remains to be elucidated. A study by Oka et al. found that altered Perm1 expression also affected *ERRa* expression. They also suggested that Perm1 may play a role in stabilizing PGC-1a expression through post-transcriptional modifications and keep PGC-1a within the optimal range of abundance [16]. These findings confirmed the interaction between Perm1 and PGC-1a/ERRa in the heart. A recent study by Cho et al. looked at Perm1 in cardiac muscle. They found that the Perm1 protein is abundant in mitochondrial fractions than cytosolic or nuclear fractions [14]. This was supported by another study that provided evidence of Perm1 being localized on the outer mitochondrial membrane (OMM) facing the cytosol [15]. The same study also suggested that Perm1 acts as a sensor on the mitochondrial membrane to monitor the energy demand on the mitochondria. Cho et al. suggested that Perm1 localization to mitochondria is facilitated by interactions with other mitochondrial proteins [14], though the protein that may play this role has yet to be determined.

Regarding whether the role of Perm1 relates to mitochondrial bioenergetics in cardiac muscle (CM), Cho et al., found that the Perm1 protein was continuously upregulated throughout the developmental stages, parallel to the expression patterns of mtDNA, OxPhos, *PGC-1*, and *ERR* genes [14]. Perm1, mtDNA, and OxPhos continued to increase as the heart matured

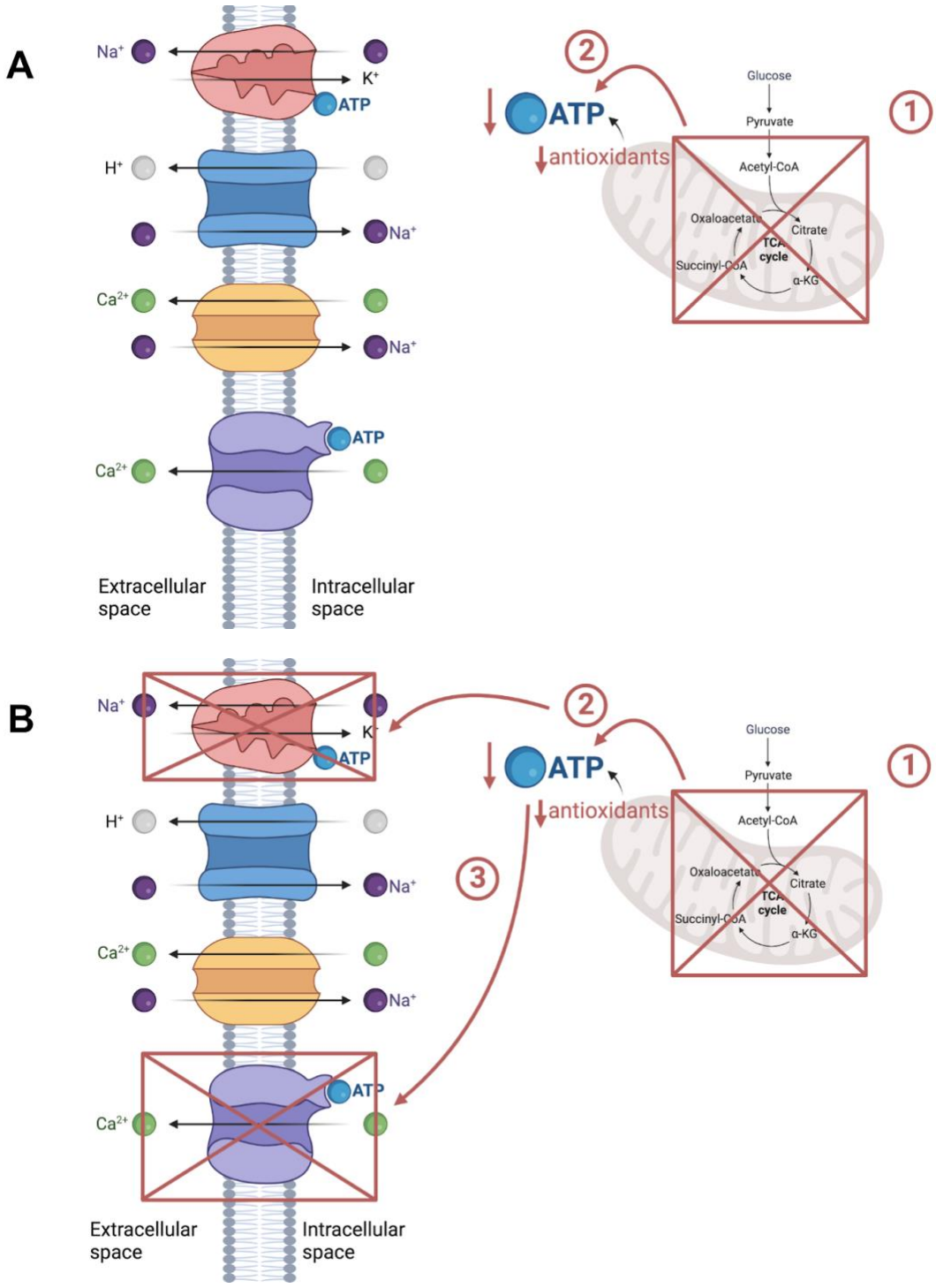
postnatally [14], suggesting that Perm1 contributes to heart maturation. While a different study found that knockdown of *Perm1* had no effect on mtDNA, mitochondrial proteome, and respiratory capacity of the heart [15], Cho et al. found that Perm1 protein overexpression in cardiomyocytes increased mtDNA content, OxPhos protein expression, *PGC-1* and *ERR* expression [14]. Oka et al. provided evidence that the protein positively regulates the expression of genes encoding OxPhos complex subunits. Depleting Perm1 resulted in downregulating gene expression of key enzymes in the TCA cycle and ETC [16]. These findings indicate the involvement of Perm1 in mitochondrial biogenesis and oxidative metabolism. Cho et al. also performed an experiment in which they subjected cardiomyocytes to hypoxia-reoxygenation and found that Perm1 overexpression reduced cellular damage and apoptosis [14]. Oka, et al. also performed a similar experiment, in which cardiomyocytes were subjected to phenylephrine-induced hypertrophic stress, and Perm1 was capable of restoring the expression of genes involved in mitochondrial energetics [16]. Though these studies suggest that Perm1 plays a cardioprotective role and regulates mitochondrial bioenergetics, the full role of Perm1 and how it functions in the heart remain to be further elucidated.

My project aims to gain further insights into the Perm1 function in the cardiac tissue, especially following pathological stress induced by ischemia-reperfusion. Specifically, I explored mitochondrial proteins that may be involved in the Perm1 pathway by looking at how cardiac-specific Perm1 overexpression may affect the protein expression of these proteins. Hearts from transgenic mice, in which Perm1 is selectively over-expressed in the heart, and wild-type mice were subjected to ischemia-reperfusion or sham procedures. Protein lysates from these hearts were then run on western blots with antibodies of different proteins. I sought the possible interactions between Perm1 and proteins involved in mitochondrial bioenergetics and oxidative

metabolism, mitochondrial structure and dynamics, and cellular apoptosis. Trends have shown that elevating Perm1 levels may preserve the function of specific OxPhos complexes and certain core proteins of mitochondrial dynamics while suppressing the activation of apoptosis-inducing protein kinases. As more experiments continue to probe the pathway through which Perm1 functions, our findings may support the possible involvement of Perm1 in novel therapeutic strategies against heart failure and other cardiomyopathies.

Figures

Figure 1.1. Mechanism of ischemia-induced damage in the cell.
During ischemia-reperfusion, ischemia-induced injury sets the stage for further damage and apoptosis during reperfusion. *A.* (1) Ischemia subjects cells to hypoxia. Oxidative phosphorylation through the electron transport chain and the preceding tricarboxylic acid cycle can no longer be supported, leading the mitochondria to rely on energy production by anaerobic metabolism. (2) Glycolysis produces much less ATP compared to oxidative phosphorylation. Thus, cellular concentration of ATP decreases. Production of antioxidants also decreases during anaerobic metabolism. *B.* (3) Decreased concentration of cellular ATP leads to dysfunction of some ion channels that require ATP activation. *C*. (4) Dysfunction of these ion channels affect the flow of ions in and out of the cell, affecting the function of other ion channels and eventually leading to accumulation of ions such as calcium, sodium, and hydrogen. *D.* This accumulation in the cell and mitochondria sets the stage for further damage during reperfusion. (5) It also causes influx of water into the cell, leading to cell swelling. Increased concentration of hydrogen ions in the cell also lowers cellular pH, leading to impaired enzyme activity. [5]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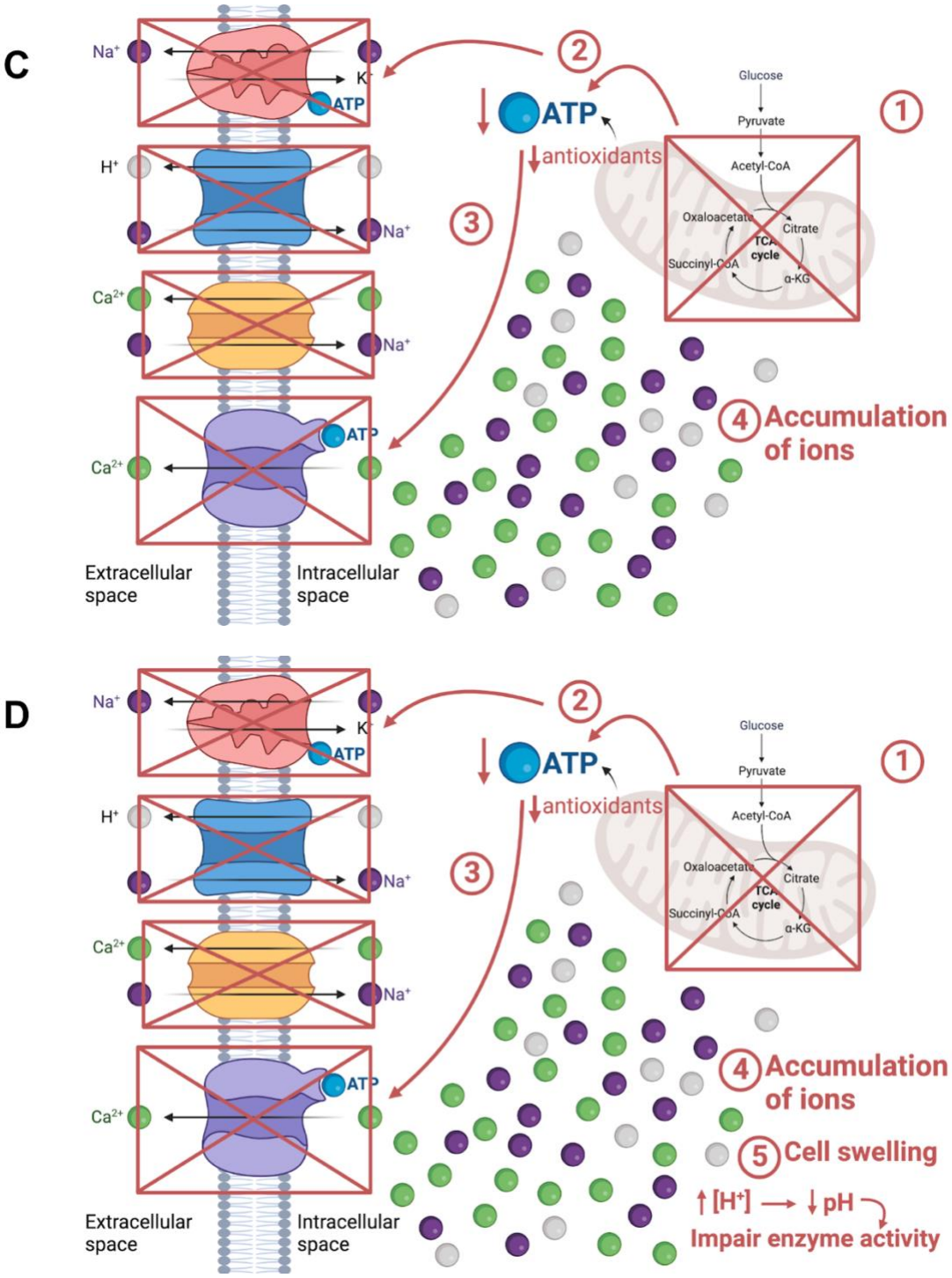
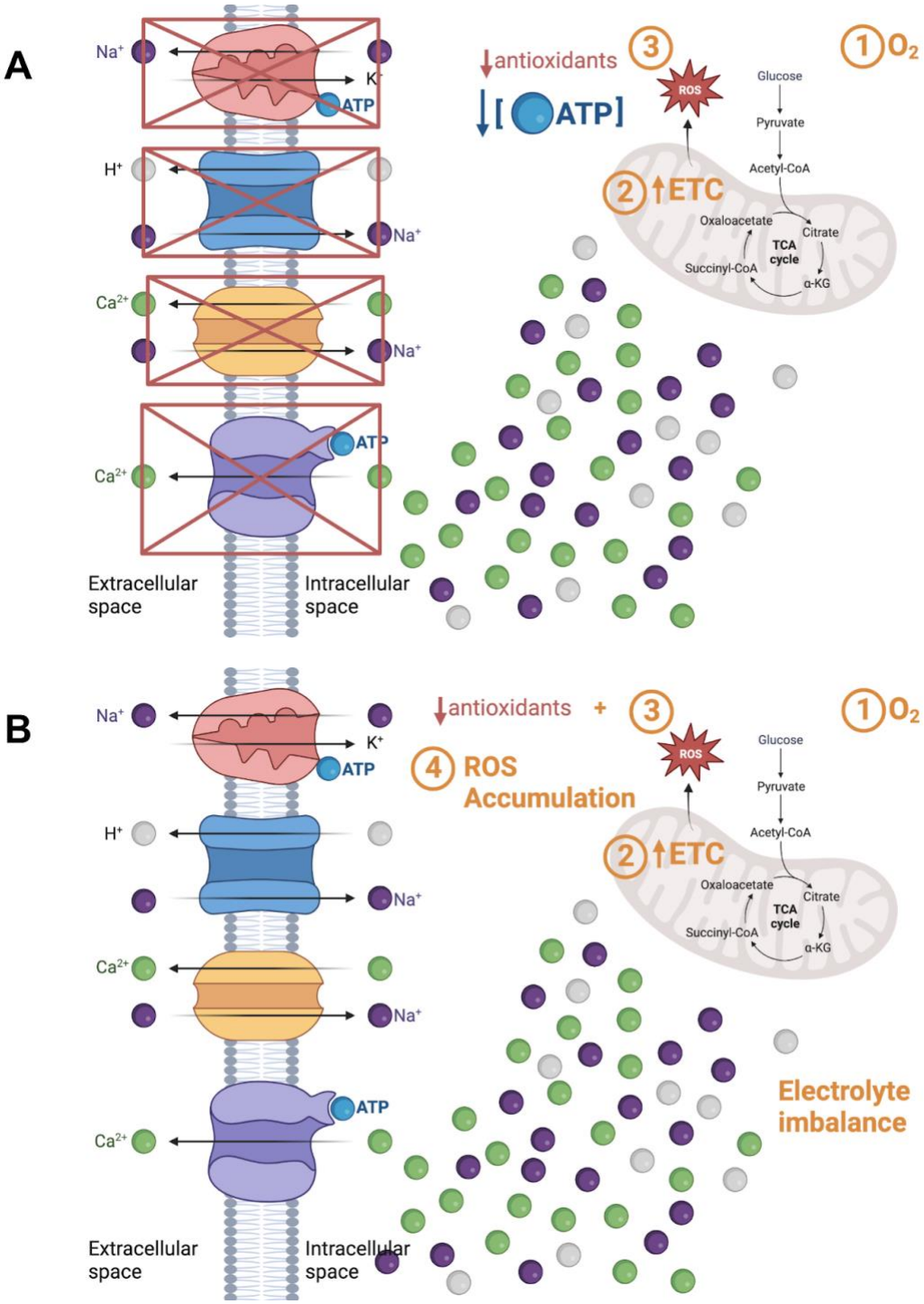


Figure 1.1. Mechanism of ischemia-induced damage in the cell (Continued) .

Figure 1.2. Mechanism of reperfusion-induced damage in the cell following ischemia. At the onset of reperfusion, ischemia had set the stage for further damage with accumulation of electrolytes and decreased concentration of antioxidants and ATP. *A.* (1) As blood flow returns to cardiac tissue, cardiomyocytes gain access to oxygen and other nutrients. (2) Energy production returns to rely on oxidative phosphorylation as the electron transport chain continues to function. (3) In the process, reactive oxygen species (ROS) is produced as a byproduct of electron transfer. *B.* (4) Due to low levels of antioxidants that usually disintegrate the ROS, ROS accumulates in the cell and mitochondria. *C.* (5) In the mitochondria, ROS and calcium ions are overloaded due to the accumulation of these two elements. ROS and calcium ions trigger the opening of mitochondria permeability transition pores (mPTP), releasing cytochrome c from mitochondria cristae into the cytosol. *D.* (6) In the cytosol, cytochrome c activates apoptosomes that further activate the caspase cascade, eventually leading to apoptosis. [5]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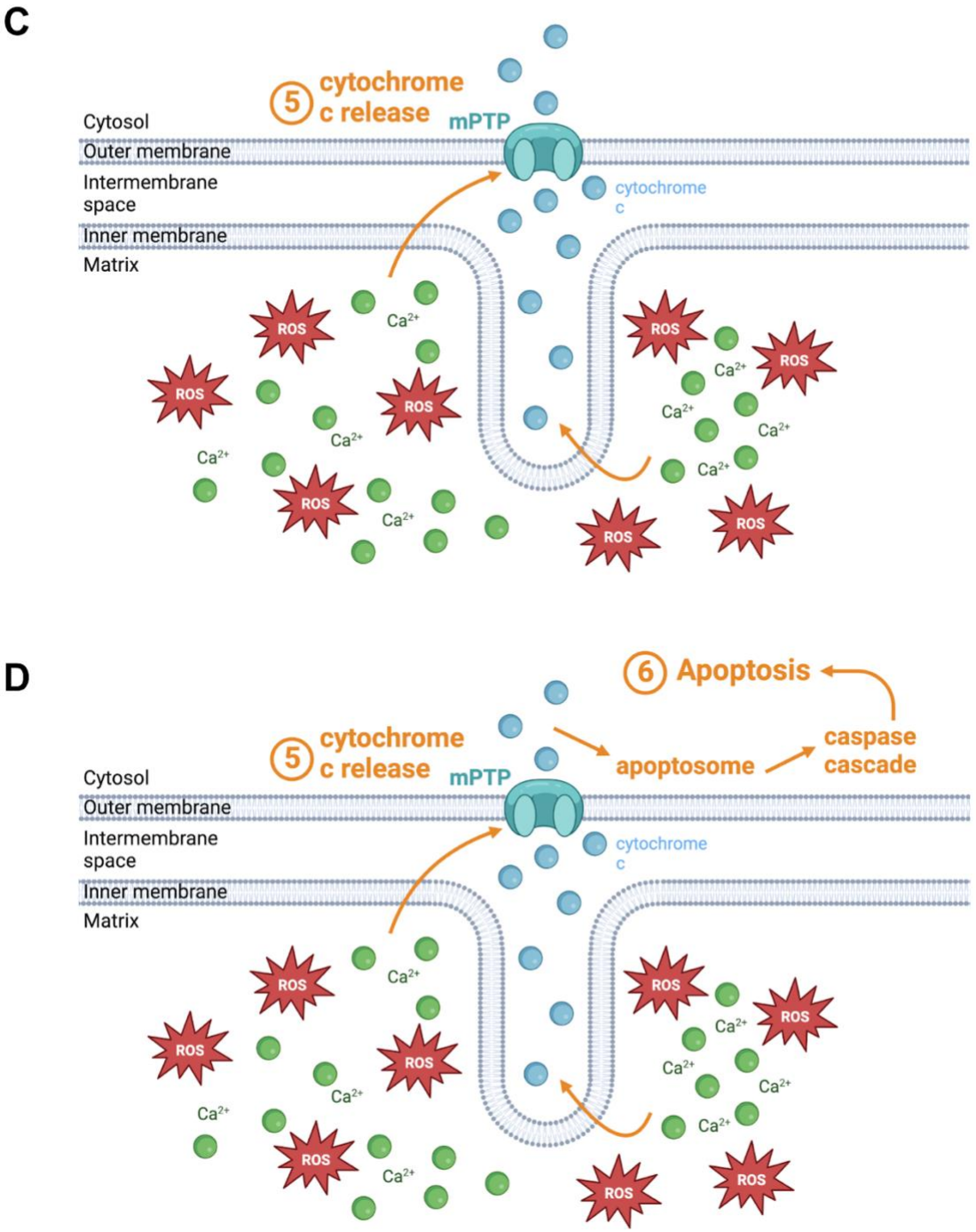


Figure 1.2. Mechanism of reperfusion-induced damage in the cell following ischemia (Continued)

REFERENCES

[1] Stanley, W. C., Recchia, F. A., & Lopaschuk, G. D. (2005). Myocardial substrate metabolism in the normal and failing heart. *Physiological reviews*, 85(3), 1093–1129. https://doi.org/10.1152/physrev.00006.2004

[2] Zhou, B., & Tian, R. (2018). Mitochondrial dysfunction in pathophysiology of heart failure. *The Journal of clinical investigation*, 128(9), 3716–3726. https://doi.org/10.1172/JCI120849

[3] Lopaschuk, G. D., Karwi, Q. G., Tian, R., Wende, A. R., & Abel, E. D. (2021). Cardiac Energy Metabolism in Heart Failure. *Circulation research*, 128(10), 1487–1513. https://doi.org/10.1161/CIRCRESAHA.121.318241

[4] Zhou, M., Yu, Y., Luo, X., Wang, J., Lan, X., Liu, P., Feng, Y., & Jian, W. (2021). Myocardial Ischemia-Reperfusion Injury: Therapeutics from a Mitochondria-Centric Perspective. *Cardiology*, 146(6), 781–792. https://doi.org/10.1159/000518879

[5] Wu, M. Y., Yiang, G. T., Liao, W. T., Tsai, A. P., Cheng, Y. L., Cheng, P. W., Li, C. Y., & Li, C. J. (2018). Current Mechanistic Concepts in Ischemia and Reperfusion Injury. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology*, 46(4), 1650–1667. https://doi.org/10.1159/000489241

[6] Sihag, S., Cresci, S., Li, A. Y., Sucharov, C. C., & Lehman, J. J. (2009). PGC-1alpha and ERRalpha target gene downregulation is a signature of the failing human heart. *Journal of molecular and cellular cardiology*, 46(2), 201–212. https://doi.org/10.1016/j.yjmcc.2008.10.025

[7] Knutti, D., Kressler, D., & Kralli, A. (2001). Regulation of the transcriptional coactivator PGC-1 via MAPK-sensitive interaction with a repressor. *Proceedings of the National Academy of Sciences of the United States of America*, 98(17), 9713–9718. https://doi.org/10.1073/pnas.171184698

[8] Huss, J. M., Imahashi, K., Dufour, C. R., Weinheimer, C. J., Courtois, M., Kovacs, A., Giguère, V., Murphy, E., & Kelly, D. P. (2007). The nuclear receptor ERRalpha is required for the bioenergetic and functional adaptation to cardiac pressure overload. *Cell metabolism*, 6(1), 25–37. https://doi.org/10.1016/j.cmet.2007.06.005

[9] Alaynick, W. A., Kondo, R. P., Xie, W., He, W., Dufour, C. R., Downes, M., Jonker, J. W., Giles, W., Naviaux, R. K., Giguère, V., & Evans, R. M. (2007). ERRgamma directs and maintains the transition to oxidative metabolism in the postnatal heart. *Cell metabolism*, 6(1), 13–24. https://doi.org/10.1016/j.cmet.2007.06.007

[10] Arany, Z., Novikov, M., Chin, S., Ma, Y., Rosenzweig, A., & Spiegelman, B. M. (2006). Transverse aortic constriction leads to accelerated heart failure in mice lacking PPAR-gamma coactivator 1alpha. *Proceedings of the National Academy of Sciences of the United States of America*, 103(26), 10086–10091. https://doi.org/10.1073/pnas.0603615103

[11] Cho, Y., Hazen, B. C., Russell, A. P., & Kralli, A. (2013). Peroxisome proliferator-activated receptor γ coactivator 1 (PGC-1)- and estrogen-related receptor (ERR)-induced regulator in muscle 1 (Perm1) is a tissue-specific regulator of oxidative capacity in skeletal muscle cells. *The Journal of biological chemistry*, *288*(35), 25207–25218. https://doi.org/10.1074/jbc.M113.489674

[12] Cho, Y., Hazen, B. C., Gandra, P. G., Ward, S. R., Schenk, S., Russell, A. P., & Kralli, A. (2016). Perm1 enhances mitochondrial biogenesis, oxidative capacity, and fatigue resistance in adult skeletal muscle. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, *30*(2), 674–687. https://doi.org/10.1096/fj.15-276360

[13] Cho, Y., Tachibana, S., Hazen, B. C., Moresco, J. J., Yates, J. R., 3rd, Kok, B., Saez, E., Ross, R. S., Russell, A. P., & Kralli, A. (2019). Perm1 regulates CaMKII activation and shapes skeletal muscle responses to endurance exercise training. *Molecular metabolism*, *23*, 88–97. https://doi.org/10.1016/j.molmet.2019.02.009

[14] Cho, Y., Tachibana, S., Lam, K., Arita, Y., Khosrowjerdi, S., Zhang, O., Liang, A., Li, R., Andreyev, A., Murphy, A. N., & Ross, R. S. (2021). Perm1 promotes cardiomyocyte mitochondrial biogenesis and protects against hypoxia/reoxygenation-induced damage in mice. *The Journal of biological chemistry*, *297*(1), 100825. https://doi.org/10.1016/j.jbc.2021.100825

[15] Aravamudhan, S., Türk, C., Bock, T., Keufgens, L., Nolte, H., Lang, F., Krishnan, R. K., König, T., Hammerschmidt, P., Schindler, N., Brodesser, S., Rozsivalova, D. H., Rugarli, E., Trifunovic, A., Brüning, J., Langer, T., Braun, T., & Krüger, M. (2021). Phosphoproteomics of the developing heart identifies PERM1 - An outer mitochondrial membrane protein. *Journal of molecular and cellular cardiology*, *154*, 41–59. https://doi.org/10.1016/j.yjmcc.2021.01.010

[16] Oka, S. I., Sabry, A. D., Horiuchi, A. K., Cawley, K. M., O'Very, S. A., Zaitsev, M. A., Shankar, T. S., Byun, J., Mukai, R., Xu, X., Torres, N. S., Kumar, A., Yazawa, M., Ling, J., Taleb, I., Saijoh, Y., Drakos, S. G., Sadoshima, J., & Warren, J. S. (2020). Perm1 regulates cardiac energetics as a downstream target of the histone methyltransferase Smyd1. *PloS one*, *15*(6), e0234913. https://doi.org/10.1371/journal.pone.0234913

[17] Lesnefsky, E. J., Chen, Q., Tandler, B., & Hoppel, C. L. (2017). Mitochondrial Dysfunction and Myocardial Ischemia-Reperfusion: Implications for Novel Therapies. *Annual review of pharmacology and toxicology*, 57, 535–565. https://doi.org/10.1146/annurev-pharmtox-010715-103335

CHAPTER II: Effect of Perm1 Overexpression on Proteins involved in Mitochondrial Bioenergetics

Introduction

Energy production primarily occurs in the mitochondria through oxidative phosphorylation. With glucose as the most efficient source of fuel for energy [1], the process of ATP production by oxidative phosphorylation begins with glycolysis [2], which produces a small amount of ATP and glycolytic intermediate pyruvate [1]. Pyruvate is further processed into acetyl-CoA, which is also produced by fatty acid oxidation and becomes a substrate for the tricarboxylic acid (TCA) cycle [1,4]. The tricarboxylic acid cycle produces reduced NADH and FADH2 [2,3,4], which are later oxidized while electrons are transferred to the electron transport chain (ETC) [2,3]. The electron transport chain (ETC, also referred to as the phosphorylation apparatus) includes four protein complexes located on the cristae membrane. Complex I contains NADH dehydrogenase, which oxidizes NADH while reducing and transferring the electrons to mobile electron carrier coenzyme Q (CoQ) [3, 4]. Complex II oxidizes its substrate succinate and $FADH_2$ while also reducing CoQ [3,4]. While complex III oxidizes CoQ, electrons are transferred to another mobile electron carrier, cytochrome c [3]. Cytochrome c is further oxidized by complex IV, also known as cytochrome oxidase, which eventually transfers those electrons to oxygen to form water [3]. While complexes I, III, and IV oxidize their respective substrates, the energy produced from transferring electrons is used to transport protons into the intermembrane space from the matrix, generating a membrane potential across the inner mitochondrial membrane [2,3]. Complex V, ATP synthase, phosphorylates ADP to ATP through the support of this proton gradient [2, 4]. Maintaining the functionality of ETC is thus crucial to continue supplying energy for cardiac function. Apart from the proteins of the TCA cycle and

ETC, other proteins of the mitochondria also support energy production. In this study, the expression of succinate dehydrogenase, mitochondrial creatine kinase 2, and ETC protein complexes were examined in response to Perm1 overexpression and pressure overload by ischemia-reperfusion.

