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SAN DIEGO STATE UNIVERSITY

The Effect of Pim Kinases Upon Mitochondrial Dynamics and Metabolism

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

Doctor of Philosophy

in

Biology

by

Shabana Naz Din

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2014

The Dissertation of Shabana Naz Din is approved, and it is acceptable
in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

San Diego State University

2014

DEDICATION

To my family and friends for all their support, inspiration, motivation, and unconditional love.

EPIGRAPH

The world breaks every one and afterward many are strong at the broken places.

Ernest Hemingway

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LIST OF ABBREVIATIONS

Drp1	Dynamin-related protein 1
sl	Simulated ischemia
sl/R	Simulated ischemia/Reperfusion
PUMA	p53 upregulated modulator of apoptosis
PimWT mice	α -MHC Pim1 overexpression
PDN mice	Pim-1 dominant negative overexpressing mice
adPumaDN	BH3-domain defective PUMA adenovirus
adPimWT	EGFP-Pim1 adenovirus
adPDN	EGFP-Pim1 dominant negative adenovirus
NRCMs	Neonatal rat cardiac myocytes
NTG	Non-transgenic wild type
PTKO	Pim Triple KnockOut
PGC-1 α	PPAR γ coactivator 1 alpha
PGC-1 β	PPAR γ coactivator 1 beta
Err- α	Estrogen-related receptor alpha
NRF1	Nuclear respiratory factor 1
TFAM	Mitochondrial transcription factor A
AMPK	AMP-activated protein kinase
Nppa	Atrial natriuretic peptide
Nppb	Brain natriuretic peptide
Acta1	α -skeletal actin
Myh7	β -myosin heavy chain
LVIDd	Left ventricle inner diameter diastole
LVAWd	Left ventricle anterior wall diastole
%EF	Percent ejection fraction
iWT MEFs	Immortalized wild-type murine embryonic fibroblast
iPTKO MEFs	Immortalized Pim Triple KnockOut murine embryonic fibroblast

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Chapter II, in full, has been submitted for publication in the journal Circulation Research, 2014. Metabolic dysfunction consistent with premature aging results from deletion of Pim Kinases: Shabana Din, Mathias H. Konstandin, Bevan Johnson, Andrea De La Torre, Mirko Voelkers, Jacqueline Emathinger, Haruhiro Toko, Brett Collins, Lucy Ormachea, Kaitlen Samse, Dieter A. Kubli, Andrew S. Kraft, Asa B. Gustafsson, Daniel P. Kelly, and Mark A. Sussman. The dissertation author was the primary author and investigator for this manuscript.

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- 2) **Din S**, Mason M, Volkers M, Johnson B, Cottage CT, Wang Z, Joyo AY, Quijada P, Erhardt P, Magnuson NS, Konstandin MH, Sussman MA. Pim-1 preserves mitochondrial morphology by inhibiting dynamin-related protein 1 translocation. *Proceedings of the National Academy of Sciences of the United States of America*. 2013;110:5969-5974
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- General molecular biology, microbiological and biochemical analyses including but not limited to: DNA/RNA purification, PCR, RT-PCR, Q-PCR, sequence analysis, cloning, immunoblot, transformation, transduction, transfection, genotyping, immunoprecipitation, proximity ligation assay, telomeric analysis by FISH, and cellular fractionation.
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ABSTRACT OF DISSERTATION

The Effect of Pim Kinases Upon Mitochondrial Dynamics and Metabolism

by

Shabana Naz Din

Doctor of Philosophy in Biology

University of California, San Diego, 2014

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Myocardial aging is an independent risk factor for cardiovascular disease. Cardiac aging promotes adverse myocardial remodeling and the accumulation of poorly functioning senescent cells, leading to a decline in cardiac performance. Pathological remodeling is associated, in part, with changes occurring at the mitochondrial level exacerbating heart disease. Mitochondrial alteration during heart failure includes cellular changes in fuel utilization and alterations in mitochondrial dynamics, implicating mitochondrial biology as an important facet of cardiac aging biology. Pim kinases are protective in a cardiac context, in part by maintaining mitochondrial integrity. However, Pim protein expression diminishes during cardiac aging. Therefore, cardiac mitochondrial dynamics and metabolism were investigated in relationship to Pim kinases.