Succinate dehydrogenase (SDH) plays two roles in the mitochondria. In the TCA cycle, it oxidizes succinate to fumarate. At the same time, in the ETC, it is also complex II and oxidizes $FADH_2$ to transfer electrons to coenzyme Q [3,4,5], as previously mentioned. Succinate dehydrogenase contains a catalytic core domain, composed of subunits A and B, and a membrane anchor domain, composed of subunits C and D [5]. While the membrane anchor domain sequesters succinate dehydrogenase to the inner mitochondrial membrane [5], the catalytic core domain is crucial for SDH function. SDH substrates succinate and $FADH_2$ bind to subunit A (SDHA) where they are oxidized, while subunit B (SDHB) contains iron-sulfur centers that mediate electron transfer activity [5]. The catalytic core domain is primarily assembled by SDH assembly factor 4 (SDHAF4), which is downregulated during cardiac stress [6]. Decreased levels or depletion of SDHAF4 results in decreased assembly and impaired activity of SDH, leading to accumulation of succinate and decreased activity of the TCA cycle, reduced expression of PGC-1α and other bioenergetic regulators, and can lead to dilated cardiomyopathy [6]. Given the role of succinate dehydrogenase as complex II in ETC, the maintenance of its structure is also crucial to ETC function and ATP generation [3]. Succinate dehydrogenase thus plays an important role in maintaining the ATP generation required for normal cardiac function.

Creatine kinase (CK) and mitochondrial creatine kinase (Ckmt2) support phosphorylation apparatus function by circulating inorganic phosphate between creatine and ATP. Ckmt2 localizes in the intermembrane space by binding its octameric structure to cardiolipin in the inner

mitochondrial membrane [7, 8, 9]. Adenine nucleotide translocators (ANT) of inner mitochondrial membrane transport ATP into the intermembrane space [3,7], where Ckmt2 dephosphorylates ATP into ADP while phosphorylating creatine (Cr) into phospho-creatine (PCr) [7, 8, 9]. ANT returns ADP to the matrix [3,7] as voltage-dependent anion channels (VDAC) transports PCr into the cytosol [7,9]. PCr is stored to be used as a temporary energy buffer. During sudden increases in energy demand, PCr is converted to ATP by CK [7,9]. This process also maintains an internal pool of ADP in the mitochondria, keeping the concentration of ADP high to continue driving ATP synthase activity. At the same time, ATP concentration is kept high in the cytosol to continuously support ATP utilization [7,9].

While deficits to the CK system may not affect the heart at rest, these deficits can be significantly detrimental during times of increased energy demand or cardiac stress [9]. A recent study has shown that dephosphorylation of Ckmt2 amino acids may occur during ischemia, causing the breakdown of Ckmt2 structure [10]. Given the role of Ckmt2 in maintaining the mitochondrial membrane potential and the loss of Ckmt2 structural integrity leads to decreased mitochondrial membrane potential and increased production of ROS [10]. Ckmt2 also limits reactive oxygen species (ROS) production by ETC during baseline conditions, and decrease in Ckmt2 activity results in increased levels of ROS [10]. In turn, Ckmt2 structure is also vulnerable to breakdown induced by (ROS) as oxidation of select amino acids of the protein can lead to inactivation of the enzyme active site and reduce Ckmt2 activity [7,10]. Deficiency in CK system activity also contributes to the accumulation of Ca^{2+} ions [9] that can lead to mitochondria-induced apoptosis. On the other hand, increased Ckmt 2 expression correlates with the upregulation of regulators of the antioxidant defense system, reducing ROS levels and cellular damage [8]. Ckmt2 overexpression maintains outer mitochondrial membrane

permeability, reducing proton leak and Ca^{2+} accumulation and decreasing the opening of mitochondrial permeability transition pores (mPTP) [8, 9]. A study also showed that Ckmt2 overexpression can help modulate the function of ETC complexes I and II during oxidative stress, effectively preserving cardiac contractile function [8]. In many ways, Ckmt2 and the CK system play an essential role in maintaining cardiomyocyte viability and energy production..

Our previous study of Perm1 in cardiac muscle found that Perm1 was continuously upregulated in the developing heart from mice embryonic stages to birth and continued to increase in expression as the heart matured postnatally. This expression pattern paralleled ETC protein complexes expression during development [11]. In isolated cardiomyocytes under baseline conditions, increased Perm1 expression correlated with increased expression of nuclear-encoded genes of the ETC complexes and protein complexes expression [11]. When ETC was uncoupled from the phosphorylation apparatus, Perm1 overexpression was also able to enhance the oxygen consumption rate [11]. Another recent study found that knocking down the *Perm1* gene resulted in decreased expression of various genes that encode ETC protein complexes subunits, such as *Ndfb8* and *Ndufs8,* which encode for subunits of complex I, *Cox5a*, *Cox8a* and *Cox6a1*, which encode subunits of complex IV, and *Coq2* and *Coa10b,* which encode subunits of coenzyme Q [12]. The study also found that Perm1 inhibition correlated with downregulation of *Ndufv1* and *Ndufs1*, which encode subunits of complex I [12]. Perm1 overexpression in isolated cardiomyocytes also correlated with increased expression of Ckmt2 and *Ckmt2*, another target gene of PGC-1α/ERRα [11].

In this study, I further explored the correlation between the expression of Perm1 and that of the electron transport chain protein complexes, ATP synthase, and Ckmt2. Utilizing a gain-of-function model, I aimed to determine whether cardiac-specific Perm1 overexpression would

correlate with altered expression of the mentioned proteins in hearts exposed to global ischemia-reperfusion injury. I hypothesize that increasing Perm1 expression will show upregulated protein expression of ETC complexes and Ckmt2 in hearts after ischemia-reperfusion injury.

Materials and Methods

Animal Models

C57BL/6 mice were housed under a 12-h light/dark cycle at constant temperature and given food and water ad libitum [11]. Demonstrated in **Figure 2.1**, mice that had knocked-in Flag-tagged full-length *Perm1* gene with a STOP cassette were interbred with mice that had aMHC-nuclear Cre, producing heterozygous mice with cardiac-specific Cre that removed the STOP cassette. These heterozygous mice were named Perm1cTG transgenic (TG) mice, in which Perm1 was selectively overexpressed in the heart. The wild-type mice group, which did not have elevated cardiac expression of Perm1, and the transgenic group each comprised 12 male mice at 3 to 4 months of age. Among the 12 mice, 8 mice were subjected to ex vivo ischemia-reperfusion, and the remaining 4 mice served as sham controls. Entire hearts were sampled. All experimental procedures performed on animals were approved by the University of California San Diego Institutional Animal Care and Use Committee (IACUC).

Echocardiography in mice

Echocardiography was performed on wild-type and transgenic mice at 12-16 weeks of age (n = 7-8) to determine cardiac performance and physical dimensions of the left ventricle prior to heart isolation. Mice were put under isoflurane anesthesia. Echocardiographic results were measured in M-mode using a Vevo 2100 machine (Visualsonics, Toronto, Canada) [13,14].

Left ventricle (LV) interventricular septal thickness (IVS), internal diameter (LVID), posterior wall thickness (LVPW), ejection fraction (EF), fractional shortening (FS), and heart rate (HR) were measured.

Ischemia/reperfusion by Langendorff apparatus

Mice at 12-16 weeks of age were heparinized with intraperitoneal injection of sodium heparin (1000 U/kg). Fifteen minutes after the heparin injection, the animals were euthanized by cervical dislocation. A laporotomy was performed, and the heart was rapidly excised and placed into ice-cold Krebs-Henseleit buffer containing, NaCl, KCl, KH_2PO_4, $MgSO_4.7H_2O$, $CaCl_2$-$2H_2O$, $NaHCO_3$, EDTA, glucose, and $CaCl_2$ [15]. Within 10 minutes following the excision of the heart, the isolated heart was cannulated and mounted onto the Langendorff heart perfusion apparatus. The hearts were perfused in a retrograde fashion via the aorta with oxygenated Krebs-Henseleit buffer in an atmosphere of 95% O_2 and 5% CO_2 at 37ºC [15]. The hearts underwent at least 10 minutes of stabilization, then subjected to 30 minutes of global ischemia followed by 60 minutes of reperfusion. Sham hearts were subjected to the same procedure with no global ischemia and 90 minutes of perfusion following stabilization (**Figure 2.2**).

TTC staining and infarct size quantification

Following reperfusion, isolated hearts were maintained at -20ºC for 20 minutes. To determine the infarct size, the hearts (n = 4-6) were then sliced into four 2 mm thick sections from base to apex for staining with triphenyl tetrazolium chloride (TTC) at 37ºC for 15 minutes. The myocardium of the infarct area was pale in color while the active myocardium was red.

Infarct size was quantified and the percent infarct area was calculated by taking the ratio of the total infarct area to the total left ventricular volume [16,17].

Western Blot

Following completion of the ischemia-reperfusion protocol, total protein was extracted from the ventricular tissue by homogenesis with homogenizing buffer containing 50 mM Tris-HCL, 1mM EDTA, 1mM EGTA, 0.3M sucrose and supplied with 1% phosphatase protein inhibitors and proteases. Protein concentrations were determined using DC protein assay kit (Bio-Rad Laboratories, Inc.).

Following preparation of total protein lysates, proteins were separated on 8%-14% SDS polyacrylamide gels or 4%-10% commercial gels (Bio-Rad Laboratories, Inc.) and electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% milk in Tris-buffered saline with 0.1% Tween 20 (TBST) at room temperature for 1 hour. The primary antibodies, diluted at 1:1000, were as follows: Perm1, SDHA, Ckmt2, and OXPHOS. GAPDH was used as a loading control. Membranes were incubated with primary antibody overnight at 4ºC. Subsequently, membranes were washed 3 times 10 minutes each with TBST then incubated with horseradish peroxidase-conjugated rabbit or mouse secondary antibody for 45 minutes at room temperature. The membranes were washed again 3 times 10 minutes each in TBST and protein bands were detected by enhanced chemiluminescence. Band intensity was measured using BioRad software, and the results were presented as normalized expression levels relative to that of the wild-type sham group.

Statistical analysis

All data are shown as means ± standard deviations. Student's t-test using 2-tail distribution and unpaired, equal variance was used to evaluate differences between wild-type sham, transgenic sham, wild-type ischemia-reperfusion, and transgenic ischemia-reperfusion groups. Differences $p < 0.05$ were considered to be statistically significant.

Results

Perm1cTG mice showed normal heart function compared to control mice

To examine whether over-expressing Perm1 can affect cardiac function under baseline conditions, echocardiography was performed prior to heart isolation and ischemia-reperfusion. Wild-type and transgenic mice were anesthetized with isoflurane and left ventricle (LV) dimensions (septal thickness, internal diameter, and posterior wall thickness) were measured to compare left ventricle mass and size. As shown in **Figure 2.3**, wild-type and transgenic mice hearts had LV dimensions within the expected range of values [18]. LV dimensions of transgenic mice hearts were comparable to that of wild-type hearts. Ejection fraction (EF), heart rate (HR), and fractional shortening (FS) were measured to assess cardiac function. Similarly to our findings for LV dimensions, EF, HR and FS of transgenic mice hearts displayed values within the standard range [19] and were comparable to that of wild-type mice (**Figure 2.3**). Normal heart size and function in both wild-type and transgenic mice demonstrated that increasing expression of Perm1 did not alter cardiac function and morphology under baseline conditions.

Perm1cTG mice showed elevated Perm1 expression

Perm1 expression was analyzed in transgenic and wild-type mice hearts upon ischemia-reperfusion. Sham hearts showed significantly elevated Perm1 levels compared to hearts subjected ischemia-reperfusion in both wild-type and transgenic mice, as expected (**Figure 2.4**). Among hearts subjected to ischemia-reperfusion, transgenic mice showed significantly elevated Perm1 expression compared to wild-type hearts (**Figure 2.4**). A similar result was observed among sham hearts, indicating that global ischemia-reperfusion induced a reduction in Perm1 expression that is elevated by transgenic expression.

Perm1cTG mice showed reduced infarct size upon ex vivo ischemia-reperfusion injury

To examine possible cardioprotective effects of overexpressing Perm1, TTC staining was used to evaluate infarct size of the transgenic and wild-type mice upon ischemia-reperfusion. Infarct area was quantified and calculated as the ratio of the total infarct size to the total left ventricular volume. Transgenic mice hearts demonstrated significantly smaller infarct size (**Figure 2.5**), suggesting a reduction of cardiac muscle damage by Perm1 overexpression following ischemia-reperfusion injury.

Analysis of succinate dehydrogenase subunit A (SDHA) expression

SDH serves as both an enzyme of the TCA cycle and complex II in the ETC [3,4,5]. SDH subunit A (SDHA) is the binding site for SDH substrates, thus is essential for SDH function as part of the energy production apparatus [5]. Using Western blots and normalizing band intensity to that of WT Sham, I examined whether SDHA expression in the mitochondria is altered by Perm1 overexpression in transgenic mice. WT IR hearts showed lower average normalized band

volume (0.707) than WT sham (1.000) (**Figure 2.6B**). TG sham showed an average normalized band volume of 0.780 while TG IR showed 1.541. The difference in normalized band volumes between WT sham and WT IR, TG sham and TG IR, TG IR and WT IR were not statistically significant ($p > 0.05$).

Analysis of OXPHOS complexes II, III and V expression

The ETC, consisting of complexes I to IV and ATP synthase, is essential to energy generation. Previously, Cho et al. showed increasing expression of ETC complexes that paralleled Perm1 expression throughout the development and maturation of mice hearts [11]. Aiming to examine whether increasing Perm1 expression may result in increased expression of OxPhos complexes in isolated hearts, the expression of complexes II, III and V in transgenic mice were specifically analyzed.

Complex II is succinate dehydrogenase, as previously mentioned. As shown in **Figure 2.7B**, WT IR showed a normalized average band volume of 1.075, close to that of WT sham (1.000). TG sham showed lower normalized average band volume at 0.680 while TG IR showed 0.965. The differences in normalized band volumes between the four heart sample groups were not statistically significant ($p > 0.05$).

Complex III, Q-cytochrome c oxidoreductase, transfers the electrons from CoQ to cytochrome C while contributing to the proton gradient [3,4]. For this ETC complex, WT sham, WT IR, and TG sham showed very similar average normalized band volumes at 1, 0.948, and 0.999 respectively (**Figure 2.7C**). TG IR showed a higher average of 1.167, but differences in normalized band volumes between the groups were not statistically significant ($p > 0.05$).

Complex V is ATP synthase which uses the proton gradient to generate ADP [2,3,4]. WT sham showed an average normalized band volume of 1, higher than that of WT IR (0.7232) and comparable to that of TG sham (1.001). TG IR showed an average of 1.078 (**Figure 2.7D**). The difference in normalized band volumes between WT sham and WT IR, TG sham and TG IR, TG IR and WT IR were not statistically significant ($p > 0.05$).

Analysis of mitochondrial creatine kinase (Ckmt2) expression

Ckmt2 plays a crucial role in regulating ATP production by maintaining the mitochondrial membrane potential and ADP concentration [7,8,9]. Previously, Cho et al. demonstrated that Perm1 overexpression in isolated baseline cardiomyocytes led to increased expression of the *Ckmt2* gene [11]. I aimed to determine whether Perm1 overexpression may lead to an increase in Ckmt2 protein expression in isolated hearts. In **Figure 2.8B**, WT IR showed an average normalized band volume of 1.120 while TG sham showed 0.881. TG IR showed an average of 0.988, comparable to that of WT sham (1.000). The differences in normalized band volumes between WT sham and WT IR, TG sham and TG IR, TG IR and WT IR were not statistically significant ($p > 0.05$).

Discussion

Previous studies of the Perm1 protein in the heart found that increasing Perm1 protein expression in isolated cardiomyocytes in the absence of stress is associated with increased expression of OxPhos proteins (complexes I to IV) and nuclear-encoded OxPhos genes [11], while decreasing Perm1 expression is associated with reduced expression of those genes [12]. The current experiment aimed to further advanced our understanding of Perm1 function by

analyzing how cardiac-specific Perm1 overexpression in a transgenic mouse model alters the expression of other mitochondrial proteins involved in oxidative metabolism.

We confirmed elevated Perm1 protein levels in transgenic mice hearts upon ischemia-reperfusion. These hearts also showed significantly smaller infarct size that that shown by wild-type hearts upon ischemia-reperfusion. This demonstrated that in isolated hearts, Perm1 overexpression also showed cardioprotection.

Oxidative phosphorylation, being the primary mechanism of energy generation for cardiac function, mainly occurs through the functions of the tricarboxylic acid (TCA) cycle, the electron transport chain (ETC), and ATP synthase. One protein complex, succinate dehydrogenase (SDH), plays a role in both processes by oxidizing its substrate succinate and FADH2. While succinate is converted to fumarate in the TCA cycle [5], electrons from FADH2 enter the ETC by being transferred to electron carrier coenzyme Q [3,4]. SDH contains four subunits, with A (SDHA) being the subunit that allows the binding of its substrates [5]. During ischemia, reversal of SDH function [20] or decrease in SDH activity [3] leads to accumulation of succinate. Some studies show that this accumulation not only stalls the TCA cycle but may also induce reverse electron flow from complex II to complex I, leading to the production of reactive oxygen species (ROS) [3]. Other studies suggest this ROS production induced by reverse electron flow occurs during reperfusion, when succinate is rapidly oxidized to keep the Coenzyme Q reduced and to drive continuous electron transfer throughout the ETC [20,21].

The observed differences in SDHA expression between the heart sample groups were not statistically significant. As a result, the data could not be interpreted. The lack of significance is likely due variation in the form of batch effects, as one batch of data showed much lower normalized band volumes than the other batch in all four heart sample groups. If more heart

samples are experimented and normalized band volume from additional samples clustered primarily with either batch, statistical significance may be able to be established to show elevated SDHA expression in IR-subjected hearts with Perm1 overexpression.

As the primary site of oxidative phosphorylation, ETC stands at the intersection between maintaining cardiomyocyte viability and inducing cardiomyocyte damage during IR. As mentioned previously, ETC becomes more susceptible to electron leakage during ischemia and serves as the primary source of ROS production during ischemia [3, 22]. On the other hand, reversibly inhibiting ETC activity during ischemia can preserve its viability and function, reducing the ETC-induced mitochondrial damage during reperfusion [3]. In addition, proteins that play a protective role against cardiac stress associate with ETC protein complexes to maintain ETC structural integrity [23].

As previously mentioned, in isolated cardiomyocytes at baseline, Perm1 overexpression lead to increased OxPhos gene and protein expression [11] while knocking out Perm1 lead to global downregulation of genes encoding ETC protein subunits [12]. In the current study, I analyzed the expression of ETC complexes II, III and V in isolated hearts with or without Perm1 overexpression in the presence of cardiac stress. Similar to SDHA, the observed differences in OxPhos complex II expression between the heart sample groups were not statistically significant. Thus, a valid interpretation could not be made and the data could not be used to corroborate the results of SDHA expression. Variation was present, though not necessarily a result of obtaining two batches of data since batch effects were not as obvious. More samples are needed to determine whether variation is due to batch effects and to reduce variation. Directly using the SDH antibody than OxPhos antibody to analyze complex II expression may also produce clearer results.

Similar to complex II, differences in complexes V and III expression between the heart sample groups were not statistically significant. Again, a valid interpretation cannot be made with the current data. In contrast to the data analysis presented for complex II expression, batch effects were an obvious source of variation in the analyses of complex V and III band intensities. One batch showed much lower normalized band volumes than the other. If additional samples were experimented and data primarily clustered with either batch, they may provide more valid support for the hypothesis that Perm1 overexpression can restore OxPhos complex V and III upon IR. Similar to complex II, directly using anti-ATP synthase and anti-Q-cytochrome c oxidoreductase to analyze protein expression may produce clearer results.

Moreover, a previous study demonstrated decreased activity of the OxPhos complexes despite increased expression in hearts with coronary artery disease [24]. This suggests that the degree of ETC complex expression may not reflect activity. Activity assays can be used to investigate OxPhos activity in IR-subjected hearts with Perm1 overexpression

Oxidative phosphorylation is supported by many other proteins and mitochondrial processes, one of which is the creatine kinase (CK) system. The CK system, comprising of cytosolic CK and mitochondria CK (Ckmt2), circulates inorganic phosphate between ADP and creatine (Cr) to generate an abundant ATP store at the myofibrils of cardiomyocytes in preparation for increases in energy demand [6,7,8]. At the same time, the CK system mediates mitochondrial and cytosolic [ADP]/[ATP] ratios to maintain the drive for ATP synthase activity [7,8,9]. Loss of the CK system can be significantly detrimental to cardiac function and impair recovery of contractile function following ischemia-reperfusion injury [9]. Furthermore, Ckmt2 is sensitive to damage by ROS [7,9], and dephosphorylation of Ckmt2, which may occur during ischemia, can also result in a significant reduction in its activity [10].

A previous study by Cho et al. demonstrated that overexpressing Perm1 in isolated cardiomyocytes is associated with increased Ckmt2 gene and protein expression and suggested that Perm1 may be recruited onto *Ckmt2* promoters [11]. Surprisingly, my results failed to show statistically significant differences in Ckmt2 expression across the four isolated heart sample groups. As a result, the data could not be interpreted and could not corroborate the findings from the previous study. Batch effects were present and could have contributed to the lack of statistical significance in the differences. However, additional variation was also present between the data batches. Additional samples are needed to reduce variation and obtain clearer, more valid results. Experiments investigating Ckmt2 mRNA expression can also be conducted to interrogate Ckmt2 gene and protein expression changes.

Variation between the data batches may be attributed to unintentional ischemic preconditioning (IPC). IPC involves subjecting the heart to brief periods of ischemia followed by reperfusion immediately before prolonged ischemia for cardioprotection [3]. The Langendorff method involving heart isolation and cannulation prior to attachment to the hanging heart apparatus subjects the heart to temporary ischemia before perfusion [25]. Evidence has shown that longer temporary ischemia period can induce IPC on the heart [25]. IPC involves signaling to modulate mitochondrial function and cardioprotection which includes preservation of OxPhos [3]. IPC can also preserve the functional coupling between Ckmt2 and adenine nucleotide translocase (ANT) on the mitochondrial inner membrane [26]. Thus, unintentional IPC may have occurred during the experiment, and the cardioprotective effects may have contributed variation in Ckmt2 levels in the individual heart samples. *Ex vivo* experiments with more samples and shorter temporary ischemia or *in vivo* experiments using transaortic constriction can be conducted to minimize or eliminate the effects of IPC that confound with the results.

Figures

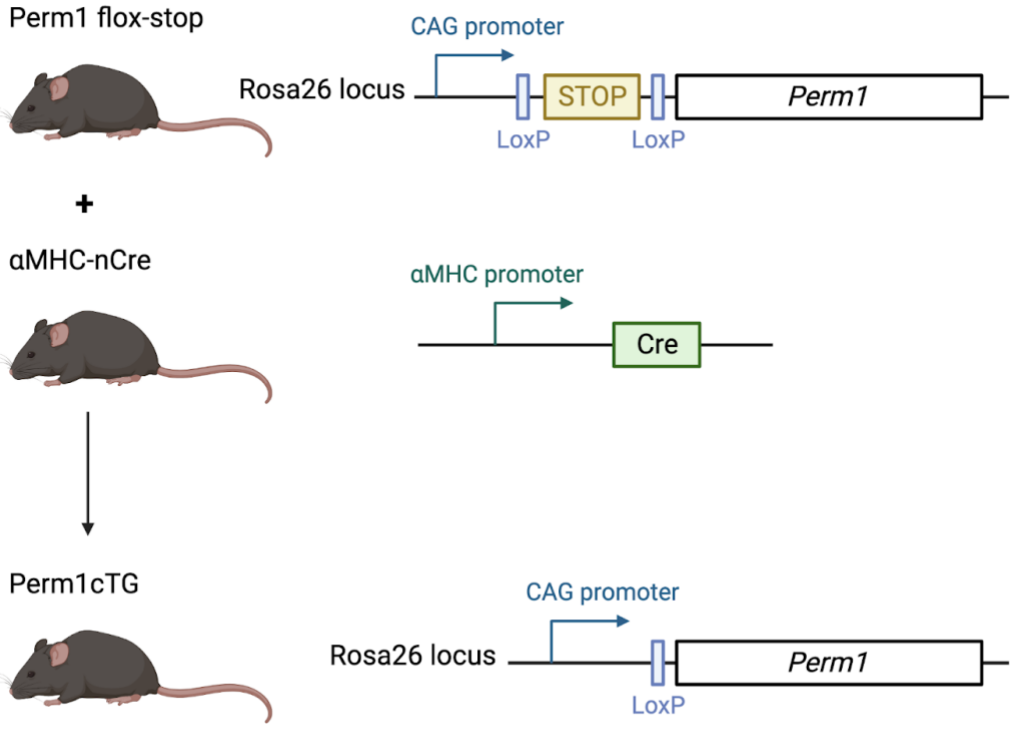


Figure 2.1. Generation of transgenic mice with cardiac-specific Perm1 overexpression. Perm1 flox-stop mice were first generated by knocking in a full length Flag-tagged *Perm1* gene with a STOP cassette into the Rosa26 locus with a CAG promoter. Perm1 flox-stop mice were interbred with mice that expressed Cre on the alpha-myosin heavy chain promoter. This interbreeding produced heterozygous mice in which Cre removed the STOP cassette on the Rosa26 locus to allow overexpression of *Perm1* in the heart only. These mice were labeled Perm1cTG and are referred to as transgenic (TG) mice.

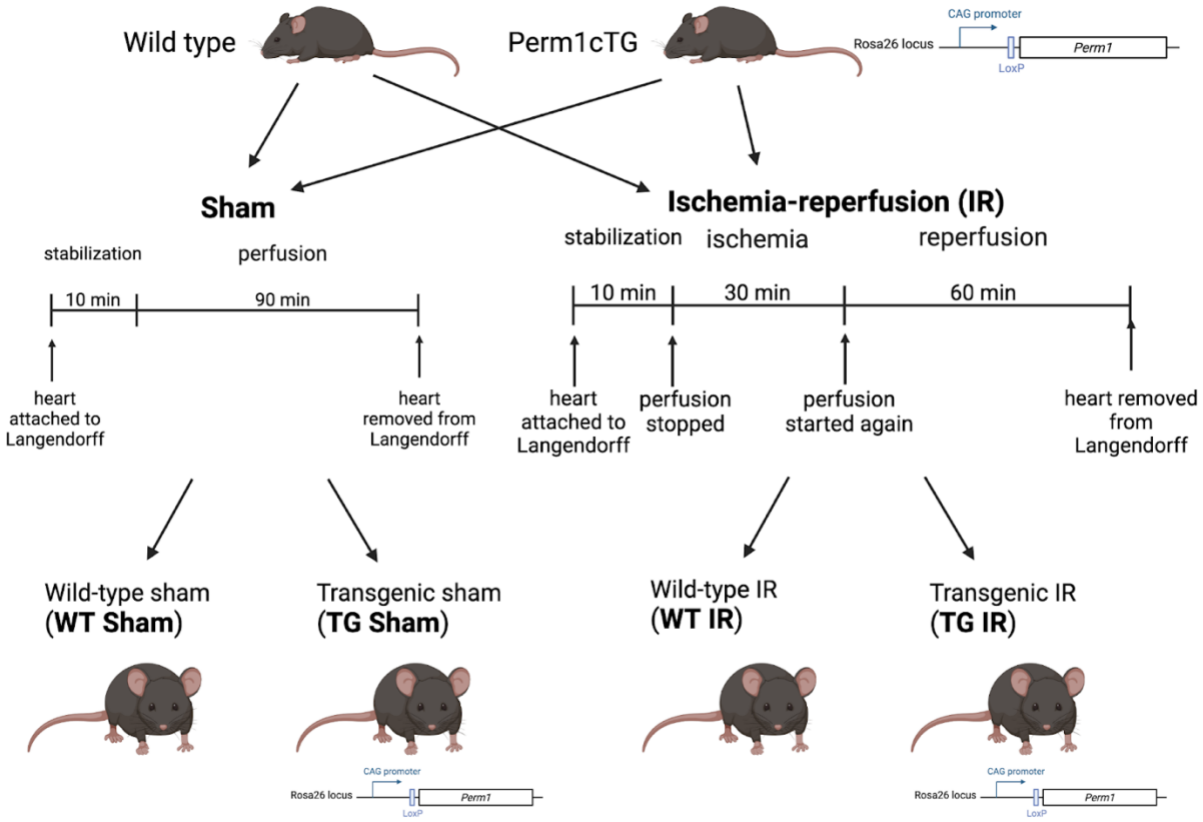


Figure 2.2. Generation of sham control and ischemia-reperfusion groups.
Hearts from wild-type (WT) mice without cardiac-specific Perm1 overexpression and transgenic (TG) mice were subjected to perfusion control procedure or ischemia-reperfusion. After extraction and cannulation, hearts were attached to the Langendorff hanging heart system. Hearts were perfused for 10 minutes for stabilization. To simulate ischemia-reperfusion, perfusion through the heart was paused for 30 minutes, the continued for 60 minutes before the hearts were removed from the Langendorff apparatus. The hearts were labeled as IR. Sham hearts were perfused for 90 minutes following stabilization. Four groups of heart samples were subsequently analyzed: WT Sham, WT IR, TG Sham, and TG IR.

	WT	TG
IVSd (mm)	0.55	0.56
IVSs (mm)	0.68	0.70
LVIDd (mm)	3.54	3.20
LVIDs (mm)	2.17	1.87
LVPWd (mm)	0.72	0.75
LVPWs (mm)	1.13	1.09
EF%	71.15	73.94
HR (bpm)	554.00	549.75
%FS	40.00	40.54

Average, n = 7-8, age 12-16 wks

Figure 2.3. Perm1 transgenic and wild type mice showed normal heart function
Perm1 transgenic and wild type mice (*n* = 7-8) were anesthetized with isoflurane and echocardiography was performed. Analyses of interventricular septal thickness, LV internal diameter, and LV posterior wall thickness in diastole and systole demonstrated little difference between LV dimensions in wild type and transgenic mice. Ejection fraction, heart rate, and fractional shortening also showed minimal difference between the two mice groups. *IVSd* = interventricular septal thickness in diastole; *IVSs* = interventricular septal thickness in systole; *LVIDd* = LV internal diameter in diastole; *LVIDs* = LV internal diameter in systole; *LVPWd* = LV posterior wall in diastole; *LVPWs* = LV posterior wall in systole; *EF* = ejection fraction; *HR* = heart rate; *FS* = fractional shortening.

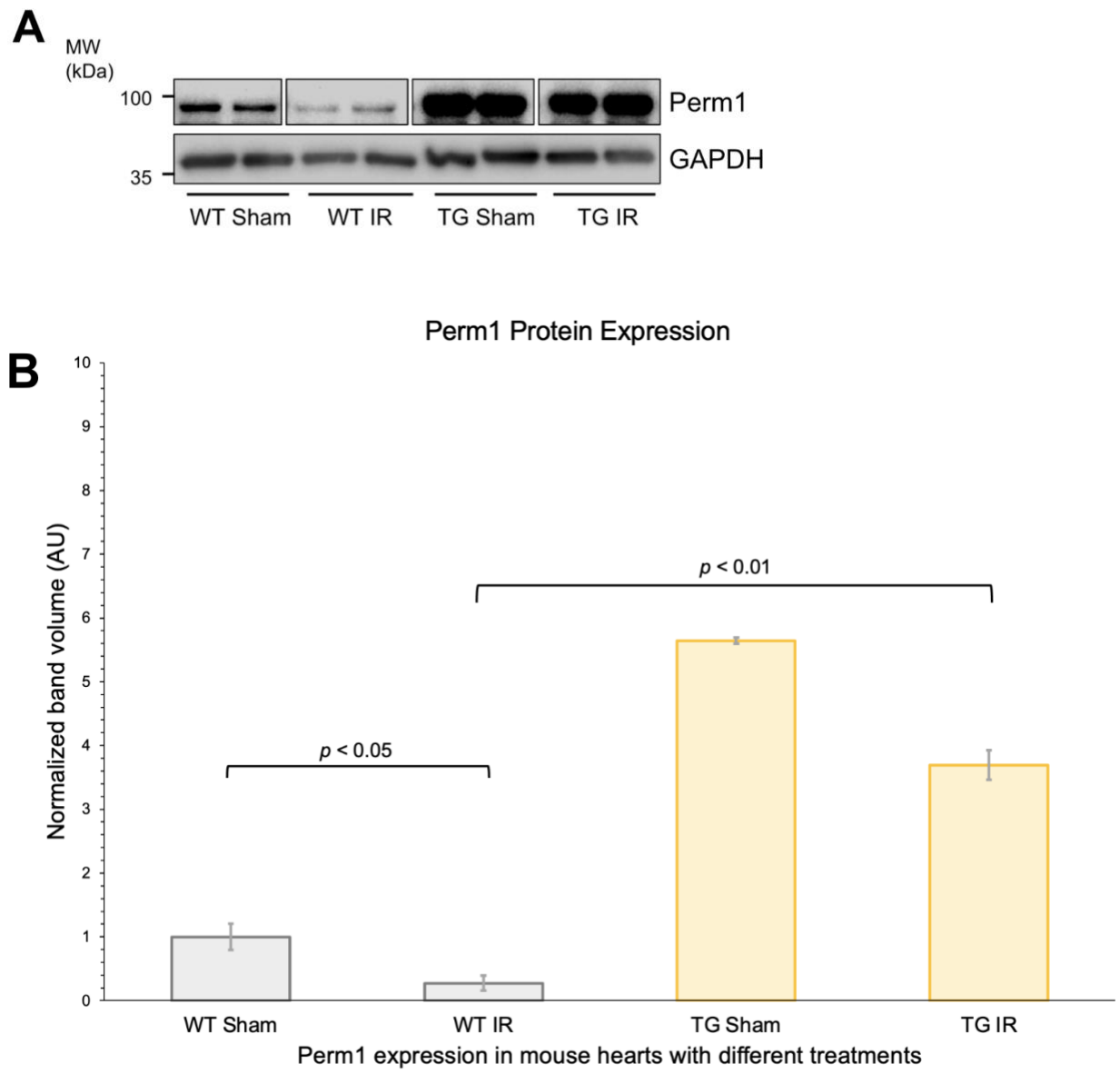


Figure 2.4. Perm1 protein expression in transgenic mice hearts.
Hearts from wild-type (WT) mice and transgenic (TG) mice with cardiac-specific Perm1 overexpression were subjected to perfusion only (sham control) or ischemia-reperfusion (IR) via the Langendorff hanging heart system. Perm1 expression in the hearts were determined by Western blot. Western blot band volumes of each group were normalized to that of the WT Sham group, and normalized band volumes represent the degree of Perm1 expression in the relative heart samples. Each symbol represents band volume of one heart sample (n = 2 per group) while bars indicate average band volume. A student's t-test with 2-tailed distribution and equal variance was used to analyze the statistical differences between WT Sham and WT IR, TG Sham and TG IR, and WT IR and TG IR. *Statistical significance $p < 0.05$.

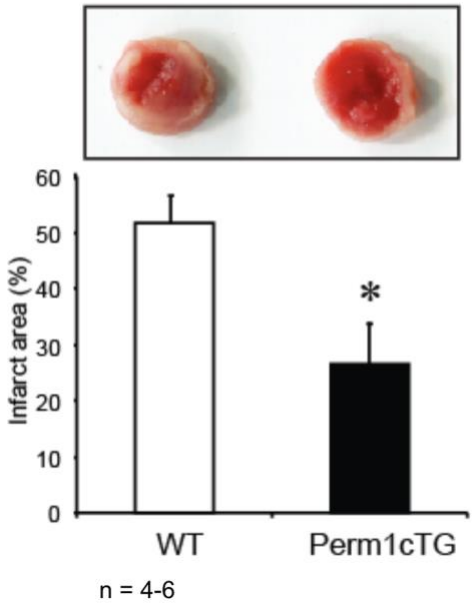


Figure 2.5. Perm1 overexpressed mice hearts showed reduced infarct size following ischemia reperfusion.
Hearts from wild-type (WT) mice and transgenic (TG) mice with cardiac-specific Perm1 overexpression were subjected to perfusion only (sham control) or ischemia-reperfusion (IR) via the Langendorff hanging heart system. Follow reperfusion, hearts were removed from the Langendorff hanging heart system and kept at -20ºC for 20 minutes, then cut into four, 2 mm thick sections from base to apex. Sections were stained with TTC to determine infarct size. *Top panel*, representation image of stained heart sections. Infarcted myocardium appeared pale in color while active myocardium appeared red. *Bottom panel*, infarct size was quantified from the heart sections. Percent infarct area was calculated by taking the ratio of total infarct area to total left ventricular volume.

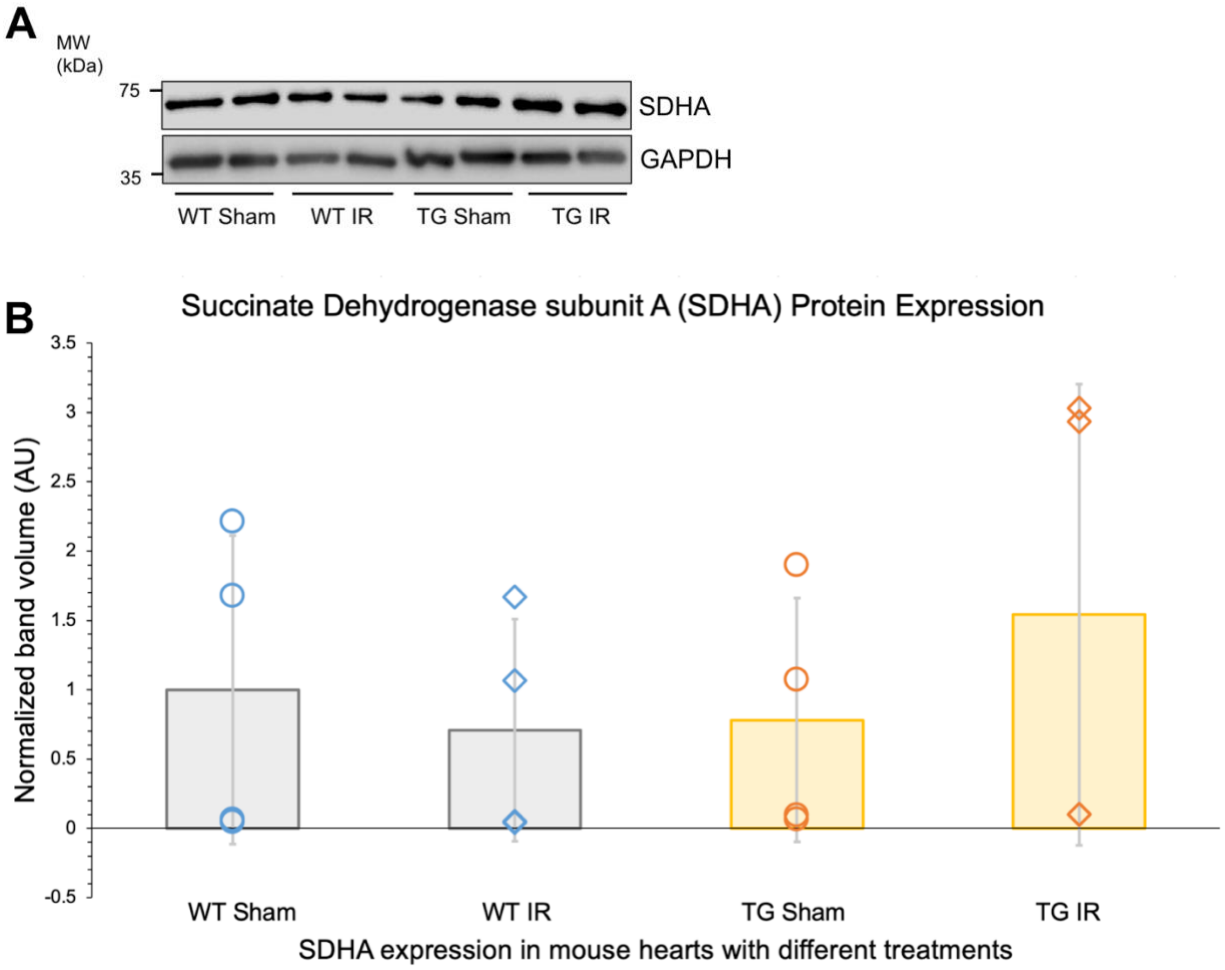
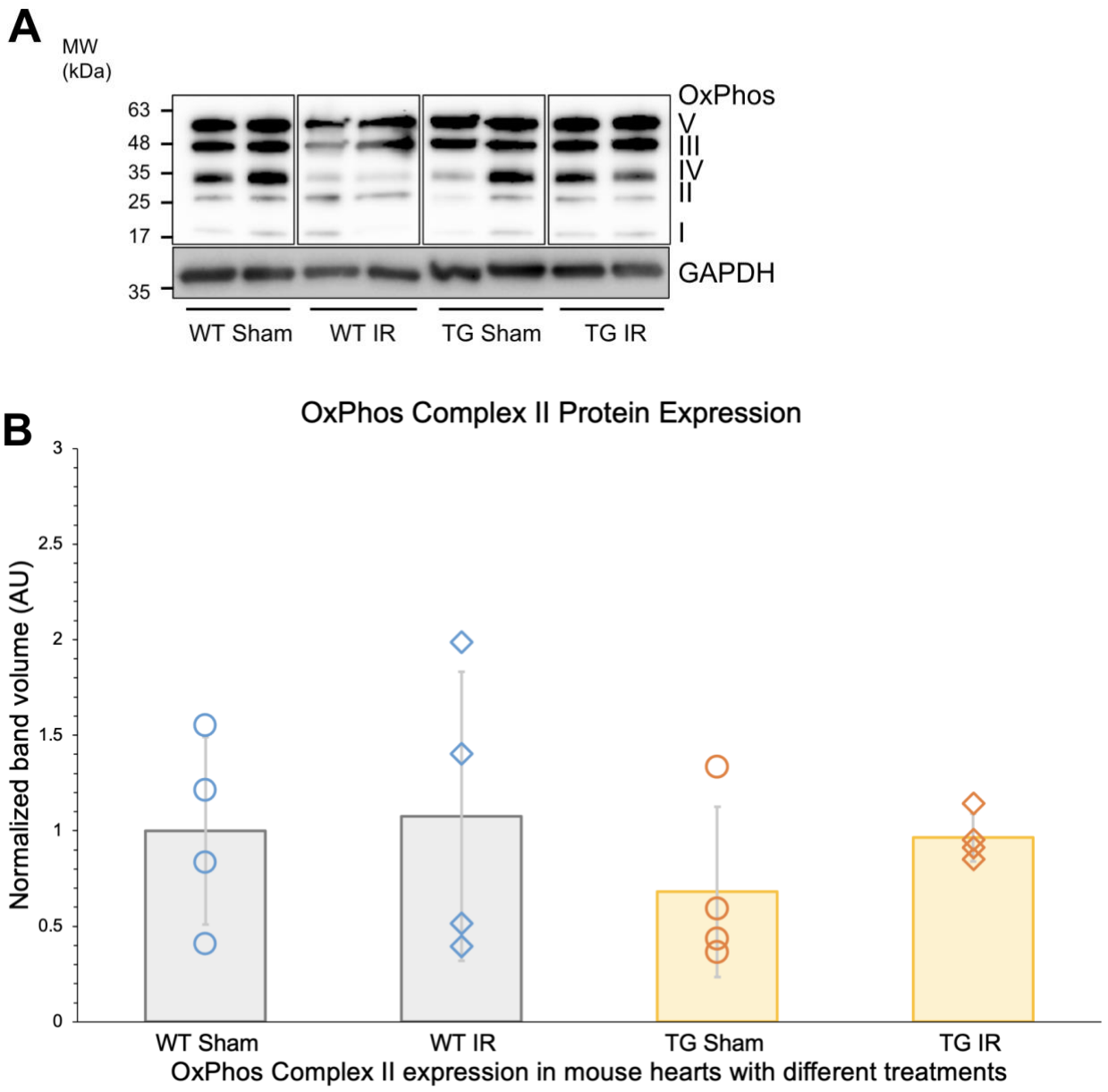


Figure 2.6. Analysis of Succinate dehydrogenase subunit A (SDHA) protein expression in Perm1-overexpressed mice hearts.
Hearts from wild-type (WT) mice and transgenic (TG) mice with cardiac-specific Perm1 overexpression were subjected to perfusion only (sham control) or ischemia-reperfusion (IR) via the Langendorff hanging heart system. SDHA expression in the hearts were determined by Western blot. Western blot band volumes of each group were normalized to that of the WT Sham group, and normalized band volumes represent the degree of SDHA expression in the relative heart samples. Each symbol represents band volume of one heart sample (n = 4 per group) while bars indicate average band volume. A student's t-test with 2-tailed distribution and equal variance was used to analyze the statistical differences between WT Sham and WT IR, TG Sham and TG IR, and WT IR and TG IR. Statistical significance $p < 0.05$.

Figure 2.7. Analysis of Protein expression oxidative phosphorylation (OxPhos) protein complexes in Perm1-overexpressed mice hearts.
Hearts from wild-type (WT) mice and transgenic (TG) mice with cardiac-specific Perm1 overexpression were subjected to perfusion only (sham control) or ischemia-reperfusion (IR) via the Langendorff hanging heart system. OxPhos complexes expression in the hearts were determined by Western blot. Western blot band volumes OxPhos complexes II (*B*), III (*C*), and V (*D*) of each group were normalized to that of the WT Sham group for each respective protein complex, and normalized band volumes represent the degree of OxPhos protein complex expression in the relative heart samples. Each symbol represents band volume of one heart sample ($n = 4$ per group) while bars indicate average band volume. A student's t-test with 2-tailed distribution and equal variance was used to analyze the statistical differences between WT Sham and WT IR, TG Sham and TG IR, and WT IR and TG IR. Statistical significance $p < 0.05$.