The relationship between Pim1 and Dynamin Related Protein 1 (Drp1) was assessed as a novel mechanism to prevent Drp1 mediated fission. Drp1 mediates fission by mitochondrial localization during pathological challenge, sensitizing cardiomyocytes to apoptosis. Overexpressing Pim1 decreased total Drp1 levels, increased phosphorylation of Drp1 at serine 637, and inhibited Drp1 localization to mitochondria while preserving reticular morphology after simulated ischemia. Overexpression of Pim1 dominant negative (PDN) increased total mitochondrial Drp1, reduced phospho Drp1, and increased mitochondrial fragmentation. PDN hearts exhibit upregulation of BH3 only protein p53 upregulated modulator of apoptosis (PUMA) that mediates mitochondrial Drp1 accumulation and increased sensitivity to apoptotic stimuli. Therefore, Pim1 activity prevents Drp1 compartmentalization to the mitochondria and preserves reticular mitochondrial morphology.

Cellular pathological hypertrophic remodeling and fetal gene program activation was evident in Pim Triple KnockOut (PTKO) mice phenotypic of cardiac aging. Cardiomyocyte senescence manifested by increased expression of cell cycle inhibitors and decreased telomere lengths. Changes in expression of PPAR γ coactivator-1 (PGC-1) α and β led to alterations in mitochondrial ultrastructure and metabolism. An energy-starved phenotype was determined with decreased ATP and increased pAMPK:AMPK ratio, confirming changes in the PPAR signaling circuit. Overexpression of PGC-1 α and c-Myc rescued changes in metabolism and restored energy homeostasis. These studies confirm the significant impact of Pim kinases on mitochondrial biology and support the notion to utilize Pim as a tool to prevent cardiac aging by preserving mitochondrial dynamics and metabolism.

INTRODUCTION OF THE DISSERTATION

Cardiovascular Disease

Cardiovascular disease remains the leading cause of death in the western world, afflicting the majority of the elderly population (1). Myocardial aging is an independent risk factor for cardiovascular disease that leads to impaired cardiac function (2). At present, there is no current clinical therapy to inhibit or prevent the progression of aging. Aging is a multifactorial process caused by accumulated damage to multiple cellular components (2, 3). Cardiac senescence is characterized by a multitude of changes on the cellular level that occur during normal physiological aging. Current dogma states that cardiac aging is irreversible and leads to increased senescence of the cardiomyocyte population and the cardiac progenitor cell (CPC) population. In post-natal development, expansion of the cardiomyocyte pool is quickly down regulated after birth and de novo regeneration of myocytes in response to aging is severely limited (4-6). The lack of cardiomyocyte proliferation with aging leads to pathological hypertrophic remodeling to manage the functional demands of the heart (7). In addition to hypertrophic remodeling, cardiac aging triggers vascular dysfunction, which leads to arterial stiffness leading to ischemic heart disease causing reduced blood perfusion throughout the heart (8). Ischemic heart disease and pathological hypertrophic remodeling eventually are independent characteristics of cardiovascular disease and significantly increase the risk of heart failure. Predisposition to either hypertrophic or ischemic heart disease can be dictated by risk factors such as familial history, diet, smoking, gender, etc.

Ischemic Heart Disease

Ischemic heart disease is characterized by decreased oxygen supply in the myocardium as a result of reduced blood flow (9). Oxygen supply is imperative for mitochondrial function to meet the energy expensive demands of the heart and therefore

bear the consequences of ischemic insult. Ischemia disrupts the electron transport chain and ATP synthase causing a reduction in ATP and promoting the utilization of anaerobic glycolytic metabolism leading to an accumulation of lactic acid. Reduced cellular energy stores instigate global cellular changes including the increase in cytosolic cations (Na^{2+} and Ca^{2+}) promoting cellular swelling and eventual membrane rupture (9). Furthermore, increases in Ca^{2+} can activate the mitochondrial permeability transition pore (MPTP) initiating the release of mitochondrial cytochrome c and activating the cellular apoptotic cascade causing cell death.