A
MW
(kDa)
63
48
35
25
17
35
OxPhos
V
III
IV
II
I
GAPDH
WT Sham
WT IR
TG Sham
TG IR
B
OxPhos Complex II Protein Expression
Normalized band volume (AU)
3
2.5
2
1.5
1
0.5
0
WT Sham
WT IR
TG Sham
TG IR
OxPhos Complex II expression in mouse hearts with different treatments

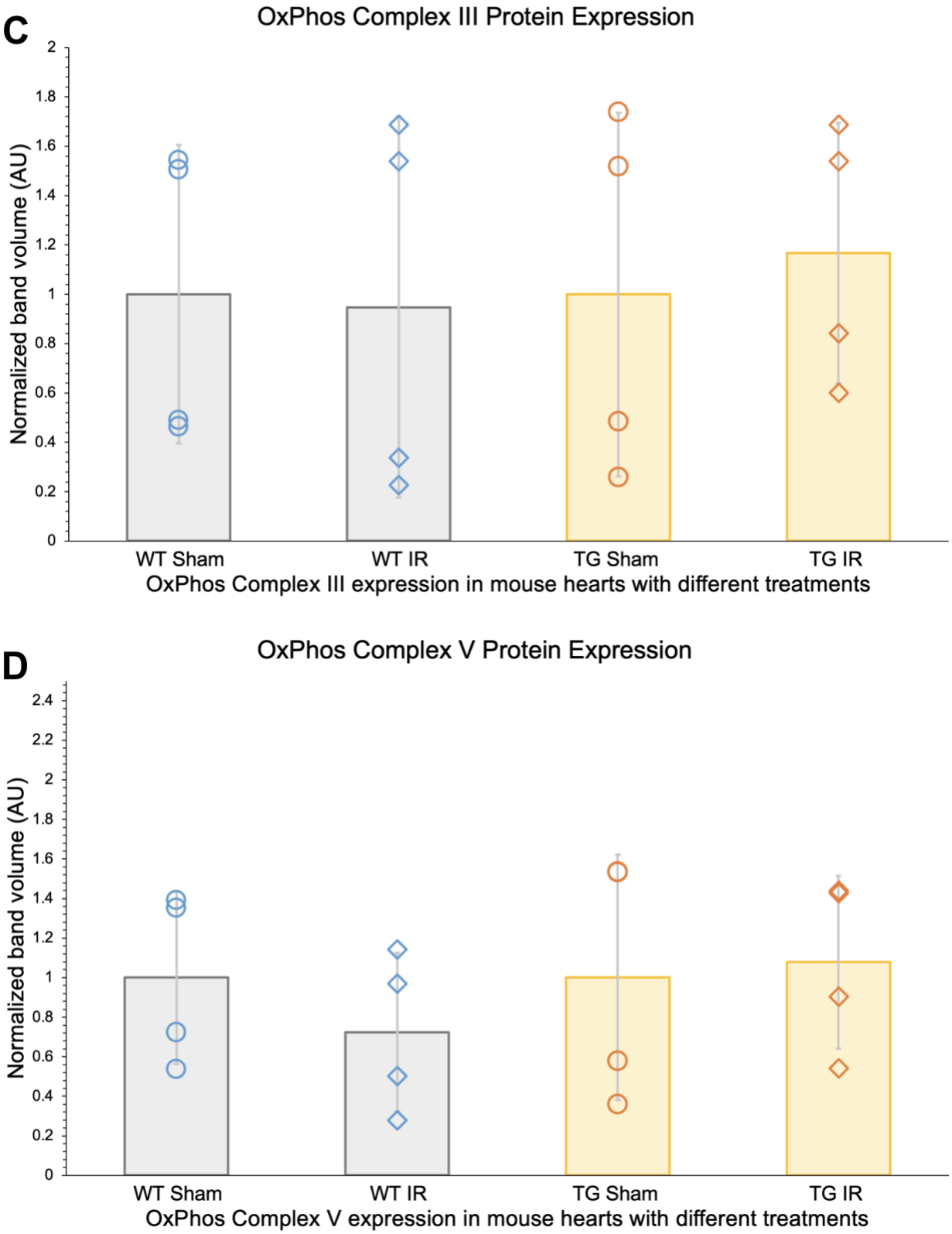


Figure 2.7. Analysis of Protein expression oxidative phosphorylation (OxPhos) protein complexes in Perm1-overexpressed mice hearts (Continued)

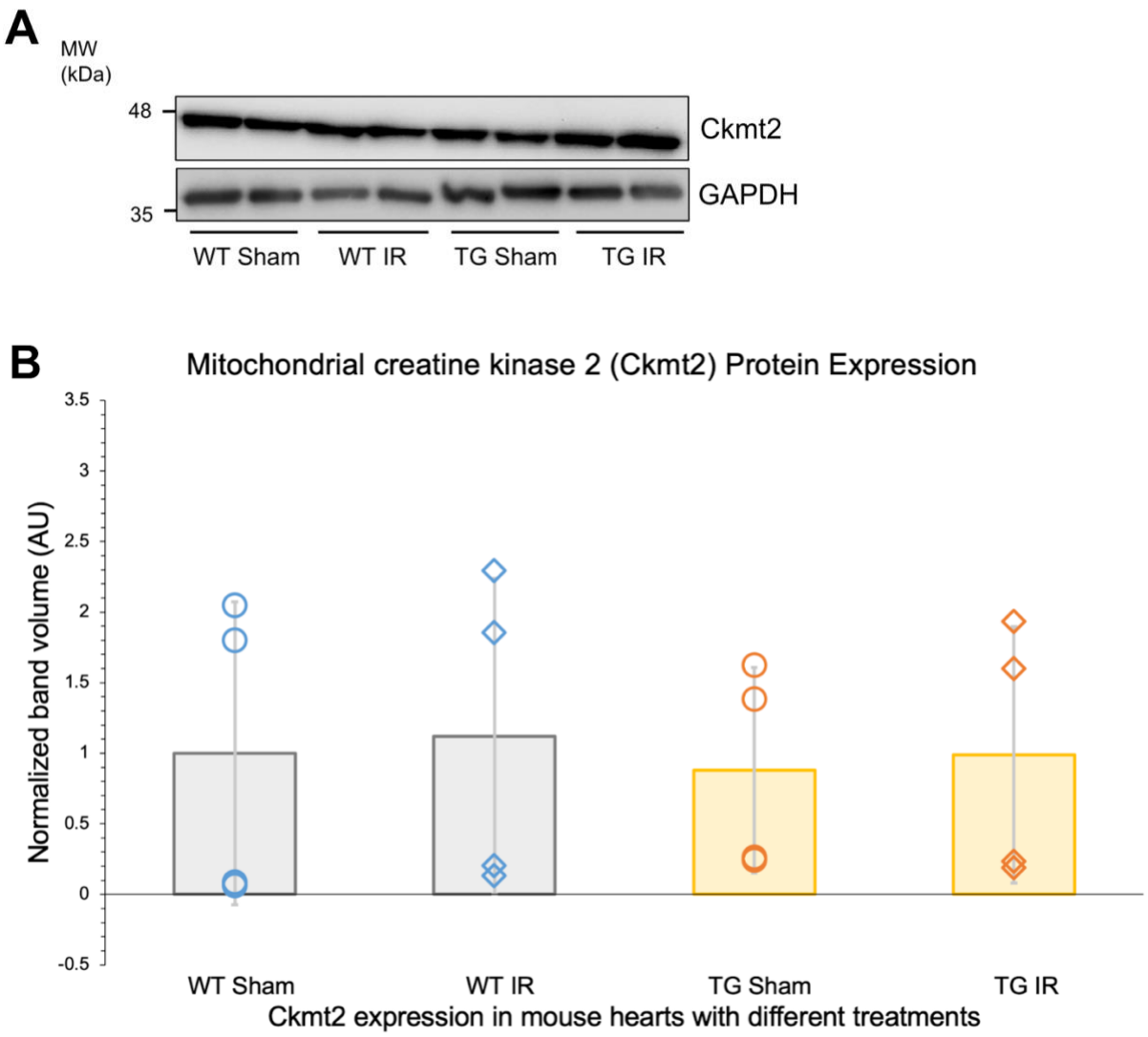


Figure 2.8. Analysis of mitochondrial creatine kinase 2 (Ckmt2) protein expression in Perm1-overexpressed mice hearts.
Hearts from wild-type (WT) mice and transgenic (TG) mice with cardiac-specific Perm1 overexpression were subjected to perfusion only (sham control) or ischemia-reperfusion (IR) via the Langendorff hanging heart system. Ckmt2 expression in the hearts were determined by Western blot. Western blot band volumes of each group were normalized to that of the WT Sham group, and normalized band volumes represent the degree of Ckmt2 expression in the relative heart samples. Each symbol represents band volume of one heart sample (n = 4 per group) while bars indicate average band volume. A student's t-test with 2-tailed distribution and equal variance was used to analyze the statistical differences between WT Sham and WT IR, TG Sham and TG IR, and WT IR and TG IR. Statistical significance $p < 0.05$.

Acknowledgement

Chapter 2 of this thesis also contains unpublished data co-authored with Yoshitake Cho and Shizuko Tachibana of the Cho lab at UCSD. The thesis author was the primary author of this chapter.

REFERENCES

[1] Lopaschuk, G. D., Karwi, Q. G., Tian, R., Wende, A. R., & Abel, E. D. (2021). Cardiac Energy Metabolism in Heart Failure. *Circulation research*, 128(10), 1487–1513. https://doi.org/10.1161/CIRCRESAHA.121.318241

[2] Zhou, B., & Tian, R. (2018). Mitochondrial dysfunction in pathophysiology of heart failure. *The Journal of clinical investigation*, 128(9), 3716–3726. https://doi.org/10.1172/JCI120849

[3] Lesnefsky, E. J., Chen, Q., Tandler, B., & Hoppel, C. L. (2017). Mitochondrial Dysfunction and Myocardial Ischemia-Reperfusion: Implications for Novel Therapies. *Annual review of pharmacology and toxicology*, 57, 535–565. https://doi.org/10.1146/annurev-pharmtox-010715-103335

[4] Tang, J. X., Thompson, K., Taylor, R. W., & Oláhová, M. (2020). Mitochondrial OXPHOS Biogenesis: Co-Regulation of Protein Synthesis, Import, and Assembly Pathways. *International journal of molecular sciences*, 21(11), 3820. https://doi.org/10.3390/ijms21113820

[5] Rutter, J., Winge, D. R., & Schiffman, J. D. (2010). Succinate dehydrogenase - Assembly, regulation and role in human disease. *Mitochondrion*, 10(4), 393–401. https://doi.org/10.1016/j.mito.2010.03.001

[6] Wang, X., Zhang, X., Cao, K., Zeng, M., Fu, X., Zheng, A., Zhang, F., Gao, F., Zou, X., Li, H., Li, M., Lv, W., Xu, J., Long, J., Zang, W., Chen, J., Gao, F., Ding, J., Liu, J., & Feng, Z. (2022). Cardiac disruption of SDHAF4-mediated mitochondrial complex II assembly promotes dilated cardiomyopathy. *Nature communications*, 13(1), 3947. https://doi.org/10.1038/s41467-022-31548-1

[7] Schlattner, U., Tokarska-Schlattner, M., & Wallimann, T. (2006). Mitochondrial creatine kinase in human health and disease. *Biochimica et biophysica acta*, 1762(2), 164–180. https://doi.org/10.1016/j.bbadis.2005.09.004

[8] Keceli, G., Gupta, A., Sourdon, J., Gabr, R., Schär, M., Dey, S., Tocchetti, C. G., Stuber, A., Agrimi, J., Zhang, Y., Leppo, M., Steenbergen, C., Lai, S., Yanek, L. R., O'Rourke, B., Gerstenblith, G., Bottomley, P. A., Wang, Y., Paolocci, N., & Weiss, R. G. (2022). Mitochondrial Creatine Kinase Attenuates Pathologic Remodeling in Heart Failure. *Circulation research*, 130(5), 741–759. https://doi.org/10.1161/CIRCRESAHA.121.319648

[9] Cao, F., Zervou, S., & Lygate, C. A. (2018). The creatine kinase system as a therapeutic target for myocardial ischaemia-reperfusion injury. *Biochemical Society transactions*, 46(5), 1119–1127. https://doi.org/10.1042/BST20170504

[10] Park, N., Marquez, J., Garcia, M. V. F., Shimizu, I., Lee, S. R., Kim, H. K., & Han, J. (2021). Phosphorylation in Novel Mitochondrial Creatine Kinase Tyrosine Residues Render Cardioprotection against Hypoxia/Reoxygenation Injury. *Journal of lipid and atherosclerosis*, 10(2), 223–239. https://doi.org/10.12997/jla.2021.10.2.223

[11] Cho, Y., Tachibana, S., Lam, K., Arita, Y., Khosrowjerdi, S., Zhang, O., Liang, A., Li, R., Andreyev, A., Murphy, A. N., & Ross, R. S. (2021). Perm1 promotes cardiomyocyte mitochondrial biogenesis and protects against hypoxia/reoxygenation-induced damage in mice. *The Journal of biological chemistry*, 297(1), 100825. https://doi.org/10.1016/j.jbc.2021.100825

[12] Oka, S. I., Sabry, A. D., Horiuchi, A. K., Cawley, K. M., O'Very, S. A., Zaitsev, M. A., Shankar, T. S., Byun, J., Mukai, R., Xu, X., Torres, N. S., Kumar, A., Yazawa, M., Ling, J., Taleb, I., Saijoh, Y., Drakos, S. G., Sadoshima, J., & Warren, J. S. (2020). Perm1 regulates cardiac energetics as a downstream target of the histone methyltransferase Smyd1. *PloS one*, 15(6), e0234913. https://doi.org/10.1371/journal.pone.0234913

[13] Manso, A. M., Li, R., Monkley, S. J., Cruz, N. M., Ong, S., Lao, D. H., Koshman, Y. E., Gu, Y., Peterson, K. L., Chen, J., Abel, E. D., Samarel, A. M., Critchley, D. R., & Ross, R. S. (2013). Talin1 has unique expression versus talin 2 in the heart and modifies the hypertrophic response to pressure overload. *The Journal of biological chemistry*, 288(6), 4252–4264. https://doi.org/10.1074/jbc.M112.427484

[14] Kaiser, R. A., Bueno, O. F., Lips, D. J., Doevendans, P. A., Jones, F., Kimball, T. F., & Molkentin, J. D. (2004). Targeted inhibition of p38 mitogen-activated protein kinase antagonizes cardiac injury and cell death following ischemia-reperfusion in vivo. *The Journal of biological chemistry*, *279*(15), 15524–15530. https://doi.org/10.1074/jbc.M313717200

[15] Ma, X. L., Kumar, S., Gao, F., Louden, C. S., Lopez, B. L., Christopher, T. A., Wang, C., Lee, J. C., Feuerstein, G. Z., & Yue, T. L. (1999). Inhibition of p38 mitogen-activated protein kinase decreases cardiomyocyte apoptosis and improves cardiac function after myocardial ischemia and reperfusion. *Circulation*, *99*(13), 1685–1691. https://doi.org/10.1161/01.cir.99.13.1685

[16] Ren, F., Mu, N., Gao, M., Sun, J., Zhang, C., Sun, X., Li, L., Li, J., Liu, T., Tse, G., & Dong, M. (2018). Role of JNK signalling pathway and platelet-lymphocyte aggregates in myocardial ischemia-reperfusion injury and the cardioprotective effect of ischemic postconditioning in rats. *Molecular medicine reports*, *18*(6), 5237–5242. https://doi.org/10.3892/mmr.2018.9545

[17] Matsui, T., Tao, J., del Monte, F., Lee, K. H., Li, L., Picard, M., Force, T. L., Franke, T. F., Hajjar, R. J., & Rosenzweig, A. (2001). Akt activation preserves cardiac function and prevents injury after transient cardiac ischemia in vivo. *Circulation*, *104*(3), 330–335. https://doi.org/10.1161/01.cir.104.3.330

[18] Vinhas, M., Araújo, A. C., Ribeiro, S., Rosário, L. B., & Belo, J. A. (2013). Transthoracic echocardiography reference values in juvenile and adult 129/Sv mice. *Cardiovascular ultrasound*, *11*, 12. https://doi.org/10.1186/1476-7120-11-12

[19] Gao, S., Ho, D., Vatner, D. E., & Vatner, S. F. (2011). Echocardiography in Mice. *Current protocols in mouse biology*, *1*, 71–83. https://doi.org/10.1002/9780470942390.mo100130

[20] Chouchani, E. T., Pell, V. R., Gaude, E., Aksentijević, D., Sundier, S. Y., Robb, E. L., Logan, A., Nadtochiy, S. M., Ord, E. N. J., Smith, A. C., Eyassu, F., Shirley, R., Hu, C. H., Dare, A. J., James, A. M., Rogatti, S., Hartley, R. C., Eaton, S., Costa, A. S. H., Brookes, P. S., Davidson, S.M., Duchen, M.R., Saeb-Parsy, K., Shattock, M.J., Robinson, A.J., Work, L.M., Frezza, C., Krieg, T., Murphy, M. P. (2014). Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. *Nature*, *515*(7527), 431–435. https://doi.org/10.1038/nature13909

[21] Andrienko, T. N., Pasdois, P., Pereira, G. C., Ovens, M. J., & Halestrap, A. P. (2017). The role of succinate and ROS in reperfusion injury - A critical appraisal. *Journal of molecular and cellular cardiology*, *110*, 1–14. https://doi.org/10.1016/j.yjmcc.2017.06.016

[22] Kuzmiak-Glancy, S., Glancy, B., & Kay, M. W. (2022). Ischemic damage to every segment of the oxidative phosphorylation cascade elevates ETC driving force and ROS production in cardiac mitochondria. *American journal of physiology. Heart and circulatory physiology*, *323*(3), H499–H512. https://doi.org/10.1152/ajpheart.00129.2022

[23] Ren, D., He, Z., Fedorova, J., Zhang, J., Wood, E., Zhang, X., Kang, D. E., & Li, J. (2021). Sestrin2 maintains OXPHOS integrity to modulate cardiac substrate metabolism during ischemia and reperfusion. *Redox biology*, *38*, 101824. https://doi.org/10.1016/j.redox.2020.101824

[24] Ait-Aissa, K., Blaszak, S. C., Beutner, G., Tsaih, S. W., Morgan, G., Santos, J. H., Flister, M. J., Joyce, D. L., Camara, A. K. S., Gutterman, D. D., Donato, A. J., Porter, G. A., Jr, & Beyer, A. M. (2019). Mitochondrial Oxidative Phosphorylation defect in the Heart of Subjects with Coronary Artery Disease. *Scientific reports*, *9*(1), 7623. https://doi.org/10.1038/s41598-019-43761-y

[25] Motayagheni N. (2017). Modified Langendorff technique for mouse heart cannulation: Improved heart quality and decreased risk of ischemia. *MethodsX*, *4*, 508–512. https://doi.org/10.1016/j.mex.2017.11.004

[26] Laclau, M. N., Boudina, S., Thambo, J. B., Tariosse, L., Gouverneur, G., Bonoron-Adèle, S., Saks, V. A., Garlid, K. D., & Dos Santos, P. (2001). Cardioprotection by ischemic preconditioning preserves mitochondrial function and functional coupling between adenine nucleotide translocase and creatine kinase. *Journal of molecular and cellular cardiology*, *33*(5), 947–956. https://doi.org/10.1006/jmcc.2001.1357

CHAPTER III: Effect of Perm1 Overexpression on Mitochondrial Dynamics and Cristae Morphology

Introduction

Mitochondrial function is closely associated with mitochondrial morphology. Mitochondria structure needs to be capable of supporting efficient energy production [1]. Maintenance of mitochondrial morphology relies on processes such as mitochondrial dynamics and proteins that support mitochondrial structure, such as the mitochondrial contact site and cristae organizing system (MICOS).

To maintain organelle quality and metabolic output, the mitochondria change their size and structure through cycles of fusion and fission, generally termed mitochondrial dynamics [2]. The fusion process, which facilitates the exchange of mitochondrial DNA and metabolites between two mitochondria [1], starts with fusion of the outer mitochondrial membrane followed by fusion of the inner mitochondrial membrane [2]. Outer mitochondrial membrane fusion is facilitated by the complementation of mitofusin proteins 1 and 2, while inner mitochondrial membrane fusion is facilitated by optic atrophy protein 1 (Opa1) [1,2]. On the other hand, the fission process involves the removal of damaged mitochondrial components from the healthy mitochondria and sending the daughter mitochondria with damaged components to be disintegrated through mitophagy [3]. This process is mainly facilitated by dynamin-related protein 1 (Drp1), a cytosolic protein that translocates to the outer mitochondrial membrane with the help of other fission factors and receptors on the outer mitochondrial membrane, such as mitochondrial fission factor (MEF), mitochondrial dynamics proteins of 49 kDa and 51 kDa (MiD49 and MID51), and fission 1 (Fis1) [1,2].

Disruption of mitochondrial dynamics processes contributes to cardiomyopathies and cardiovascular diseases. Studies have shown that loss of MFN proteins in the mitochondria led to a lack of fusion-induced content mixing necessary for mitochondrial DNA expansion, resulting in a lack of mitochondrial DNA which, in combination with mutations, left the oxidative phosphorylation complex impaired and unable to fulfill its responsibility in respiratory capacity and ATP production [4]. In the heart, MFN protein expression was shown to be downregulated during oxidative stress and cardiomyopathies [5]. On the contrary, the fission process is upregulated during ischemia-reperfusion, contributing to the production of reactive oxygen species (ROS) and apoptosis [3,6]. In turn, high levels of ROS and calcium ion overload can further upregulate mitochondrial fission [3,6]. Previous studies have shown that inhibiting Drp1 through pharmacological inhibition or other means can decrease apoptosis and myocardial infarct size upon cardiac ischemia and reperfusion [3]. However, some studies have also demonstrated that complete ablation of Drp1 or excessive mitochondrial fission can induce severe metabolic defects and cardiomyopathies [3,5]. Maintaining the balance between fusion and fission is thus crucial for effective mitochondria function.

Mitochondrial morphology is also regulated by proteins supporting mitochondrial structure. The inner mitochondrial membrane consists of numerous projecting, tube-like structures called cristae. They are constructed by the cristae membrane and are connected to the rest of the inner mitochondrial membrane, termed the inner boundary membrane (IBM), through cristae junctions [2,7]. Cristae are dynamic structures that take on different forms of organization depending on the energy demand and the location of the mitochondria [9]. In tissues with higher energy demand, such as skeletal and cardiac muscles, cristae are generally densely packed in a parallel, thin shape, termed lamellar cristae [9]. In contrast, tubular cristae have wider shapes and

make up a more disorganized mitochondrial internal structure [10]. In the myocardium, mitochondria localized between myofibrils (interfibrillar mitochondria) are also made up of lamellar cristae [6].

The cristae membrane is the site where the electron transport chain (ETC) proteins reside and where oxidative phosphorylation occurs [8] (**Figure 3.1**). Maintenance of cristae structure is thus crucial to maintaining energy production. Cristae structure is supported by various proteins, with the main ones consisting of Opa1, ATP synthase (complex V), and the mitochondrial contact site and cristae organizing system (MICOS) [8]. Opa1, localized at the sides of the cristae membrane at the close to the cristae junctions, contains short and long isoforms that work together to restrict the width of the cristae and prevent the release of cytochrome c and other metabolites from the cristae lumen into the intermembrane space [1,2,7,8] (**Figure 3.2**). While the cristae membrane provides a site of assembly for complex V [1,2], the dimerization of ATP synthase helps to establish the curvature of the cristae membrane at the peak of the cristae [2,7] (**Figure 3.1**).

MICOS is a multi-subunit protein complex situated at the cristae junctions that plays a crucial role in maintaining proper cristae structure [7,8,9]. MICOS consists of seven subunits, Mic10, Mic13, Mic19, Mic25, Mic26, Mic27, and Mic60 [2,7]. These subunits make up two core subcomplexes, the Mic60 and the Mic10 subcomplexes [7,9]. Mic60 and Mic10 are the most crucial components of the complex, with their deletion resulting in the most severe cristae defects [7,9]. Apart from Mic60, the Mic60 subcomplex also consists of Mic19 and Mic25, members of the Coiled-Coil-Helix-Coiled-Coil-Helix (CHCHD) family that facilitate and maintain the assembly and stability of the MICOS complex itself [7,9]. Apart from Mic10, the Mic10 subcomplex also consists of Mic26 and Mic27, apolipoproteins that work together to

regulate cristae structure [7,9]. Mic13 bridges and stabilizes the two subcomplexes; loss of Mic13 results in MICOS instability characterized by upregulation of the Mic60 subcomplex accompanied by degradation of the Mic10 subcomplex [7,9]. MICOS has the capacity to be involved in apoptosis and mitophagy through interactions with other proteins, establishing contact sites between the inner and outer mitochondrial membranes, and facilitating cristae fusion and fission within the mitochondria [7]. Disrupted cristae structure can also affect mitochondrial dynamics as well as the generation of ROS, leading to impairment of mitochondrial bioenergetic metabolism [9].

The role of the MICOS complex in heart pathophysiology has not yet been extensively studied. A recent study about cardiac muscle changes with age demonstrated that depletion of *Mic60, Mic19,* or *Mic25* genes resulted in an increased count of fragmented mitochondria, decreased basal oxygen consumption rate, and decreased respiratory ability during increased energy demand [11]. They also demonstrated that depletion of *Mic25* genes in cardiomyocytes resulted in increased concentration of ROS, suggesting a link between MICOS and genetic factors relating to heart failure and membrane potential modulation [11]. Another study on hypoplastic left heart syndrome using *Drosophila* found a relationship between cardiac-specific *Chchd3/6* gene (homologous to *Mic19/25*), cardiac contractility, and sarcomeric F-Actin viability [12]. Their findings through cardiac-specific knock-out of *Chchd3/6* and ATP synthase subunits further supported the link between MICOS and mitochondrial dynamics, oxidative phosphorylation protein complex abundance and assembly, and ATP production [12].

Mitochondrial fission and cristae structure stability are important factors that contribute to the ability of cardiac mitochondria to support the high energy demand required for proper cardiac function. Studies found that the PGC-1α pathway is associated with mitochondrial fusion

and fission and mitophagy in the myocardium [5,13]. Another study on skeletal muscle found evidence that Perm1 physically interacts with components of the MICOS and mitochondrial intermembrane bridging complex (MIB), suggesting that Perm1 may play a role in stabilizing this complex [14]. The role that MICOS plays in cardiac muscle is largely unknown, and the relation between Perm1 and mitochondrial fission and cristae remodeling in the myocardium has yet to be explored.

In this study, I aimed to determine whether cardiac-specific Perm1 overexpression would correlate with altered mitochondrial fission and cristae remodeling by interrogating the expression of the Drp1 and MICOS proteins in isolated hearts exposed to global ischemia-reperfusion injury. I hypothesize that increasing Perm1 expression will show decreased Drp1 protein expression and increased or stabilized expression of MICOS subunits, suggesting a role for Perm1 in suppressing mitochondrial fission while stabilizing cristae morphology to reduce cardiomyocyte damage and apoptosis upon ischemia-reperfusion.

Materials and Methods

Animal Models

C57BL/6 mice were housed and transgenic mice were generated through methods previously described. All experimental procedures perform in animals were approved by the University of California San Diego Institutional Animal Care and Use Committee (IACUC).

Ischemia/reperfusion by Langendorff apparatus

The wild-type mice and the transgenic mice of 12-16 weeks of age were grouped into two with 12 male mice each, with 8 mice subjected to ex vivo ischemia-reperfusion and the

remaining 4 mice serving as sham controls using the Langendorff apparatus and procedure previously described. Entire hearts were sampled.

Western Blot

Following completion of the ischemia-reperfusion protocol, total protein lysates were prepared and Western blots were run as previously described. was extracted from the ventricular tissue by homogenesis with homogenizing buffer as previously described. The primary antibodies, diluted at 1:1000, were as follows: Drp1, Mic60, Mic19, Mic25, and Mic27. GAPDH was used as a loading control. Protein bands were detected by enhanced chemiluminescence (ECL), and band intensity was measured using BioRad software. The results were presented as normalized expression levels relative to that of the wild-type sham group.

Statistical analysis

All data are shown as means ± standard deviations. Student's t-test using 2-tail distribution and unpaired, equal variance was used to evaluate differences between wild-type sham, transgenic sham, wild-type ischemia-reperfusion, and transgenic ischemia-reperfusion groups. Differences $p<0.05$ were considered to be statistically significant.

Results

Analysis of dynamic-related protein 1 (Drp1) expression

Multiple studies have demonstrated that excessive fission occurs during IR and contributes to IR injury [15]. With Drp1 as the main facilitator of mitochondrial fission [16], I examined Drp1 expression in transgenic mice hearts with Perm1 overexpression upon IR to

determine whether Perm1 plays a role in mediating mitochondrial fission. In wild-type hearts, IR hearts showed significantly reduced levels of Drp1 relative to that in sham hearts (**Figure 3.3B**, $p < 0.05$). At the same time, TG sham showed an average normalized band volume of 0.915 and TG IR had an average of 0.825. The difference in normalized band volumes between these two groups were not statistically significant. The difference between WT IR and TG IR also did not reach statistical significance ($p > 0.05$).

Analysis of MICOS Mic60 expression

Mic60 is critical for the assembly of the MICOS complex [17] and maintenance of cristae junctions [18]. Studies have shown that Mic60 levels decrease upon IR injury [19]. I investigated whether increasing Perm1 expression may alter Mic60 protein expression upon IR. As shown in **Figure 3.4B**, WT IR showed an average normalized band volume that is 0.623, lower than that of WT sham (1.000). TG sham had an average normalized band volume of 0.976 and TG IR had an average of 1.029. The differences between WT sham and WT IR, TG sham and TG IR, and WT IR and TG IR did not reach statistical significance ($p > 0.05$).

Analysis of MICOS Mic19 expression

Mic19, a member of the Mic60 subcomplex, plays a role in stabilizing the MICOS complex and mitochondrial inner membrane morphology [20]. Multiple previous studies have demonstrated that decreased levels of Mic60 exhibited a decreased abundance of Mic19 in the mitochondria of kidney and liver cells [18,20,21]. I investigated whether Mic19 levels will be altered in transgenic hearts upon IR. **Figure 3.4C** shows that WT IR, TG sham, and TG IR had average normalized band volumes of 1.044, 1.072, and 1.030 respectively. These averages did

not show statistically significant differences ($p > 0.05$) with one another and with that of WT sham (1.000).

Analysis of MICOS Mic25 expression

Apart from Mic19, the Mic60 subcomplex also consists of Mic25 [9]. The full role of Mic25 is still unclear, but studies have suggested that it may contribute to cristae maintenance by bridging the sorting and assembly machinery proteins on the outer mitochondrial membrane with the MICOS complex on the inner membrane [22]. Studies have also found that Mic60 expression levels are coordinately regulated with that of Mic25 [23]. I investigated whether Mic25 abundance may be altered upon IR of the heart and Perm1 overexpression. WT IR showed an average normalized band volume of 1.492 (**Figure 3.4D**). TG sham and TG IR showed averages of 1.714 and 1.279 respectively, with no statistically significant difference ($p > 0.05$). There was also no statistically significant difference between the averages of WT sham, WT IR, and TG IR ($p > 0.05$).

Analysis of MICOS Mic27 expression

The other subcomplex of MICOS, the Mic10 subcomplex, also contributes to cristae morphology and consists of Mic10, Mic26, and Mic27 [9]. Mic26 (apolipoprotein O) and Mic27 (apolipoprotein O-like) are apolipoproteins that typically bind and transport lipids [24]. In the mitochondria, Mic26 and Mic27 are associated with cardiolipin [24], a mitochondrial membrane-specific phospholipid involved in many mitochondrial functions associated with the mitochondrial membrane [25]. I examined how the Mic27 expression changed in hearts upon IR and Perm1 overexpression. WT IR, TG sham, and TG IR showed average normalized band

volumes of 1.122, 1.279, and 1.222 respectively (**Figure 3.4E**). Differences in the averages between one another and with that of WT sham (1.000) did not reach statistical significance ($p > 0.05$).

Discussion

Previous studies showed that Perm1 gene and protein expression are associated with the expression of nuclear-encoded OxPhos genes and ETC protein expression [26,27]. Although this current study did not find significant alterations in OxPhos protein expression in isolated Perm1 transgenic hearts upon global IR, I continued aiming to advance our understanding of Perm1 function by examining the expression of proteins involved in mitochondria dynamics and morphology. Mitochondria dynamics and morphology are crucial components that maintain mitochondria structural and functional integrity, mitochondrial fission serves to maintain viability of the mitochondria [3] and cristae present as the primary site of oxidative phosphorylation [8].

Although mitochondrial fission is crucial to maintaining mitochondrial functional integrity, excessive fission can occur during IR and cause mitochondrial dysfunction [28]. Mitochondrial fission is primarily facilitated by Drp1, and studies have shown that mitochondrial translocation of Drp1 increases upon IR, and Drp1 expression is elevated in infarcted regions [15,16,29]. At the same time, inhibition of Drp1 activation or translocation is cardioprotective [15,16,29]. Contrary to expectations from these findings, my results showed significantly lower Drp1 expression in wild-type hearts upon ischemia-reperfusion. This observation may present a lack of validity in establishing controls due to experimental error, requiring experimentation with more samples to establish a valid control. The difference in Drp1 western blot band intensity

between WT sham and TG sham was not statistically significant, and statistical significance was also lacking in the difference in Drp1 expression between TG sham and TG IR. Although this may suggest that Drp1 expression was maintained in hearts with Perm1 overexpression, it is difficult to be confident in this interpretation due to batch effects, though not large, and the lack of valid comparison with Drp1 expression in wild-type hearts subjected to IR.

A possible reason for the observed Drp1 levels in wild-type IR hearts may be the length of reperfusion. In the experiment conducted by Distanik et al., Drp1 expression increased significantly at 10 minutes of reperfusion but returned to basal levels after 60 minutes of reperfusion [15]. In my experiment, Drp1 expression was analyzed after the hearts were reperfused for 60 minutes, when Drp1 levels would have returned to baseline as observed in the experiment by Distanik et al. Further experiments involving shorter reperfusion times can be performed to better analyze changes in Drp1 expression.

Ischemic preconditioning (IPC) can also affect Drp1 expression. As mentioned in the previous chapter, temporary ischemia between heart extraction and cannulation in the Langendorff procedure can induce cardioprotective effects of IPC [30], leading to inaccurate results. Ismail and colleagues showed that Drp1 gene expression was significantly downregulated in hearts subjected to IPC [31]. These findings suggest that unintentional IPC may have been induced during my Langendorff experiment leading to downregulated Drp1 expression.

Drp1 is a cytosolic protein that translocates to the mitochondria with the help of other fission factors and receptors on the outer mitochondrial membrane [1,2]. Studies demonstrated that mitochondrial dynamics protein of 51 kDa (MiD51) serves as a mitochondria-specific, primary outer-membrane binding partner of Drp1 [28,32]. Otera et al. showed that the close

proximity of the Drp1-MiD51 complex to cristae remodeling systems is critical to cristae remodeling and cytochrome c release that induces apoptosis [32]. Gao and colleagues showed that MiD51 knockdown did not induce any changes in Drp1 mitochondrial translocation or apoptosis under basal conditions but alleviated myocardial injury and cardiac dysfunction upon ischemia-reperfusion [28]. Their findings demonstrated MiD51 to be a Drp1 fission receptor only activated during ischemia-reperfusion. Expression of mitochondrial MiD51 can be interrogated to better understand alterations in Drp1 translocation to the mitochondria in hearts with increased Perm1 expression.

Mitochondrial dynamics involves a balance between fission and fusion processes [33]. Fusion of the outer mitochondrial membrane is facilitated by mitofusion proteins 1 and 2 (MFN1 and MFN2), while inner membrane fusion is facilitated by Opa1 [1,2]. Many studies have shown that decreased levels of MFN1 and 2 are associated with reduced mitochondrial DNA stability, decreased respiratory capacity, and impaired mitochondrial function as well as cardiomyopathies [4,5,33]. Future experiments can examine the expression of fusion-facilitating proteins to provide insight to how the fusion may be affected in hearts with increased Perm1 expression.

In addition to mitochondrial dynamics, the integrity of cristae structure is also crucial to mitochondrial function. Cristae are supported at the cristae junctions by the MICOS complex [7]. Mic60 has been considered as a core component of MICOS that is crucial to the stability of the complex itself [17]. Mic60 knockout experiments have shown tremendous loss of cristae junctions, decreased number of cristae, and decreased protein levels of other MICOS components such as Mic19, Mic10, and Mic25 [18]. Cells with downregulated Mic60 expression showed decreased activity of electron transport chain subunits that are encoded by nuclear DNA [34], increased sensitivity to intrinsic apoptotic stimuli, and increased release of cytochrome c

[35]. On the other hand, Thapa and colleagues showed that Mic60 overexpression preserved the activity of electron transport chain complexes I, III, IV, and ATP synthase and restored respiration capacity in diabetes mellitus-associated hearts [36]. These studies show that Mic60 plays an essential role in mediating energy production, mitochondrial and cristae morphology, and even apoptosis.

Tombo et al. showed that Mic60 decreases during reperfusion, with the degree of decrease corresponding to the length of ischemia [19]. Although my results showed a lower average normalized band volume in WT IR hearts compared with the average of WT sham, the difference was not statistically significant and an interpretation cannot be made to indicate that my results aligned with previous findings. The transgenic sham and IR hearts showed Mic60 expression that were not statistically significantly different. While this may suggest that Mic60 expression was maintained in transgenic hearts upon IR, it is difficult to be confident in this interpretation. A valid comparison with WT IR can not be made due to the lack of statistical significance in the difference in Mic60 levels between WT IR and TG IR. Lack of statistical significance in the differences among the heart sample groups can be attributed to the large variations in the data. Although batch effects appear to be present, separation of the two batches are not clear and it is difficult to attribute the large variations to primarily batch effects. More samples are needed to reduce variation and show clearer results prior to further interpretation. Mic60 mRNA expression can also be analyzed to gain more understanding of how Mic60 protein expression changes upon Perm1 overexpression and IR.

Overexpression of Mic60 has also been indicated to have an additive effect on cardiac hypertrophy and increased production of reactive oxygen species in response to hypertrophic stimuli [37]. This finding indicates that Mic60 levels may need to be maintained within a specific

range for optimal cell function. If statistical significance can be established to support the hypothesis that Mic60 is restored in Perm1 transgenic hearts upon IR, further research can investigate Perm1 might play a role in maintaining Mic60 within an optimal range.

Mic60 forms the Mic60 subcomplex with Mic19 and Mic25 to maintain the stability of MICOS [9]. Mic19 has been shown to play an essential role in cristae junction stability. Sakowska et al. showed that oxidized Mic19 is required for MICOS stability [21], while Darshi et al. demonstrated that knocking out Mic19 resulted in reduced cristae content and cristae junction opening diameter [20]. However, little is known about how Mic19 functions in the heart and how myocardial affects Mic19 activity and expression.

Although there has not been previous research conducted on the effects of myocardial IR on Mic19, multiple studies have demonstrated that Mic19 expression decreases with decreased Mic60. Knocking out Mic60 decreased the abundance of Mic19 [17,21] and knocking out Mic19 also decreased Mic60 protein levels [20]. Thus, Mic60 and Mic19 can be considered to be coordinately regulated. While I expected to observe Mic19 expression patterns similar to that of Mic60, my results showed that Mic19 expression remained comparable with no statistically significant differences across all four types of heart samples. Although this result may suggest that Mic19 protein expression is not affected during myocardial IR nor by Perm1 overexpression, I can not be confident in this interpretation due to the small sample size and large variation from batch effects. Further research needs to be conducted to gain understanding of whether myocardial IR induces changes in Mic19 activity and expression. Then, how Perm1 overexpression may affect Mic19 expression can be further interrogated. //

Mic25 is another component of the Mic60 subcomplex that is crucially involved in cristae junction integrity [2]. Cells with knocked-out Mic25 showed a reduced number of cristae

junctions, reduced cristae density, and abnormal cristae structures [22,23]. Similar to Mic19, little is known about how Mic25 activity and expression may be altered upon myocardial IR. However, Mic60 and Mic25 were found to physically interact with each other, and elevated Mic60 expression was observed in human colon cancer cells with overexpression of Mic25 protein [23]. Mic25 also showed similar expression patterns as Mic60 in Tafazzin knock-out hearts [38]. At the same time, Ding and colleagues demonstrated that Mic19 and Mic25 may be functionally homologous [22]. Studies using induced pluripotent stem cell-derived cardiomyocytes showed that knocking out Mic25 in cardiomyocytes resulted in reduced mitochondria size and increased production of reactive oxygen species (ROS) [11], suggesting the importance of Mic25 in mitochondrial structural and functional integrity. On the other hand, Mic25 knockout experiments showed significantly decreased ATP synthesis and oxygen consumption rate in human breast and colon cancer cells [23] but only slight reductions in HeLa cells [22]. These findings indicate that the involvement of Mic25 in mitochondrial and respiratory function.

A valid interpretation can not be made from the current results due to lack of knowledge of Mic25 expression upon myocardial IR, the small sample size, and lack of statistical significance in the observed differences of Mic25 expression among the heart sample groups. Variation in the normalized band volumes exists in addition to the batch effects present. More samples are needed for clearer results. Similar to Mic19, future experiments should first focus on investigating the effects of myocardial IR on Mic25 before conducting further research on whether Mic25 expression and activity associate with Perm1 cardioprotection.

While the Mic60 subcomplex was considered to be responsible for forming contact sites and stabilizing MICOS, the Mic10 subcomplex was considered to be involved in membrane

sculpting [39]. The Mic10 subcomplex consists of Mic10 and two apolipoproteins, Mic26 (apolipoprotein O) and Mic27 (apolipoprotein O-like) [7]. Mic26 and Mic27 are paralogues of each other [9]. Their protein expression is antagonistically regulated so that they are functionally complementary, and depletion of one of them only induces mild cristae defects [7,9]. Similar to Mic19 and Mic25, little is known about how Mic26 and Mic27 gene and protein expression change upon ischemia-reperfusion in the heart.

In the current study, only Mic27 expression was interrogated. Specifically, Mic27 contributes to stabilization of Mic10 [39], other MICOS subunits, and cristae structure [24]. I observed comparable Mic27 levels in sham and IR hearts in both transgenic and wild-type samples. The comparable Mic27 expression and lack of statistically significant difference across the heart sample groups may suggest that Mic27 expression is not significantly affected by IR nor Perm1 overexpression. However, it is difficult to be confident in this interpretation. The data sample size was small, and variation was present due to batch effects. More samples are needed. If additional samples cluster with either batch of data, a more confident interpretation may be made.

Anand and colleagues had demonstrated that Mic26 and Mic27 are required for ATP synthase stabilization by integration of F1 subunit [24], indicating that Mic26 and Mic27 play a role in respiratory capacity and oxidative phosphorylation. On the other hand, Turkieh et al. demonstrated that overexpression of Mic26 in diabetic mice induced loss of cristae, mitochondrial dysfunction, and consequential cardiac dysfunction [40]. Since Mic26 and Mic27 are functionally complementary, overexpression of Mic27 may induce similar effects, thus suggesting that Mic26 and Mic27 expression needs to be maintained at a certain range for optimal function. Future experiments should first focus on gaining a better understanding of how

Mic26 and Mic27 are affected upon myocardial IR and how the results of those changes affect cardiac function.

As apolipoproteins, Mic26 and Mic27 are involved in remodeling of cardiolipin [41]. Cardiolipin is a type of phospholipid specifically localized in the mitochondrial membrane involved in essential mitochondrial functions such as stabilization of electron transport chain protein complexes [25]. Cardiolipin also binds directly to cytochrome C in the inner mitochondrial membrane [42]. During ischemia-reperfusion, oxidative damage of cardiolipin causes cytochrome C to detach from the inner mitochondrial membrane, leading to cytochrome C release into the cytosol and subsequent apoptotic signaling [42]. Cardiolipin abundance decreases during double knockout experiments of Mic26 and Mic27 [41], suggesting that Mic26 and Mic27 play important roles in the stability of cardiolipin. Cardiolipin abundance was also shown to decrease during myocardial IR [25], which may indicate dysfunction of Mic26 and Mic27 upon IR. Future experiments can investigate alterations in the interaction between cardiolipin and the apolipoproteins during ischemia-reperfusion.

Mic10 is also a core subunit of MICOS, with the Mic10 subcomplex considered to be essential to stabilizing cristae structure, while Mic13 bridges the two subcomplexes and thus is also important for MICOS stability [9]. Further experiments should also investigate how the expression of these two proteins change with increased Perm1 expression and ischemia-reperfusion.

Figures

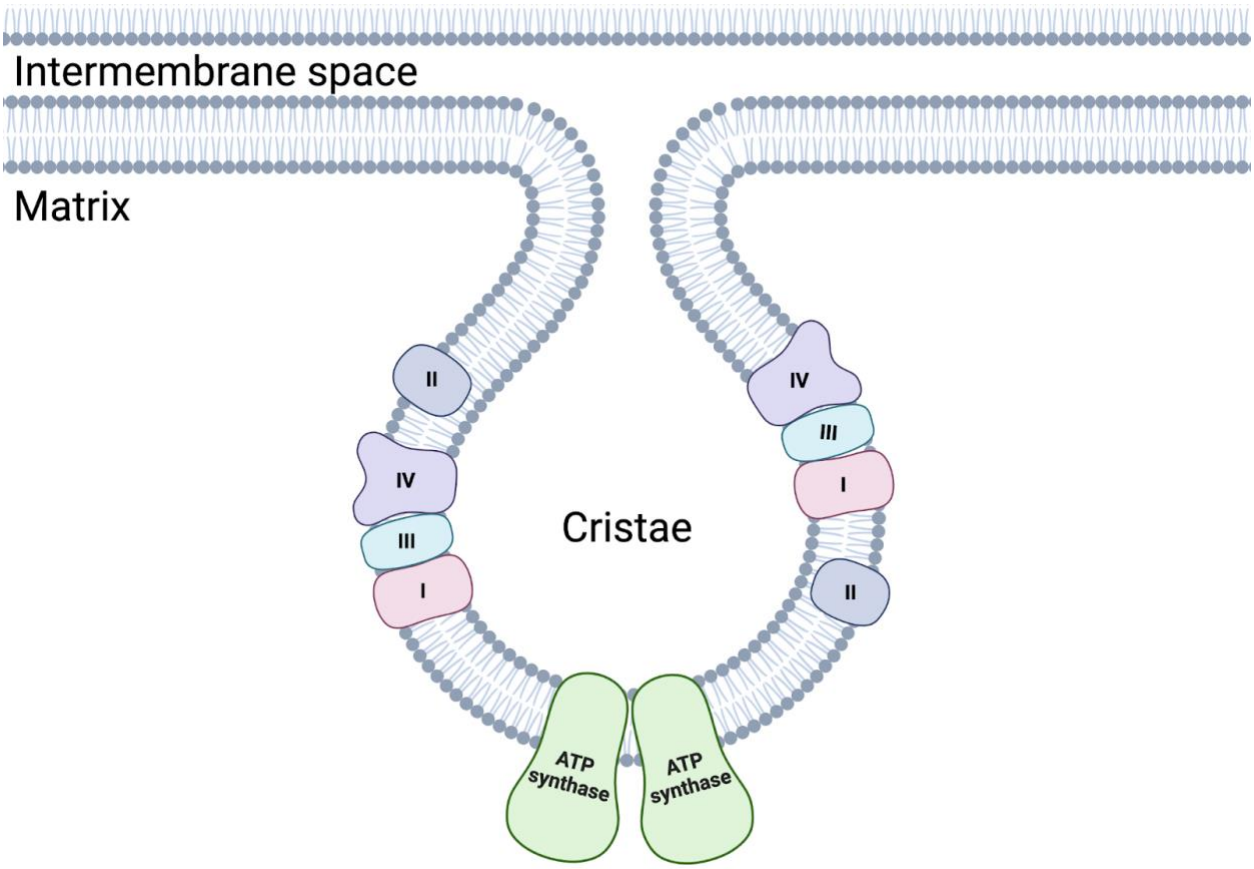


Figure 3.1. Electron transport chain resides on the cristae membrane
Cristae membrane is the site of oxidative phosphorylation. Sets of complexes I through IV are situated on the sides of the cristae, with two ATP synthases (complex V) sitting at the peak of the cristae as a dimer [7,8]. Stabilization of cristae and mitochondrial shape is essential to the organization of oxidative phosphorylation proteins and functioning electron transport chain. In turn, cristae morphology becomes crucial to energy production and cardiac contractility relying on stable energy supply.

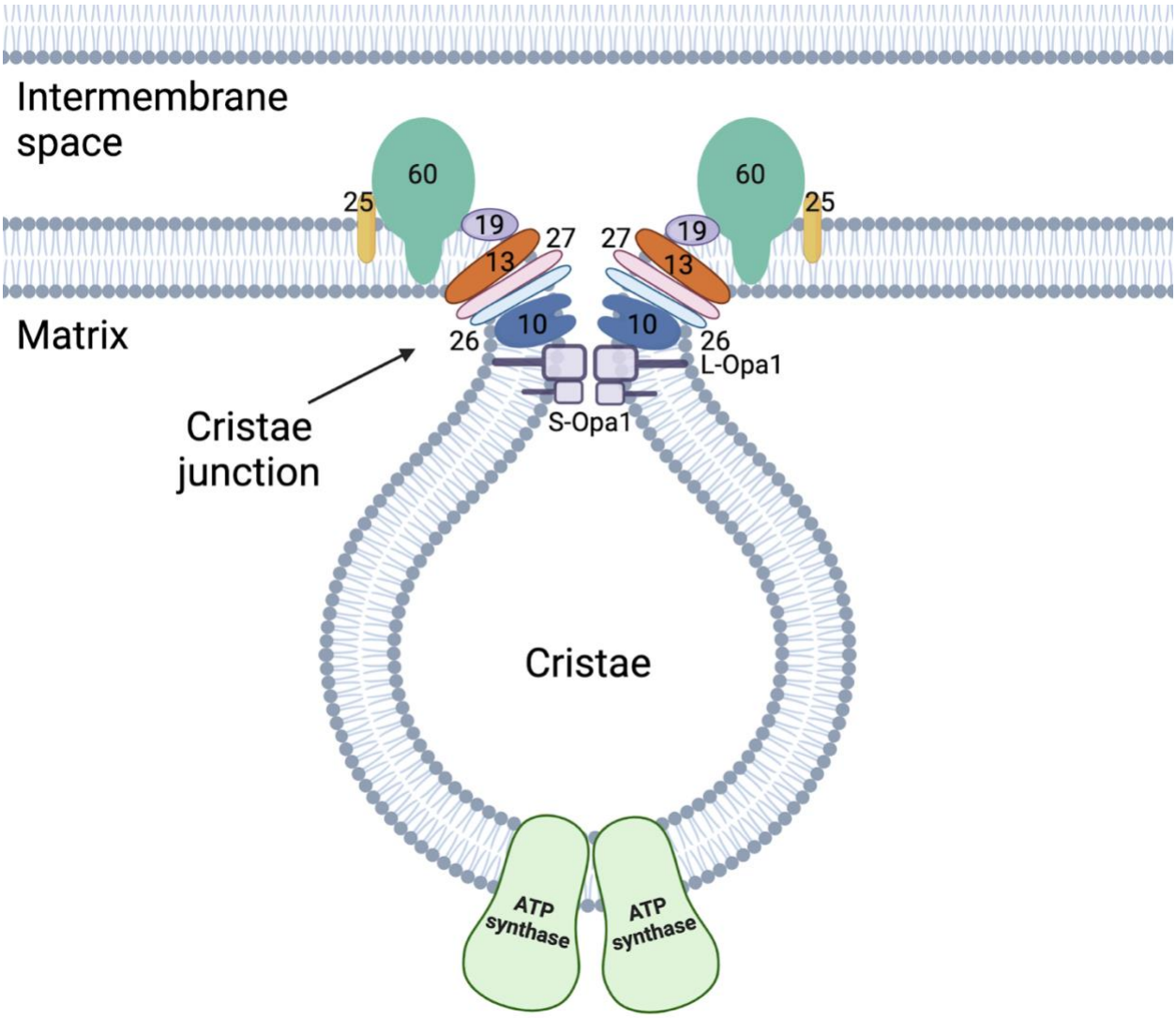


Figure 3.2. Cristae structure is supported by MICOS and Opa1 at the cristae junctions
Cristae structure is regulated by many proteins. Optic atrophy protein 1 (Opa1) keeps the cristae junctions closed, maintaining the width of the cristae and separating the cristae lumen content from the intermembrane space (L-Opa1, long isoform of Opa1; S-Opa1, short isoform of Opa1) [8]. At the same time, the dimerization of ATP synthases at the peak of the cristae maintains cristae curvature [7,8]. The mitochondrial contact site and cristae organizing system (MICOS) stabilize the cristae junctions and facilitate interaction between the inner membrane and outer membrane [8]. MICOS consists of seven subunits: Mic60, Mic19, Mic25, Mic10, Mic26, Mic27, and Mic13 [7,8]. Mic60 and Mic10 serve as the core subunits of the complex and each form two separate subcomplexes (Mic60 subcomplex consists of Mic60, Mic19, and Mic25; Mic10 subcomplex consists of Mic10, Mic26, and Mic27) that are bridged by Mic13 [7,8].