Pathological Hypertrophic Remodeling

Pathological hypertrophic remodeling occurs in response to increased mechanical stress caused by pressure accumulation during hypertension. In the initial phase, compensatory hypertrophic remodeling leads to thickening of the ventricular walls in order to sustain cardiac output. However, prolonged stress will eventually cause decompensated or pathological hypertrophy leading to hemodynamic dysfunction and heart failure, increasing mortality. Pathological hypertrophy is characterized by increased cardiomyocyte size and the activation of the fetal gene program. Clinically, two pathological hypertrophic phenotypes can be seen depending on disease etiology (10). The first, concentric hypertrophy occurs in response to pressure overload and promotes the growth of individual cardiomyocytes. Eccentric hypertrophy causes the lengthening or addition of sarcomeres promoting longitudinal cell growth. To attenuate increased ventricular size and mechanical stress, growth of ventricular walls coincides with mitochondrial biogenesis to promote increased oxidative capacity (11). However as hypertrophic remodeling transitions into pathological hypertrophy, mitochondrial biogenesis diminishes and mitochondrial oxidative capacity decreases due to metabolic alteration (12). This phenotype causes distorted mitochondrial energetics and

diminution of cellular energy stores, exacerbating the pathological hypertrophic pathology and increasing morbidity (12).

The two pathologies of ischemia and pathological hypertrophic remodeling leads to heart failure, in part due to imbalances in mitochondrial dynamics and disruption in mitochondrial function, respectively. Mitochondrial dynamics maintain mitochondrial shape and function through the processes of fission and fusion. Mitochondrial fission and fusion provide mitochondria the plasticity to adjust to biological stressors and maintain functional integrity. Imbalances in mitochondrial morphology are implicated in many disease states including Parkinson's, Alzheimer's, cardiac ischemia-reperfusion injury, and diabetes impacting pathogenesis (13). Pathological hypertrophic remodeling causes perturbations in cardiac metabolism, initiating a fuel shift away from fatty acid oxidation to glucose metabolism similar to fetal metabolism. Utilization of glucose as the main cardiac energy source produces insufficient energy to maintain heart function and leads to an energy-starved state, exacerbating cardiovascular disease etiology (14). Therefore, the ability to antagonize detrimental changes occurring at the mitochondrial level is important to blunt the cardiovascular aging phenotype and prevent heart failure.

Mitochondrial Dynamics

Mitochondrial biogenesis declines with age, resulting in a limited supply of functional mitochondria. Mitochondria are dynamic organelles constantly undergoing the process of fission and fusion to maintain a population of functional mitochondria (15, 16). Mitochondrial dynamics control the length, shape, size, and mitochondrial availability. Mitochondrial function is also maintained by mitochondrial dynamics by allowing the removal of damaged or dysfunctional mitochondria (17). Furthermore, disruption in mitochondrial dynamics during development causes embryonic lethality (18). Subsequently, imbalance of fission and fusion in adult cells sensitizes cells to numerous

diseases associated with aging (19, 20). However, mitochondrial fission dominates mitochondrial morphology during proliferation to allow the proper inheritance of maternal mitochondria to progeny cells (21). Mitochondrial fusion promotes the merging of outer and inner mitochondrial membrane causing a reticular and elongated mitochondrial shape. Conversely, mitochondrial fission promotes the scission of the outer mitochondrial membrane, promoting smaller mitochondria with punctate or fragmented appearance (22).

Mitochondria are double membrane organelles with an outer and inner mitochondrial membrane. Mitofusin 1 (Mfn1) and Mitofusin 2 (Mfn2) promote the merging of the outer mitochondrial membrane and are present on outer mitochondrial membranes of adjacent mitochondria (23). Studies have shown that Mfn1 and Mfn2 form an antiparallel-coiled coil which stabilizes adjacent mitochondria and promotes fusion of the outer membrane. Inner membrane fusion and cristae remodeling is regulated by Optic atrophy 1 (Opa1), which is present in the inner mitochondrial membrane. Opa is a large GTPase and also prevents the release of cytochrome c (24). Consequently, deficiency of mitochondrial fusion regulators causes increased mitochondrial fission (25).

Drp1 and mitochondrial proteins Fis1, Mff, and PUMA regulate mitochondrial fission. The pro-fission protein, dynamin related protein 1 (Drp1), is believed to cycle between the cytosol and scission events on mitochondria where it interacts with accessory proteins to couple GTP hydrolysis and promote membrane fission (26). Drp1 shuttling is regulated by phosphorylation on serine 616 and serine 637 (26). Drp1 phosphorylation at serine 616 is regulated by Cdk1/cyclinB, whereas Drp1 phosphorylation at serine 637 is regulated by protein kinase A and calcineurin. Phosphorylation at serine 616 is shown to regulate mitochondrial fission during mitosis

and is important for appropriate mitochondrial distribution into daughter cells (21). Phosphorylation of Drp1 at serine 637 promotes Drp1 cytosolic sequestration whereas dephosphorylation promotes Drp1 mitochondrial localization (26). Furthermore, Drp1 dephosphorylation at serine 637 is implicated in instigating the mitochondrial apoptotic cascade during pathological challenge. Drp1 will dock on the outer mitochondrial membrane by interacting with Fis1 or Mff and subsequently oligomerize into a ring structure and initiate scission of the mitochondrion into two smaller mitochondria (27). Prolonged or unrestricted fission, in particular, causes increased sensitivity to apoptotic stimuli promoting cell death.