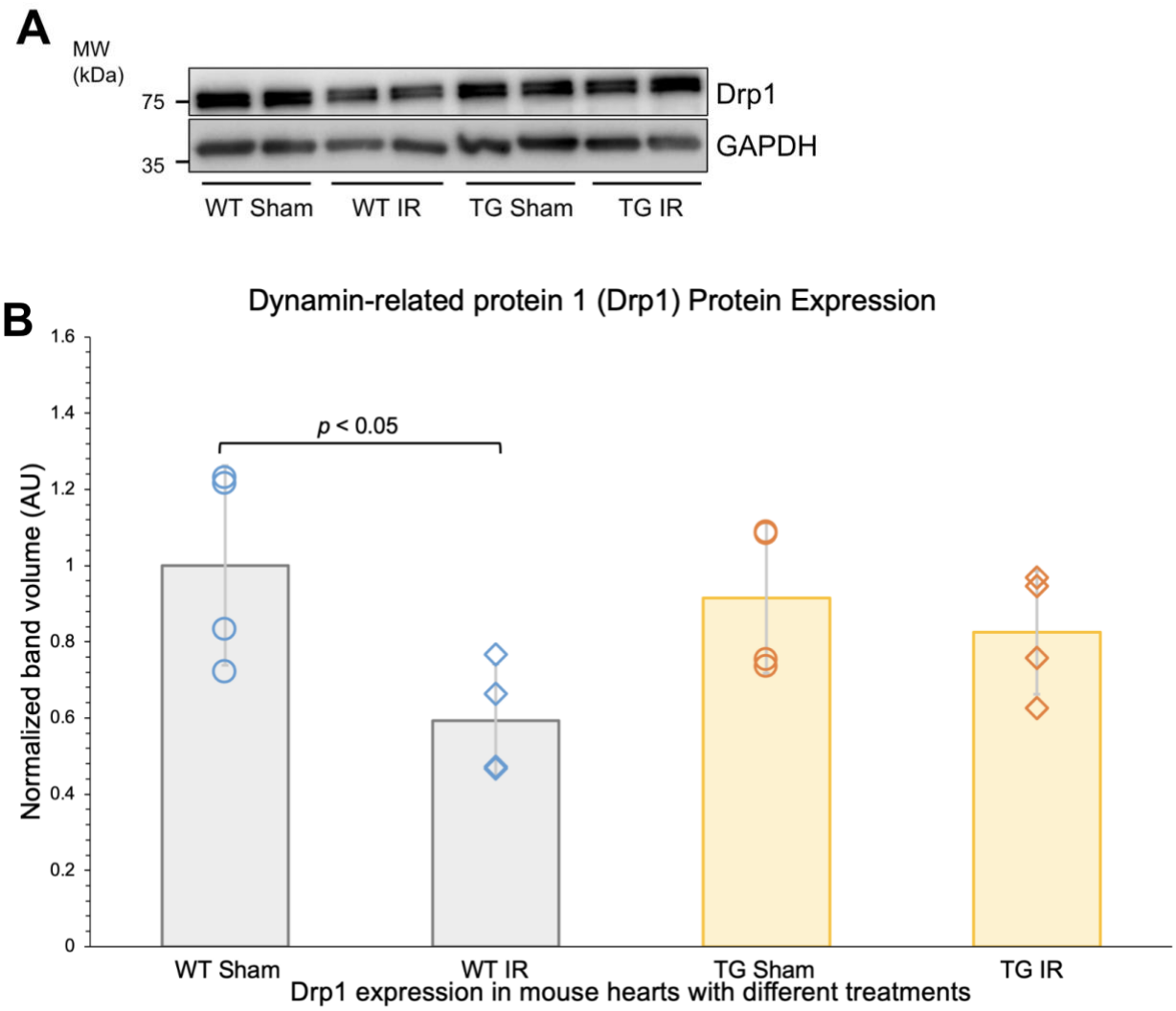
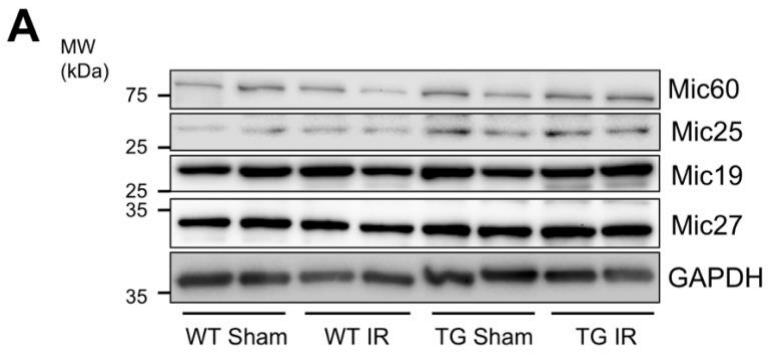
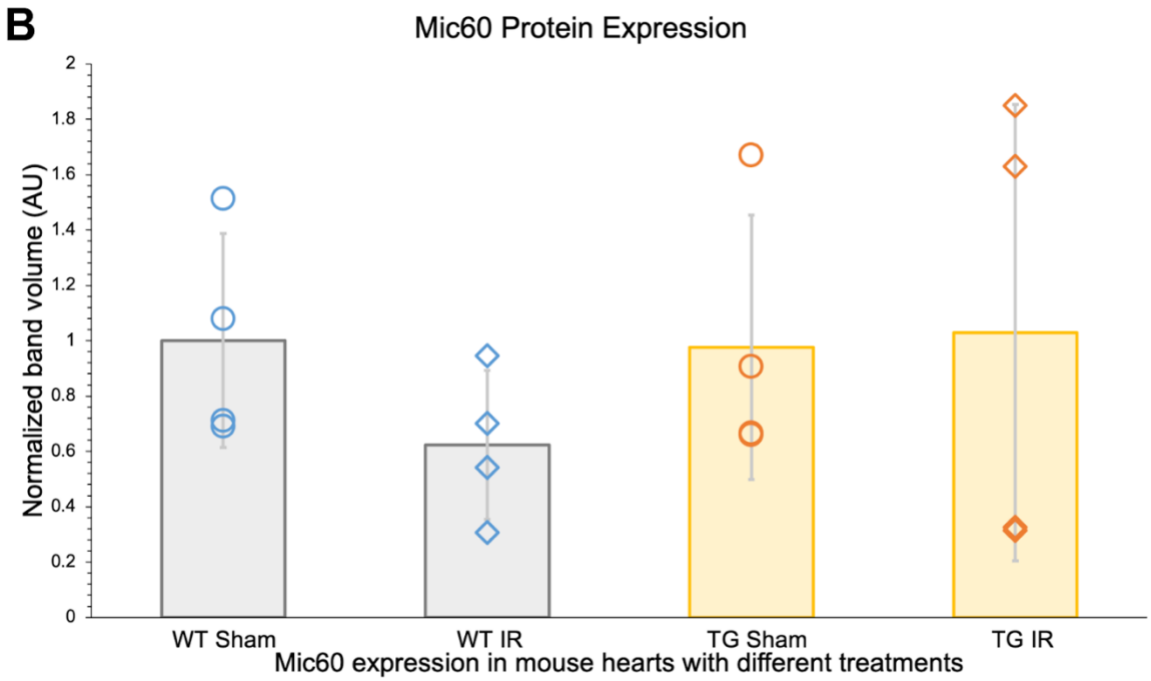


Figure 3.3. Analysis of Dynamin-related protein 1 (Drp1) protein expression in Perm1-overexpressed mice hearts.
Hearts from wild-type (WT) mice and transgenic (TG) mice with cardiac-specific Perm1 overexpression were subjected to perfusion only (sham control) or ischemia-reperfusion (IR) via the Langendorff hanging heart system. Drp1 expression in the hearts were determined by Western blot. Western blot band volumes of each group were normalized to that of the WT Sham group, and normalized band volumes represent the degree of Drp1 expression in the relative heart samples. Each symbol represents band volume of one heart sample (n = 4 per group) while bars indicate average band volume. A student's t-test with 2-tailed distribution and equal variance was used to analyze the statistical differences between WT Sham and WT IR, TG Sham and TG IR, and WT IR and TG IR. Statistical significance $p < 0.05$.

Figure 3.4. Analysis of protein expression of mitochondrial cristae and contact site organizing system (MICOS) subunits in Perm1-overexpressed mice hearts.
Hearts from wild-type (WT) mice and transgenic (TG) mice with cardiac-specific Perm1 overexpression were subjected to perfusion only (sham control) or ischemia-reperfusion (IR) via the Langendorff hanging heart system. MICOS subunit expression in the hearts were determined by Western blot. For each subunit analyzed, Western blot band volumes of each group were normalized to that of the WT Sham group, and normalized band volumes represent the degree of subunit protein expression in the relative heart samples. Each symbol represents band volume of one heart sample ($n = 4$ per group) while bars indicate average band volume. A student's t-test with 2-tailed distribution and equal variance was used to analyze the statistical differences between WT Sham and WT IR, TG Sham and TG IR, and WT IR and TG IR. Statistical significance $p < 0.05$.


A
MW
(kDa)
75
25
25
35
35
Mic60
Mic25
Mic19
Mic27
GAPDH
WT Sham
WT IR
TG Sham
TG IR


B
Mic60 Protein Expression
Normalized band volume (AU)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
WT Sham
WT IR
TG Sham
TG IR
Mic60 expression in mouse hearts with different treatments

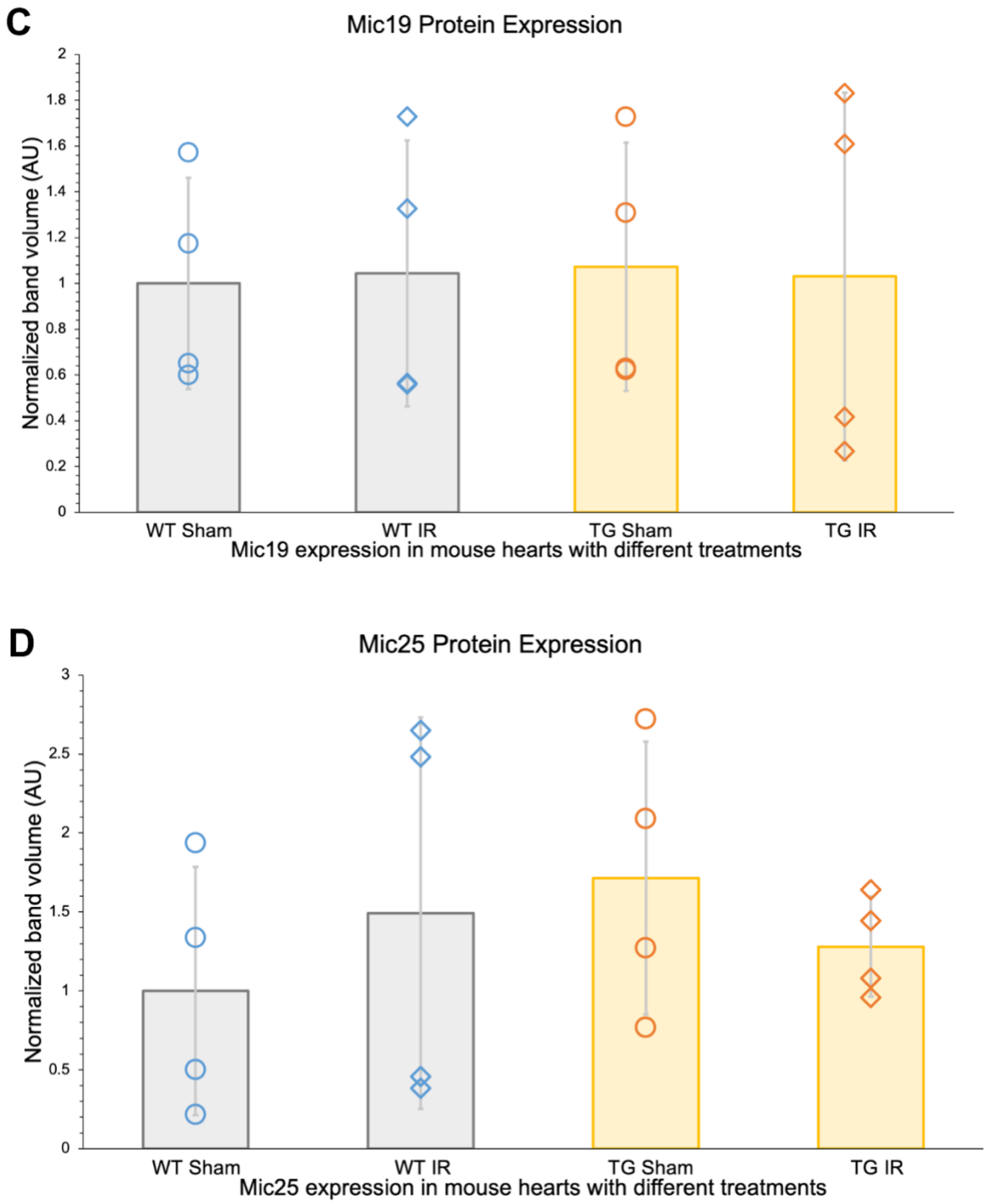


Figure 3.4. Analysis of protein expression of mitochondrial cristae and contact site organizing system (MICOS) subunits in Perm1-overexpressed mice hearts (Continued)

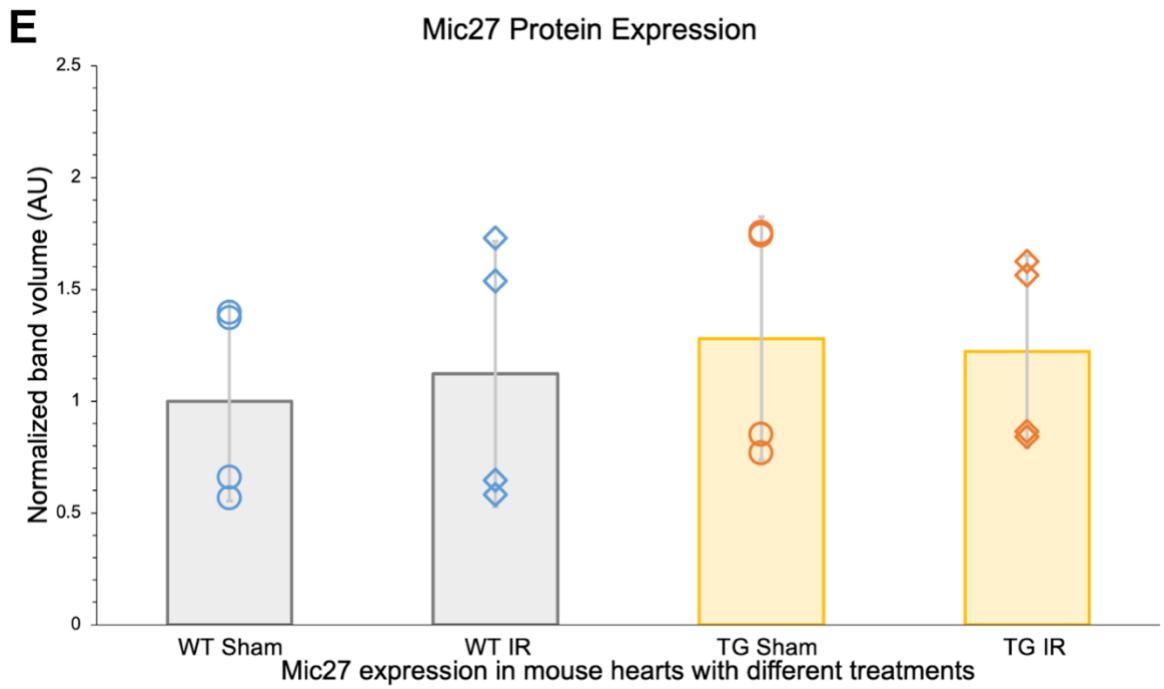


Figure 3.4. Analysis of protein expression of mitochondrial cristae and contact site organizing system (MICOS) subunits in Perm1-overexpressed mice hearts (Continued)

REFERENCES

[1] Pernas, L., & Scorrano, L. (2016). Mito-Morphosis: Mitochondrial Fusion, Fission, and Cristae Remodeling as Key Mediators of Cellular Function. *Annual review of physiology*, *78*, 505–531. https://doi.org/10.1146/annurev-physiol-021115-105011

[2] Baker, N., Patel, J., & Khacho, M. (2019). Linking mitochondrial dynamics, cristae remodeling and supercomplex formation: How mitochondrial structure can regulate bioenergetics. *Mitochondrion*, *49*, 259–268. https://doi.org/10.1016/j.mito.2019.06.003

[3] Vásquez-Trincado, C., García-Carvajal, I., Pennanen, C., Parra, V., Hill, J. A., Rothermel, B. A., & Lavandero, S. (2016). Mitochondrial dynamics, mitophagy and cardiovascular disease. *The Journal of physiology*, *594*(3), 509–525. https://doi.org/10.1113/JP271301

[4] Chen, H., Vermulst, M., Wang, Y. E., Chomyn, A., Prolla, T. A., McCaffery, J. M., & Chan, D. C. (2010). Mitochondrial fusion is required for mtDNA stability in skeletal muscle and tolerance of mtDNA mutations. *Cell*, *141*(2), 280–289. https://doi.org/10.1016/j.cell.2010.02.026

[5] G W D. (2016). Mitochondrial fission/fusion and cardiomyopathy. *Current opinion in genetics & development*, *38*, 38–44. https://doi.org/10.1016/j.gde.2016.03.001

[6] Lesnefsky, E. J., Chen, Q., Tandler, B., & Hoppel, C. L. (2017). Mitochondrial Dysfunction and Myocardial Ischemia-Reperfusion: Implications for Novel Therapies. *Annual review of pharmacology and toxicology*, 57, 535–565. https://doi.org/10.1146/annurev-pharmtox-010715-103335

[7] Anand, R., Reichert, A. S., & Kondadi, A. K. (2021). Emerging Roles of the MICOS Complex in Cristae Dynamics and Biogenesis. *Biology*, *10*(7), 600. https://doi.org/10.3390/biology10070600

[8] Cogliati, S., Enriquez, J. A., & Scorrano, L. (2016). Mitochondrial Cristae: Where Beauty Meets Functionality. *Trends in biochemical sciences*, *41*(3), 261–273. https://doi.org/10.1016/j.tibs.2016.01.001

[9] Eramo, M. J., Lisnyak, V., Formosa, L. E., & Ryan, M. T. (2020). The 'mitochondrial contact site and cristae organising system' (MICOS) in health and human disease. *Journal of biochemistry*, *167*(3), 243–255. https://doi.org/10.1093/jb/mvz111

[10] Adams, R. A., Liu, Z., Hsieh, C., Marko, M., Lederer, W. J., Jafri, M. S., & Mannella, C. (2023). Structural Analysis of Mitochondria in Cardiomyocytes: Insights into Bioenergetics and Membrane Remodeling. *Current issues in molecular biology*, *45*(7), 6097–6115. https://doi.org/10.3390/cimb45070385

[11] Vue, Z., Neikirk, K., Vang, L., Garza-Lopez, E., Christensen, T. A., Shao, J., Lam, J., Beasley, H. K., Marshall, A. G., Crabtree, A., Anudokem, J., Jr, Rodriguez, B., Kirk, B.,

Bacevac, S., Barongan, T., Shao, B., Stephens, D. C., Kabugi, K., Koh, H. J., Koh, A., Evans, C. S., Taylor B., Reddy, A. K., Miller-Fleming, T., Actkins, K. E., Zaganjor, E., Daneshgar, N., Murray, S. A., Mobley, B. C., Damo, S. M., Gaddy, J. A., Riggs, B., Wanjalla, C., Kirabo, A., McReynolds, M., Gomez, J. A., Phillips, M. A., Exil, V., Dai, D., Hinton, A., Jr (2023). Three-dimensional mitochondria reconstructions of murine cardiac muscle changes in size across aging. *American journal of physiology. Heart and circulatory physiology*, *325*(5), H965–H982. https://doi.org/10.1152/ajpheart.00202.2023

[12] Birker, K., Ge, S., Kirkland, N. J., Theis, J. L., Marchant, J., Fogarty, Z. C., Missinato, M. A., Kalvakuri, S., Grossfeld, P., Engler, A. J., Ocorr, K., Nelson, T. J., Colas, A. R., Olson, T. M., Vogler, G., & Bodmer, R. (2023). Mitochondrial MICOS complex genes, implicated in hypoplastic left heart syndrome, maintain cardiac contractility and actomyosin integrity. *eLife*, *12*, e83385. https://doi.org/10.7554/eLife.83385

[13] Chen, L., Qin, Y., Liu, B., Gao, M., Li, A., Li, X., & Gong, G. (2022). PGC-1α-Mediated Mitochondrial Quality Control: Molecular Mechanisms and Implications for Heart Failure. *Frontiers in cell and developmental biology*, *10*, 871357. https://doi.org/10.3389/fcell.2022.871357

[14] Bock, T., Türk, C., Aravamudhan, S., Keufgens, L., Bloch, W., Rozsivalova, D. H., Romanello, V., Nogara, L., Blaauw, B., Trifunovic, A., Braun, T., & Krüger, M. (2021). PERM1 interacts with the MICOS-MIB complex to connect the mitochondria and sarcolemma via ankyrin B. *Nature communications*, *12*(1), 4900. https://doi.org/10.1038/s41467-021-25185-3

[15] Zepeda, R., Kuzmicic, J., Parra, V., Troncoso, R., Pennanen, C., Riquelme, J. A., Pedrozo, Z., Chiong, M., Sánchez, G., & Lavandero, S. (2014). Drp1 loss-of-function reduces cardiomyocyte oxygen dependence protecting the heart from ischemia-reperfusion injury. *Journal of cardiovascular pharmacology*, *63*(6), 477–487. https://doi.org/10.1097/FJC.0000000000000071

[16] Disatnik, M. H., Ferreira, J. C., Campos, J. C., Gomes, K. S., Dourado, P. M., Qi, X., & Mochly-Rosen, D. (2013). Acute inhibition of excessive mitochondrial fission after myocardial infarction prevents long-term cardiac dysfunction. *Journal of the American Heart Association*, *2*(5), e000461. https://doi.org/10.1161/JAHA.113.000461

[17] Feng, Y., Madungwe, N. B., & Bopassa, J. C. (2019). Mitochondrial inner membrane protein, Mic60/mitofilin in mammalian organ protection. *Journal of cellular physiology*, *234*(4), 3383–3393. https://doi.org/10.1002/jcp.27314

[18] Li, H., Ruan, Y., Zhang, K., Jian, F., Hu, C., Miao, L., Gong, L., Sun, L., Zhang, X., Chen, S., Chen, H., Liu, D., & Song, Z. (2016). Mic60/Mitofilin determines MICOS assembly essential for mitochondrial dynamics and mtDNA nucleoid organization. *Cell death and differentiation*, *23*(3), 380–392. https://doi.org/10.1038/cdd.2015.102

[19] Tombo, N., Imam Aliagan, A. D., Feng, Y., Singh, H., & Bopassa, J. C. (2020). Cardiac ischemia/reperfusion stress reduces inner mitochondrial membrane protein (mitofilin) levels

during early reperfusion. *Free radical biology & medicine*, *158*, 181–194. https://doi.org/10.1016/j.freeradbiomed.2020.06.039

[20] Darshi, M., Mendiola, V. L., Mackey, M. R., Murphy, A. N., Koller, A., Perkins, G. A., Ellisman, M. H., & Taylor, S. S. (2011). ChChd3, an inner mitochondrial membrane protein, is essential for maintaining crista integrity and mitochondrial function. *The Journal of biological chemistry*, *286*(4), 2918–2932. https://doi.org/10.1074/jbc.M110.171975

[21] Sakowska, P., Jans, D. C., Mohanraj, K., Riedel, D., Jakobs, S., & Chacinska, A. (2015). The Oxidation Status of Mic19 Regulates MICOS Assembly. *Molecular and cellular biology*, *35*(24), 4222–4237. https://doi.org/10.1128/MCB.00578-15

[22] Ding, C., Wu, Z., Huang, L., Wang, Y., Xue, J., Chen, S., Deng, Z., Wang, L., Song, Z., Chen, S. (2015) Mitofilin and CHCHD6 physically interact with Sam50 to sustain cristae structure. *Sci Rep* 5(16064). https://doi.org/10.1038/srep16064

[23] An, J., Shi, J., He, Q., Lui, K., Liu, Y., Huang, Y., & Sheikh, M. S. (2012). CHCM1/CHCHD6, novel mitochondrial protein linked to regulation of mitofilin and mitochondrial cristae morphology. *The Journal of biological chemistry*, *287*(10), 7411–7426. https://doi.org/10.1074/jbc.M111.277103

[24] Anand, R., Kondadi, A. K., Meisterknecht, J., Golombek, M., Nortmann, O., Riedel, J., Peifer-Weiß, L., Brocke-Ahmadinejad, N., Schlütermann, D., Stork, B., Eichmann, T. O., Wittig, I., & Reichert, A. S. (2020). MIC26 and MIC27 cooperate to regulate cardiolipin levels and the landscape of OXPHOS complexes. *Life science alliance*, *3*(10), e202000711. https://doi.org/10.26508/lsa.202000711

[25] Dudek, J., Hartmann, M., & Rehling, P. (2019). The role of mitochondrial cardiolipin in heart function and its implication in cardiac disease. *Biochimica et biophysica acta. Molecular basis of disease*, *1865*(4), 810–821. https://doi.org/10.1016/j.bbadis.2018.08.025

[26] Cho, Y., Tachibana, S., Lam, K., Arita, Y., Khosrowjerdi, S., Zhang, O., Liang, A., Li, R., Andreyev, A., Murphy, A. N., & Ross, R. S. (2021). Perm1 promotes cardiomyocyte mitochondrial biogenesis and protects against hypoxia/reoxygenation-induced damage in mice. *The Journal of biological chemistry*, 297(1), 100825. https://doi.org/10.1016/j.jbc.2021.100825

[27] Oka, S. I., Sabry, A. D., Horiuchi, A. K., Cawley, K. M., O'Very, S. A., Zaitsev, M. A., Shankar, T. S., Byun, J., Mukai, R., Xu, X., Torres, N. S., Kumar, A., Yazawa, M., Ling, J., Taleb, I., Saijoh, Y., Drakos, S. G., Sadoshima, J., & Warren, J. S. (2020). Perm1 regulates cardiac energetics as a downstream target of the histone methyltransferase Smyd1. *PloS one*, 15(6), e0234913. https://doi.org/10.1371/journal.pone.0234913

[28] Gao, T., Shi, R., Liu, Z., De, D., Li, R., Chen, Y., Pei, J., & Ding, M. (2023). Ischemia/reperfusion-induced MiD51 upregulation recruits Drp1 to mitochondria and contributes to myocardial injury. *Biochemical and biophysical research communications*, *665*, 78–87. https://doi.org/10.1016/j.bbrc.2023.05.013

[29] Sharp, W. W., Fang, Y. H., Han, M., Zhang, H. J., Hong, Z., Banathy, A., Morrow, E., Ryan, J. J., & Archer, S. L. (2014). Dynamin-related protein 1 (Drp1)-mediated diastolic dysfunction in myocardial ischemia-reperfusion injury: therapeutic benefits of Drp1 inhibition to reduce mitochondrial fission. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, *28*(1), 316–326. https://doi.org/10.1096/fj.12-226225

[30] Motayagheni N. (2017). Modified Langendorff technique for mouse heart cannulation: Improved heart quality and decreased risk of ischemia. *MethodsX*, *4*, 508–512. https://doi.org/10.1016/j.mex.2017.11.004

[31] Ismail, N. I., Michel, N. A., Katwadi, K., Lim, M. M., Chan, T. K., Rahman, A., Xu, D., Ong, S. G., Hausenloy, D. J., & Ong, S. B. (2022). Ischemic Preconditioning and Postconditioning Protect the Heart by Preserving the Mitochondrial Network. *BioMed research international*, *2022*, 6889278. https://doi.org/10.1155/2022/6889278

[32] Otera, H., Miyata, N., Kuge, O., & Mihara, K. (2016). Drp1-dependent mitochondrial fission via MiD49/51 is essential for apoptotic cristae remodeling. *The Journal of cell biology*, *212*(5), 531–544. https://doi.org/10.1083/jcb.201508099

[33] Givvimani, S., Pushpakumar, S. B., Metreveli, N., Veeranki, S., Kundu, S., & Tyagi, S. C. (2015). Role of mitochondrial fission and fusion in cardiomyocyte contractility. *International journal of cardiology*, *187*, 325–333. https://doi.org/10.1016/j.ijcard.2015.03.352

[34] Yang, R. F., Sun, L. H., Zhang, R., Zhang, Y., Luo, Y. X., Zheng, W., Zhang, Z. Q., Chen, H. Z., & Liu, D. P. (2015). Suppression of Mic60 compromises mitochondrial transcription and oxidative phosphorylation. *Scientific reports*, *5*, 7990. https://doi.org/10.1038/srep07990

[35] Yang, R. F., Zhao, G. W., Liang, S. T., Zhang, Y., Sun, L. H., Chen, H. Z., & Liu, D. P. (2012). Mitofilin regulates cytochrome c release during apoptosis by controlling mitochondrial cristae remodeling. *Biochemical and biophysical research communications*, *428*(1), 93–98. https://doi.org/10.1016/j.bbrc.2012.10.012

[36] Thapa, D., Nichols, C. E., Lewis, S. E., Shepherd, D. L., Jagannathan, R., Croston, T. L., Tveter, K. J., Holden, A. A., Baseler, W. A., & Hollander, J. M. (2015). Transgenic overexpression of mitofilin attenuates diabetes mellitus-associated cardiac and mitochondria dysfunction. *Journal of molecular and cellular cardiology*, *79*, 212–223. https://doi.org/10.1016/j.yjmcc.2014.11.008

[37] Zhang, Y., Xu, J., Luo, Y. X., An, X. Z., Zhang, R., Liu, G., Li, H., Chen, H. Z., & Liu, D. P. (2014). Overexpression of mitofilin in the mouse heart promotes cardiac hypertrophy in response to hypertrophic stimuli. *Antioxidants & redox signaling*, *21*(12), 1693–1707. https://doi.org/10.1089/ars.2013.5438

[38] Zhu, S., Chen, Z., Zhu, M., Shen, Y., Leon, L. J., Chi, L., Spinozzi, S., Tan, C., Gu, Y., Nguyen, A., Zhou, Y., Feng, W., Vaz, F. M., Wang, X., Gustafsson, A. B., Evans, S. M., Kunfu,

O., & Fang, X. (2021). Cardiolipin Remodeling Defects Impair Mitochondrial Architecture and Function in a Murine Model of Barth Syndrome Cardiomyopathy. *Circulation. Heart failure*, *14*(6), e008289. https://doi.org/10.1161/CIRCHEARTFAILURE.121.008289

[39] Zerbes, R. M., Höß, P., Pfanner, N., van der Laan, M., & Bohnert, M. (2016). Distinct Roles of Mic12 and Mic27 in the Mitochondrial Contact Site and Cristae Organizing System. *Journal of molecular biology*, *428*(8), 1485–1492. https://doi.org/10.1016/j.jmb.2016.02.031

[40] Turkieh, A., Caubère, C., Barutaut, M., Desmoulin, F., Harmancey, R., Galinier, M., Berry, M., Dambrin, C., Polidori, C., Casteilla, L., Koukoui, F., Rouet, P., & Smih, F. (2014). Apolipoprotein O is mitochondrial and promotes lipotoxicity in heart. *The Journal of clinical investigation*, *124*(5), 2277–2286. https://doi.org/10.1172/JCI74668

[41] Liu, Y., Xiong, Z., Zhou, W., Chen, Y., Huang, Q., & Wu, Y. (2022). Role of apolipoprotein O in autophagy via the p38 mitogen-activated protein kinase signaling pathway in myocardial infarction. *Clinics (Sao Paulo, Brazil)*, *77*, 100046. https://doi.org/10.1016/j.clinsp.2022.100046

[42] Paradies, G., Paradies, V., Ruggiero, F. M., & Petrosillo, G. (2018). Mitochondrial bioenergetics and cardiolipin alterations in myocardial ischemia-reperfusion injury: implications for pharmacological cardioprotection. *American journal of physiology. Heart and circulatory physiology*, *315*(5), H1341–H1352. https://doi.org/10.1152/ajpheart.00028.2018

CHAPTER IV: Effects of Perm1 Overexpression on Apoptotic Signaling Pathways

Introduction

Apoptosis and necrosis are cell death processes that occur in cardiomyocytes during ischemia-reperfusion [1]. Cell death by necrosis is defined by cell rupturing and release of cellular contents, while apoptosis is defined by shrinkage and detachment of the cell from surrounding tissue and degradation of cellular components of phagocytosis by other cells [2]. While previous studies have struggled to clarify whether apoptosis is induced during ischemia and then accelerated during reperfusion or whether apoptosis is only induced during reperfusion and not ischemia [3,4], it remains clear that apoptosis plays a role in myocardial damage from ischemia-reperfusion injury.

Apoptosis can occur by the extrinsic pathway or the intrinsic pathway. The extrinsic pathway involves death ligands binding to transmembrane receptors containing death domains to transmit death signals to intracellular pathways [5]. An example includes tumor necrosis factor-alpha (TNF-α) and tumor necrosis factor receptor 1 (TNFR1). Members of the caspase family, such as caspase 3, 8, and 7, are activated to trigger the apoptotic cascade response and apoptosis execution [5]. On the other hand, the intrinsic pathway involves the mitochondria and can be activated during stressful conditions such as hypoxia [5]. In this pathway, the mitochondrial membrane potential is altered to promote the opening of the mitochondrial permeability transition pores (mPTP) [1,5]. Alterations to the cristae junctions also cause release of cytochrome c from the cristae lumen [6]. Once released into the cytosol, cytochrome c activates the caspase cascade [1], starting with caspase 9, which then promotes caspase 3 and 7 activation [5]. Outer mitochondrial membrane permeability and cytochrome c release are mediated by Bcl-2 family proteins situated on the outer mitochondrial membrane [5].

During the onset of reperfusion, levels of TNF-α and TNF-related apoptosis-inducing ligand (TRAIL) increase [5]. In the ischemic myocardium, levels of Bcl-2 family member Bax, a proapoptotic protein, also increase [5]. In addition to these components, proteins of the mitogen-activated protein kinase (MAPK) signaling pathway interact with these apoptotic signaling pathways to promote or inhibit apoptosis. These MAPK proteins include p38MAPK (also referred to as p38) and c-Jun N-terminal kinase (JNK). Both of these MAP kinases are activated by phosphorylation and show increased activity and expression during ischemia and reperfusion [2]. Increases in their activity correspond with increased DNA fragmentation, a signature of apoptosis [2]. P38 appears to be proapoptotic and can activate TNF-α production, while JNK can have both anti- and pro-apoptotic characteristics [2].

JNK plays a role in apoptosis by interacting with components of the extrinsic apoptotic signaling pathway, such as TNF-a [7,8]. JNK is also involved in the intrinsic apoptotic signaling pathway, as activation of mitochondrial JNK or translocation of JNK to the mitochondria induces apoptosis [8,9]. Within the mitochondria, JNK phosphorylates anti-apoptotic proteins Bcl-2 and Bcl-xL of the Bcl-2 family, possibly attenuating their anti-apoptotic effects [10] such as maintaining mitochondrial membrane potential, suppressing caspase activation, and preventing cytochrome c release [9]. Meanwhile, JNK can also phosphorylate Bad, a pro-apoptotic member of the Bcl-2 family [9]. Previous studies have observed that JNK promotes the generation of ROS and is also activated by ROS, Ca^{2+} influx, and ETC activity [9]. However, JNK can also be activated by MEKK1, another MAPK, which inhibits TNF-a production [2]. Disrupting MEKK1 activity can result in increased mitochondrial sensitivity to ROS [2], suggesting JNK involvement in anti-apoptosis. On the other hand, p38 regulates inflammatory responses, cell growth, cell differentiation, cell cycle, and cell death [11]. P38 activation is stimulated by

inflammatory response, growth factors, and physiological stress [11] and phosphorylation by MAPK kinases MKK3, and MKK6 [12].

Protein kinase B, also referred to as Akt, is a serine/threonine kinase belonging to the AMP/GMP protein kinases and protein kinase C family proteins [13]. Although Akt belongs to a different family of protein kinases as JNK and p38, it is also activated by phosphorylation by phosphoinositide-3-kinase (PI3K) [13]. In turn, Akt activates numerous downstream targets involved in metabolism, angiogenesis, cell growth and proliferation, cell survival, protein synthesis, transcription, and apoptosis [14]. Akt is also involved in cardioprotection. Studies have shown that Akt regulates growth factors such as insulin-like growth factor 1 (IGF-1), which decreases infarct size and apoptosis [4]. Akt functional knockdown experiments showed reduced cell survival and protective effects induced by insulin-like growth factor 1 (IGF-1) [15]. Akt also directly reduces apoptosis by inhibiting caspase 9 and activates endothelial nitric oxide synthase to maintain cardiovascular homeostasis [14].

Cho et al. demonstrated that overexpressing Perm1 in cardiomyocytes was associated with reduced cell death upon hypoxia-reoxygenation [16]. In the current study, I proceeded to examine whether overexpressing Perm1 in the myocardium associates with alterations in the expression and activation of proteins involved in apoptotic signaling. apoptosis-related proteins following ischemia-reperfusion. Levels of phosphorylated and total JNK, p38, and Akt were specifically interrogated in transgenic and wild-type mice hearts upon sham and ischemia-reperfusion.

Materials and Methods

Animal Models

C57BL/6 mice at 3 to 4 months of age were housed and transgenic mice were generated through methods previously described. All experimental procedures perform in animals were approved by the University of California San Diego Institutional Animal Care and Use Committee (IACUC).

Ischemia/reperfusion by Langendorff apparatus

The wild-type mice and the transgenic mice groups each comprised a total of 12 male mice, with 8 mice subjected to ex vivo ischemia-reperfusion and the remaining 4 mice serving as sham controls using the Langendorff apparatus and procedure previously described. Entire hearts were sampled.

Western Blot

Following completion of the ischemia-reperfusion protocol, total protein lysates were prepared and Western blots were run as previously described. was extracted from the ventricular tissue by homogenesis with homogenizing buffer as previously described. Membranes were blocked with 5% BSA in TBST. The primary antibodies, diluted at 1:1000, were as follows: p-JNK, SAPK/JNK, p-p38, p38, p-Akt, and Akt. GAPDH was used as a loading control. Protein bands were detected by enhanced chemiluminescence (ECL), and band intensity was measured using BioRad software. The results were presented as normalized expression levels relative to that of the wild-type sham group.

Statistical analysis

All data are shown as means ± standard deviations. Student's t-test using 2-tail distribution and unpaired, equal variance was used to evaluate differences between wild-type sham, transgenic sham, wild-type ischemia-reperfusion, and transgenic ischemia-reperfusion groups. Differences $p<0.05$ were considered to be statistically significant.

Results

Analysis of expression of phosphorylated JNK to total JNK

JNK is a MAP kinase involved in both the extrinsic and intrinsic apoptotic pathway [7]. During IR, JNK is activated in the cytosol and mitochondria through phosphorylation of serine residues by MAPK kinases [11,18]. I analyzed the ratio of phosphorylated JNK (p-JNK) expression to total JNK expression as a representation of JNK activation to determine whether JNK activation would decrease in response to Perm1 overexpression upon IR. **Figure 4.1B** showed that WT IR had an average normalized band volume ratio of p-JNK to total JNK expression of 2.789, higher than that of WT sham (1.000). TG sham and TG IR had an average of 1.250 and 2.494 respectively. The differences in expression ratio between WT sham and WT IR, TG sham and TG IR, and WT IR and TG IR were not statistically significant ($p > 0.05$) .

Analysis of expression of phosphorylated p38 to total p38

In addition to JNK, p38, another member of the MAPK family, is also involved in apoptosis. P38 is involved in signaling for inflammatory responses, cell growth, cell differentiation, cell death, and the cell cycle [19]. Although there has been some controversy regarding whether p38 plays a beneficial role or induces injury upon myocardial IR, majority of studies have shown that inhibition of p38 prior to ischemia-reperfusion attenuates injury and

apoptosis in the myocardium [13]. The ratio of phosphorylated p38 (p-p38) expression to total p38 expression was analyzed to determine whether p38 activation decreases in response to Perm1 overexpression upon IR, shown in **Figure 4.2B**. WT IR showed an average normalized band volume ratio of p-p38 to total p38 expression of 2.354, higher than that of WT sham (1.000) although this difference is not statistically significance ($p > 0.05$). TG sham had an average of 1.683 and TG IR had an average of 1.315. Differences between normalized expression ratios in WT IR, TG IR, and TG sham did not show statistical significance ($p > 0.05$).

Analysis of expression of phosphorylated Akt to total Akt

Protein kinase B, also called Akt, is a serine/threonine kinase involved in cell survival. Akt is activated by phosphoinositide-3-kinase (PI3K) [14] and activates numerous downstream targets involved in metabolism, angiogenesis, cell growth and proliferation, cell survival, protein synthesis, transcription, and apoptosis [20]. The PI3K/Akt pathway is involved in facilitating the cardioprotective effects exerted by numerous cellular components such as growth factors [21].

The function of Perm1 was further studied by first examining whether Perm1 overexpression affects total Akt expression upon myocardial IR. From **Figure 4.3B**, WT IR, TG sham, and TG IR showed average normalized band volumes of 0.892, 1.097, and 0.799 respectively. Differences between these averages did not reach statistical significance ($p > 0.05$).

The normalized ratio of phosphorylated Akt (p-Akt) to total Akt expression was also analyzed. **Figure 4.3C** showed that WT IR had an average ratio of 1.438, comparable to that of TG sham (1.413). TG IR showed an average expression ratio of 1.013. Differences between these averages and that of WT sham (1.000) were also not statistically significant ($p > 0.05$).

Discussion

Previously, Cho and colleagues showed that isolated cardiomyocytes with increased Perm1 expression showed decreased apoptosis upon hypoxia-reoxygenation [16]. In Chapter 2, I also showed that isolated mice hearts with Perm1 overexpression showed reduced infarct size upon global IR (**Figure 2.5**). Given these findings, I questioned whether Perm1 carries out its cardioprotective role by directly associating with the apoptotic signaling pathway. To investigate this, the activation of pro-apoptotic and survival-promoting protein kinases were analyzed in isolated hearts with increased Perm1 expression upon global ischemia-reperfusion.

c-Jun N-terminal kinase (JNK) is a member of the mitogen activated protein kinase (MAPK) family and can exert pro-apoptotic or anti-apoptotic effects through its involvement in apoptotic signaling pathways [7]. JNK can be activated by environmental stressors [10] or phosphorylation by MAPK kinases [8]. Studies have shown that JNK is activated in the cytosol and mitochondria of the myocardium during IR [11,18], and multiple studies have demonstrated that increased JNK activity primarily occurs during reperfusion [9,23]. At the same time, studies examining the effects of inhibiting JNK activity on IR injury demonstrated cardioprotective effects of JNK inhibition. Pharmacological inhibition of JNK showed decreased apoptosis [9]. Genetic knock-out and knock-down of JNK isoforms showed significantly reduced infarct area and DNA fragmentation that serves as an indicator of apoptosis [10]. Ferrandi et al. also demonstrated decreased infarct size and DNA fragmentation with lower concentrations of phosphorylated JNK through JNK specific inhibitors [23].

The expression ratio of p-JNK to total JNK did not demonstrate a statistically significant difference between WT IR and WT sham. As a result, a valid interpretation can not be made to suggest that my results align with that of previous findings. A valid, confident interpretation also

can not be made regarding the effect of Perm1 overexpression on JNK activation due to the small sample size and lack of statistical significance in the observed expression ratio differences between WT IR, TG sham, and TG IR. Batch effects were present, contributing to the large variation in the data. Experimentation with more samples are needed to reduce variation and batch effects. If additional samples cluster with either data batch and variation is reduced, clearer results can be obtained for interpretation.

JNK can also exert anti-apoptotic effects. JNK can be activated by another MAP kinase MEKK1, which can inhibit TNF-a production [2]. Disrupting MEKK1 activity can result in increased mitochondrial sensitivity to reactive oxygen species (ROS) [2], suggesting a possible role for JNK in regulating ROS-induced apoptosis. Although majority of studies demonstrated that JNK exerts pro-apoptotic effects during ischemia-reperfusion, given the anti-apoptotic functions of JNK, if Perm1 is involved in cardiomyocyte apoptotic signaling through association with JNK, the association may not necessarily be one of inhibition. More research is needed to further our understanding of whether and how Perm1 associates with JNK.

P38 is a MAP kinase involved in regulating inflammatory responses, cell growth, cell differentiation, cell cycle, and cell death [19]. P38 activation can be stimulated by physiological stress [19] and occurs through phosphorylation by MAPK kinases [13]. Ma et al. demonstrated that p38 activation peaks at 15 minutes of ischemia [24]. Although levels of activated p38 decreased starting from 30 minutes of ischemia, reactivation occurred at the onset of reperfusion and peaked at a higher level at 10 minutes of reperfusion [24]. Multiple studies have shown that inhibition of p38 through specific inhibitors or by functional knockdown of MKK6 or MKK3 demonstrates cardioprotective effects, such as preserved cell viability, decreased levels of reactive oxygen species, decreased DNA fragmentation, and decreased cardiomyocyte apoptosis

[19]. Schwertz et al. also showed that administering p38 inhibitor reduced necrotic area induced by ischemia-reperfusion [25]. Kaiser et al. demonstrated that inhibiting p38 protects the heart from lasting ischemia-reperfusion induced defects, such as intracellular fibrosis and infarct expansion, and preserves cardiac contractile function [12].

The observed differences in my results did not reach statistical significance. As a result of this lack of statistical significance and the small sample size, valid interpretations cannot be made to suggest that my results align with previous findings of p38 during IR. Interpretations regarding p38 activation in transgenic hearts also can not be made from the current results. The lack of statistical significance can be attributed to the large variations in my data. Although batch effects are present, variation appears to exist within the separate batches as well, further indicating that more samples are needed to reduce variation to establish statistical significance. If additional samples cluster around either data batch, clearer results can be obtained for more confident interpretations that can be discussed. Until then, it is difficult to hypothesize whether Perm1 overexpression suppresses p38 activation upon ischemia-reperfusion.

Zhao et al. showed that p38 substrate protein kinase (PRAK), a downstream target of p38, is essential for normal cardiac performance [27]. PRAK-knockout experiments showed deficiency in cardiac contractility, dilation, and general decreased cardiac performance both during baseline conditions and after ischemia-reperfusion [27]. This study demonstrates that p38 is also involved in maintenance of cardiac function in addition to the role it plays in promoting apoptosis. I hypothesize that since p38 appear to exert both protective and apoptotic effects, if Perm1 associates with p38, the nature of Perm1 interference with p38 activation may depend on physiological conditions.

Research can also be conducted to investigate whether Perm1 interacts with upstream regulators of JNK and p38, such as MAPK kinases (MKK) or MKK kinases (MKKK), which are further upstream of MKK. Although JNK and p38 are generally activated by different MKK, it appears that MKK4 serves as a common MAPK kinases that can activate both JNK and p38 to a certain degree [7,11]. Further studies can investigate whether Perm1 interacts with MKK4, other MKKs, or even MKKKs to gain further insight into the pathway by which Perm1 reduced cardiomyocyte apoptosis during ischemia-reperfusion injury.

Akt is a serine/threonine kinase activated by phosphorylation by phosphoinositide-3-kinase (PI3K) [13]. Akt is involved in metabolism, angiogenesis, cell growth and proliferation, cell survival, protein synthesis, transcription, and apoptosis [14]. Activation of Akt can reduce infarct size, abundance of apoptotic nuclei, and DNA fragmentation, as well as preserve systolic thickening and cardiac performance following IR [28]. Thus, the PI3K/Akt pathway is considered to be cardioprotective and enhanced to counter injury promoted by ischemia-reperfusion. However, a study by Penela et al. found that, though levels of phosphorylated Akt increased during reperfusion, total Akt levels decreased and remained low during IR [21]. They demonstrated a decrease in Akt substrate phosphorylation despite increased Akt phosphorylation [21]. They speculated that, though the Akt pathway is considered to be enhanced during IR, Akt activation of remaining total Akt that has not degraded is not sufficient to compensate for necessary Akt activity [21].

The observed differences in average total Akt expression and average expression ratio of p-Akt to total Akt between the heart sample groups were not statistically significant. Thus, an interpretation can not be made to suggest results that align with findings made by Penela et al. Due to small sample size and large variation, the data also can not be interpreted to make valid

conclusions regarding the effect of Perm1 overexpression on Akt activation. While batch effects may be present, variations appear to be large within and between the two data batches. The variations in total Akt data and expression ratio data can not be primarily attributed to batch effects, further indicating that more samples and more experimentation are needed to obtain clearer results before any interpretations can be made.

Akt has been shown to be involved in cardiac hypertrophy by promoting cell growth and contractility [13]. While Akt overexpression exerts beneficial effects, long term overexpression can lead to heart failure [13], suggesting that a balance of Akt activation may be necessary for cardiac function. As we gain a better understanding of whether Perm1 overexpression affects Akt expression from more experiments, future research may be able to investigate whether Perm1 plays a role in stabilizing Akt activation within an optimal range sufficient to counter ischemia-reperfusion induced injury.

Figures

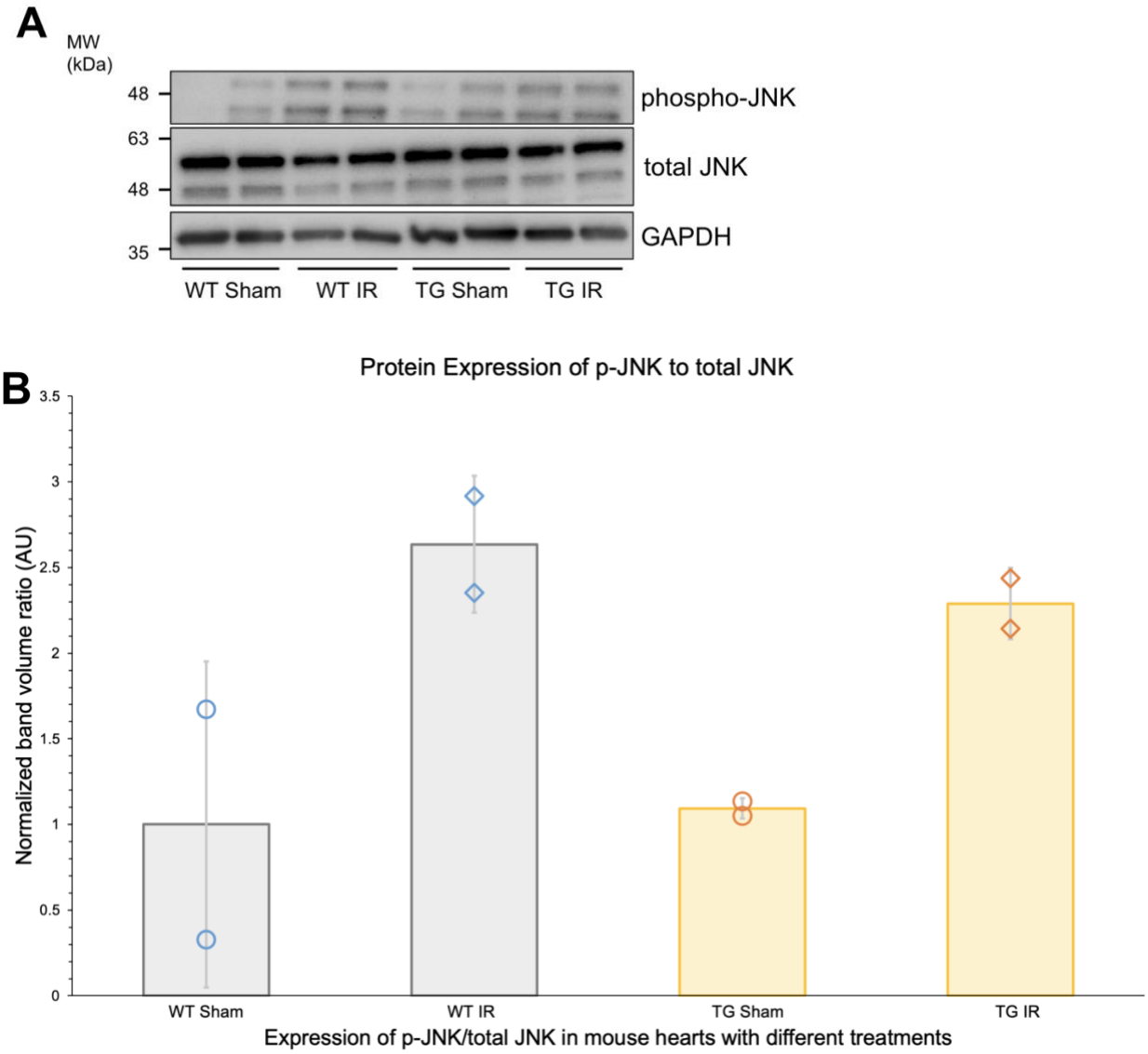


Figure 4.1. Analysis of ratio of protein expression of phosphorylated c-Jun N-terminal kinase (p-JNK) to total JNK in Perm1-overexpressed mice hearts.
Hearts from wild-type (WT) mice and transgenic (TG) mice with cardiac-specific Perm1 overexpression were subjected to perfusion only (sham control) or ischemia-reperfusion (IR) via the Langendorff hanging heart system. Phosphorylated JNK and total JNK expression in the hearts were determined by Western blot independently. For both proteins analyzed, Western blot band volumes of each group were normalized to that of the WT Sham group. The ratio of normalized p-JNK band volumes to normalized total JNK band volumes was then taken, and normalized band volumes represent the ratio of the degree of protein expression in the relative heart samples. Each symbol represents band volume of one heart sample (n = 4 for WT IR, TG Sham, and TG IR; n = 3 for WT Sham as bands could not be detected in one of the samples) while bars indicate average band volume. A student's t-test with 2-tailed distribution and equal variance was used to analyze the statistical differences between WT Sham and WT IR, TG Sham and TG IR, and WT IR and TG IR. Statistical significance $p < 0.05$.

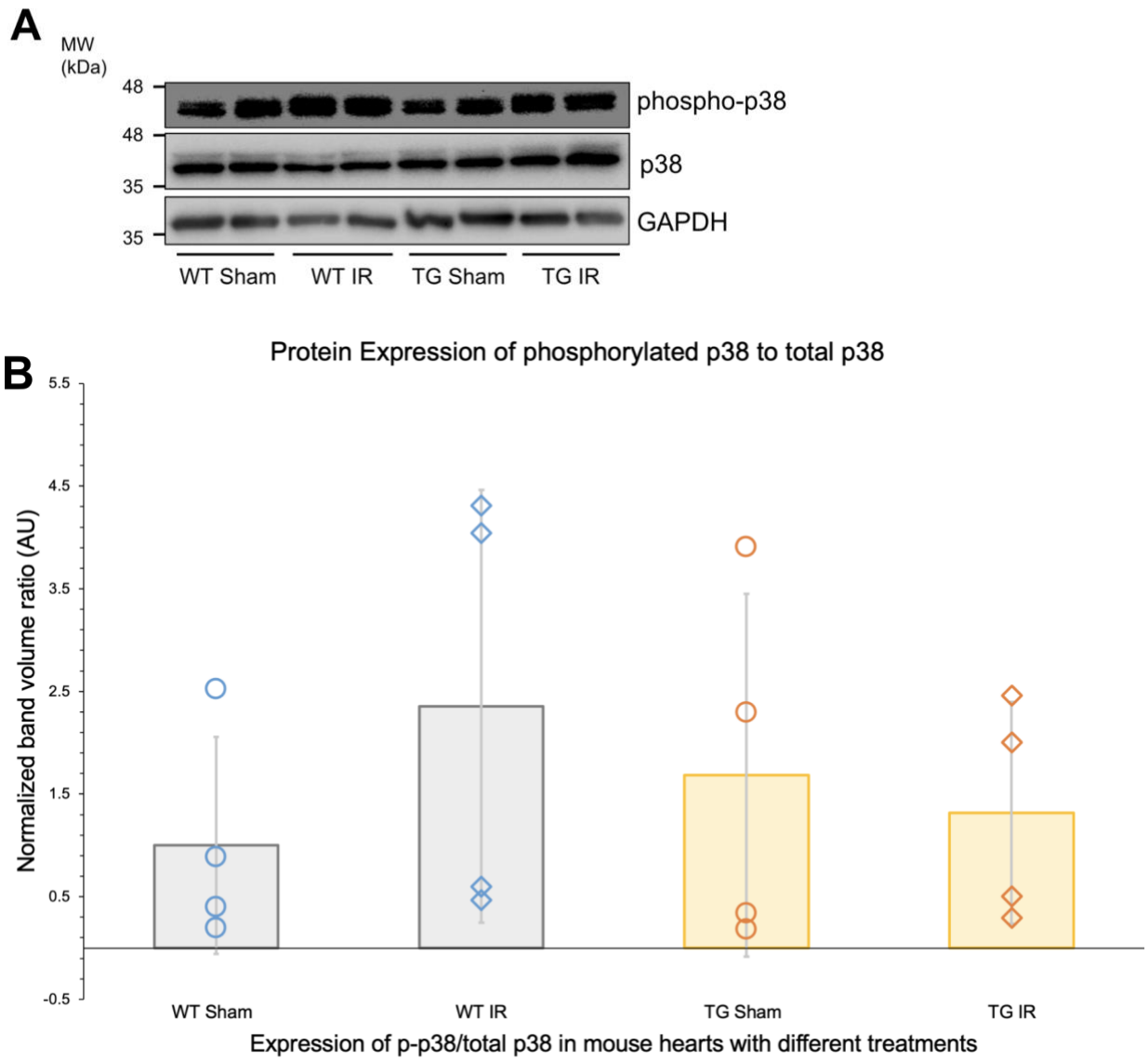
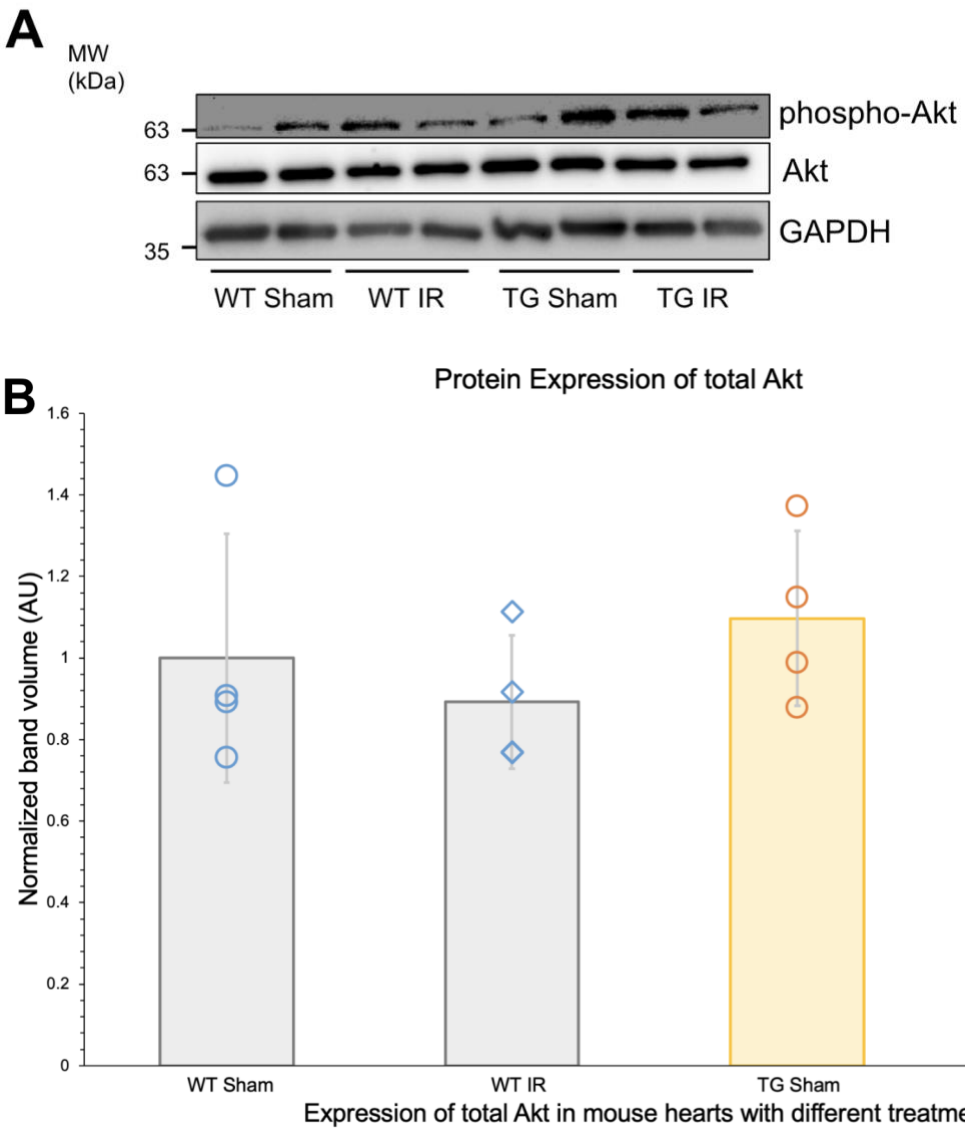


Figure 4.2. Analysis of ratio of protein expression of phosphorylated p38 (p-p38) to total p38 in Perm1-overexpressed mice hearts.
Hearts from wild-type (WT) mice and transgenic (TG) mice with cardiac-specific Perm1 overexpression were subjected to perfusion only (sham control) or ischemia-reperfusion (IR) via the Langendorff hanging heart system. Phosphorylated p38 and total p38 expression in the hearts were determined by Western blot independently. For both proteins analyzed, Western blot band volumes of each group were normalized to that of the WT Sham group. The ratio of normalized p-p38 band volumes to normalized total p38 band volumes was then taken, and normalized band volumes represent the ratio of the degree of protein expression in the relative heart samples. Each symbol represents band volume of one heart sample (n = 4) while bars indicate average band volume. A student's t-test with 2-tailed distribution and equal variance was used to analyze the statistical differences between WT Sham and WT IR, TG Sham and TG IR, and WT IR and TG IR. Statistical significance $p < 0.05$.

Figure 4.3. Analysis of ratio of protein expression of phosphorylated Akt (p-Akt) to total Akt in Perm1-overexpressed mice hearts.
Hearts from wild-type (WT) mice and transgenic (TG) mice with cardiac-specific Perm1 overexpression were subjected to perfusion only (sham control) or ischemia-reperfusion (IR) via the Langendorff hanging heart system. Phosphorylated Akt and total Akt expression in the hearts were determined by Western blot independently. For both proteins analyzed, Western blot band volumes of each group were normalized to that of the WT Sham group. The ratio of normalized p-Akt band volumes to normalized total Akt band volumes was then taken, and normalized band volumes represent the ratio of the degree of protein expression in the relative heart samples. Each symbol represents band volume of one heart sample ($n = 4$ for WT Sham, WT IR, and TG IR; $n = 3$ for TG Sham as one outlier was removed from the data) while bars indicate average band volume. A student's t-test with 2-tailed distribution and equal variance was used to analyze the statistical differences between WT Sham and WT IR, TG Sham and TG IR, and WT IR and TG IR. Statistical significance $p < 0.05$.


A
MW
(kDa)
63
63
35
phospho-Akt
Akt
GAPDH
WT Sham
WT IR
TG Sham
TG IR
B
Protein Expression of total Akt
Normalized band volume (AU)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
WT Sham
WT IR
TG Sham
TG IR
Expression of total Akt in mouse hearts with different treatments

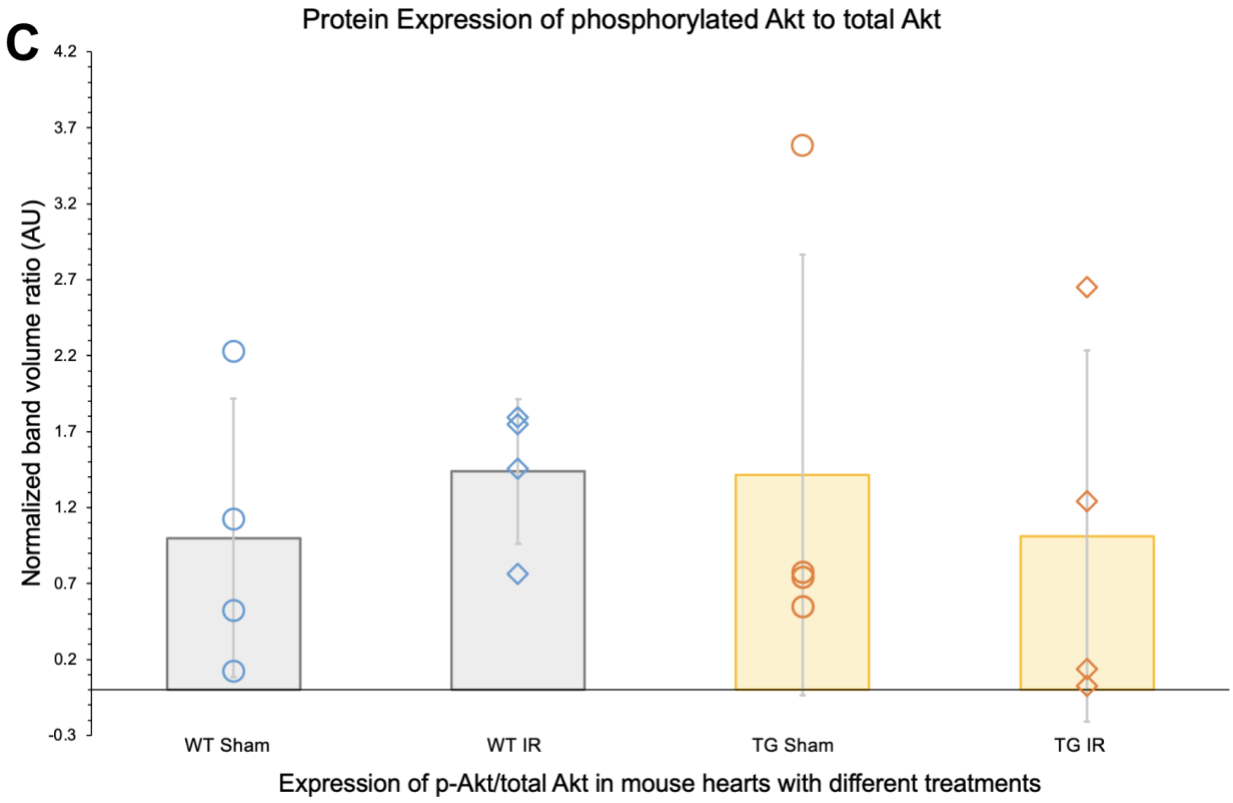


Figure 4.3. Analysis of ratio of protein expression of phosphorylated Akt (p-Akt) to total Akt in Perm1-overexpressed mice hearts (Continued)

REFERENCES

[1] Wu, M. Y., Yiang, G. T., Liao, W. T., Tsai, A. P., Cheng, Y. L., Cheng, P. W., Li, C. Y., & Li, C. J. (2018). Current Mechanistic Concepts in Ischemia and Reperfusion Injury. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology*, 46(4), 1650–1667. https://doi.org/10.1159/000489241

[2] Krijnen, P. A., Nijmeijer, R., Meijer, C. J., Visser, C. A., Hack, C. E., & Niessen, H. W. (2002). Apoptosis in myocardial ischaemia and infarction. *Journal of clinical pathology*, *55*(11), 801–811. https://doi.org/10.1136/jcp.55.11.801

[3] Fliss, H., & Gattinger, D. (1996). Apoptosis in ischemic and reperfused rat myocardium. *Circulation research*, *79*(5), 949–956. https://doi.org/10.1161/01.res.79.5.949

[4] Eefting, F., Rensing, B., Wigman, J., Pannekoek, W. J., Liu, W. M., Cramer, M. J., Lips, D. J., & Doevendans, P. A. (2004). Role of apoptosis in reperfusion injury. *Cardiovascular research*, *61*(3), 414–426. https://doi.org/10.1016/j.cardiores.2003.12.023

[5] He, J., Liu, D., Zhao, L., Zhou, D., Rong, J., Zhang, L., & Xia, Z. (2022). Myocardial ischemia/reperfusion injury: Mechanisms of injury and implications for management (Review). *Experimental and therapeutic medicine*, *23*(6), 430. https://doi.org/10.3892/etm.2022.11357

[6] Cogliati, S., Enriquez, J. A., & Scorrano, L. (2016). Mitochondrial Cristae: Where Beauty Meets Functionality. *Trends in biochemical sciences*, *41*(3), 261–273. https://doi.org/10.1016/j.tibs.2016.01.001

[7] Liu, J., & Lin, A. (2005). Role of JNK activation in apoptosis: a double-edged sword. *Cell research*, *15*(1), 36–42. https://doi.org/10.1038/sj.cr.7290262

[8] Weston, C. R., & Davis, R. J. (2007). The JNK signal transduction pathway. *Current opinion in cell biology*, *19*(2), 142–149. https://doi.org/10.1016/j.ceb.2007.02.001

[9] Shvedova, M., Anfinogenova, Y., Atochina-Vasserman, E. N., Schepetkin, I. A., & Atochin, D. N. (2018). c-Jun N-Terminal Kinases (JNKs) in Myocardial and Cerebral Ischemia/Reperfusion Injury. *Frontiers in pharmacology*, *9*, 715. https://doi.org/10.3389/fphar.2018.00715

[10] Kaiser, R. A., Liang, Q., Bueno, O., Huang, Y., Lackey, T., Klevitsky, R., Hewett, T. E., & Molkentin, J. D. (2005). Genetic inhibition or activation of JNK1/2 protects the myocardium from ischemia-reperfusion-induced cell death in vivo. *The Journal of biological chemistry*, *280*(38), 32602–32608. https://doi.org/10.1074/jbc.M500684200

[11] Ren, F., Mu, N., Gao, M., Sun, J., Zhang, C., Sun, X., Li, L., Li, J., Liu, T., Tse, G., & Dong, M. (2018). Role of JNK signalling pathway and platelet-lymphocyte aggregates in myocardial ischemia-reperfusion injury and the cardioprotective effect of ischemic

postconditioning in rats. *Molecular medicine reports*, *18*(6), 5237–5242. https://doi.org/10.3892/mmr.2018.9545

[12] Kaiser, R. A., Bueno, O. F., Lips, D. J., Doevendans, P. A., Jones, F., Kimball, T. F., & Molkentin, J. D. (2004). Targeted inhibition of p38 mitogen-activated protein kinase antagonizes cardiac injury and cell death following ischemia-reperfusion in vivo. *The Journal of biological chemistry*, *279*(15), 15524–15530. https://doi.org/10.1074/jbc.M313717200

[13] Kumphune, S., Chattipakorn, S., & Chattipakorn, N. (2012). Role of p38 inhibition in cardiac ischemia/reperfusion injury. *European journal of clinical pharmacology*, *68*(5), 513–524. https://doi.org/10.1007/s00228-011-1193-2

[14] Abeyrathna, P., & Su, Y. (2015). The critical role of Akt in cardiovascular function. *Vascular pharmacology*, *74*, 38–48. https://doi.org/10.1016/j.vph.2015.05.008

[15] Fujio, Y., Nguyen, T., Wencker, D., Kitsis, R. N., & Walsh, K. (2000). Akt promotes survival of cardiomyocytes in vitro and protects against ischemia-reperfusion injury in mouse heart. *Circulation*, *101*(6), 660–667. https://doi.org/10.1161/01.cir.101.6.660

[16] Cho, Y., Tachibana, S., Lam, K., Arita, Y., Khosrowjerdi, S., Zhang, O., Liang, A., Li, R., Andreyev, A., Murphy, A. N., & Ross, R. S. (2021). Perm1 promotes cardiomyocyte mitochondrial biogenesis and protects against hypoxia/reoxygenation-induced damage in mice. *The Journal of biological chemistry*, *297*(1), 100825. https://doi.org/10.1016/j.jbc.2021.100825

[18] He, H., Li, H. L., Lin, A., & Gottlieb, R. A. (1999). Activation of the JNK pathway is important for cardiomyocyte death in response to simulated ischemia. *Cell death and differentiation*, *6*(10), 987–991. https://doi.org/10.1038/sj.cdd.4400572

[19] Ono, K., & Han, J. (2000). The p38 signal transduction pathway: activation and function. *Cellular signalling*, *12*(1), 1–13. https://doi.org/10.1016/s0898-6568(99)00071-6

[20] Hemmings, B. A., & Restuccia, D. F. (2012). PI3K-PKB/Akt pathway. *Cold Spring Harbor perspectives in biology*, *4*(9), a011189. https://doi.org/10.1101/cshperspect.a011189

[21] Ghafouri-Fard, S., Khanbabapour Sasi, A., Hussen, B. M., Shoorei, H., Siddiq, A., Taheri, M., & Ayatollahi, S. A. (2022). Interplay between PI3K/AKT pathway and heart disorders. *Molecular biology reports*, *49*(10), 9767–9781. https://doi.org/10.1007/s11033-022-07468-0

[22] Penela, P., Inserte, J., Ramos, P., Rodriguez-Sinovas, A., Garcia-Dorado, D., & Mayor, F., Jr (2019). Degradation of GRK2 and AKT is an early and detrimental event in myocardial ischemia/reperfusion. *EBioMedicine*, *48*, 605–618. https://doi.org/10.1016/j.ebiom.2019.09.019

[23] Ferrandi, C., Ballerio, R., Gaillard, P., Giachetti, C., Carboni, S., Vitte, P. A., Gotteland, J. P., & Cirillo, R. (2004). Inhibition of c-Jun N-terminal kinase decreases cardiomyocyte apoptosis and infarct size after myocardial ischemia and reperfusion in anaesthetized rats. *British journal of pharmacology*, *142*(6), 953–960. https://doi.org/10.1038/sj.bjp.0705873

[24] Ma, X. L., Kumar, S., Gao, F., Louden, C. S., Lopez, B. L., Christopher, T. A., Wang, C., Lee, J. C., Feuerstein, G. Z., & Yue, T. L. (1999). Inhibition of p38 mitogen-activated protein kinase decreases cardiomyocyte apoptosis and improves cardiac function after myocardial ischemia and reperfusion. *Circulation*, *99*(13), 1685–1691. https://doi.org/10.1161/01.cir.99.13.1685

[25] Schwertz, H., Carter, J. M., Abdudureheman, M., Russ, M., Buerke, U., Schlitt, A., Müller-Werdan, U., Prondzinsky, R., Werdan, K., & Buerke, M. (2007). Myocardial ischemia/reperfusion causes VDAC phosphorylation which is reduced by cardioprotection with a p38 MAP kinase inhibitor. *Proteomics*, *7*(24), 4579–4588. https://doi.org/10.1002/pmic.200700734

[26] Motayagheni N. (2017). Modified Langendorff technique for mouse heart cannulation: Improved heart quality and decreased risk of ischemia. *MethodsX*, *4*, 508–512. https://doi.org/10.1016/j.mex.2017.11.004

[27] Zhao, Y. T., Du, J., Yano, N., Wang, H., Wang, J., Dubielecka, P. M., Zhang, L. X., Qin, G., Zhuang, S., Liu, P. Y., Chin, Y. E., & Zhao, T. C. (2019). p38-Regulated/activated protein kinase plays a pivotal role in protecting heart against ischemia-reperfusion injury and preserving cardiac performance. *American journal of physiology. Cell physiology*, *317*(3), C525–C533. https://doi.org/10.1152/ajpcell.00122.2019

[28] Matsui, T., Tao, J., del Monte, F., Lee, K. H., Li, L., Picard, M., Force, T. L., Franke, T. F., Hajjar, R. J., & Rosenzweig, A. (2001). Akt activation preserves cardiac function and prevents injury after transient cardiac ischemia in vivo. *Circulation*, *104*(3), 330–335. https://doi.org/10.1161/01.cir.104.3.330