The preservation of mitochondrial dynamics to protect against pathological challenge has been described in cultured neurons, fibroblasts, yeast and neonatal rat cardiomyocytes (NRCMs), however the importance of active mitochondrial dynamics in an adult cardiac context was not well understood until recently. Several studies reported the expression of mitochondrial dynamic proteins and mRNA in human, rat, and mouse cardiac tissue (28-30). While the heart is composed of many different cell types such as myocytes, smooth muscle, vascular endothelial, and fibroblasts, direct evidence of the impact of mitochondrial dynamics in an adult heart *in-vivo* was shown in an ischemic cardiac model. Ong et al. demonstrated that treatment with mdivi-1, a small pharmacological inhibitor of Drp1, during cardiac ischemia reperfusion injury blunted myocardial infarct size and protected mitochondrial integrity by preventing mitochondrial permeability transition pore opening, thereby designating mitochondrial fission as a regulator of cardiomyocyte apoptosis during ischemia (15). Further substantiating the requirement for balanced mitochondrial dynamics in an adult cardiac context are studies performed with genetically manipulated mouse models. Conditional cardiac specific ablation of Mfn1 and Mfn2 at 5 months led to altered mitochondrial ultrastructure (18).

Loss of cardiac Mfn1 and Mfn2 promoted the formation of smaller fragmented mitochondria with distorted cristae morphology caused by unopposed mitochondrial fission. Furthermore, a rapid decline in cardiac function led to a dilated cardiomyopathy just eight weeks after ablation of mitochondrial fusion.

Cumulatively these studies provide convincing evidence in support of the essential function of mitochondrial dynamics in maintaining homeostatic cardiac function. Therefore, mechanisms preserving a balance between fission and fusion during pathological challenge are important to investigate as potential therapeutic targets to prevent the development or progression of cardiovascular disease.

PPAR γ coactivator-1 and Cardiac Metabolism

The heart is an energy-demanding organ and the highest ATP consuming tissue in the body. ATP reserves in the heart are limited, as energy is consumed rapidly; therefore metabolic regulation is essential to cardiac function. The heart relies primarily on fatty acid oxidation as a major fuel source to maintain efficient contractile function (31). Cardiac metabolism is dependent on signaling from transcriptional coregulators PPAR γ coactivator-1 (PGC-1) α and β . PGC-1 α and PGC-1 β are major regulators of mitochondrial biogenesis and maintain cellular energy homeostasis in tissues with high oxidative capacity (7). PGC-1 α is the major isoform in the heart and is induced to meet the energy demands of the heart in response to physiologic or pathological conditions (32, 33).

PGC-1 α , as the name implies, promotes cardiac metabolism and ATP production through coactivation of downstream transcription factors. PGC-1 α binding to transcription factors creates a scaffold, allowing the binding of transcriptional mediators such as chromatin modifying enzymes to the amino terminus of PGC-1 α (34). The carboxy terminus of PGC-1 α binds the TRAP/Mediator complex and mRNA splicing

machinery, leading to enhanced transcription of enzymes and genes promoting fuel metabolism and mitochondrial oxidative phosphorylation (35). PGC-1 α and PGC-1 β signaling occurs through nuclear peroxisome proliferator-activated receptors (PPARs), estrogen related receptor alpha (Err α), and nuclear receptor factor (Nrf1) (14). In particular, co-activating downstream transcription factors promote increased fatty acid oxidation, angiogenesis, and electron transport chain subunit expression. In addition to regulating energy reserves PGC-1 α promotes mitochondrial biogenesis through Nrf1 induced upregulation of TFAM, increasing mitochondrial DNA transcription and replication (36).

PGC-1 α expression and activation occur in response to developmental, physiological, and dietary signals that require increased oxidative phosphorylation. In the heart postnatal development requires a burst of mitochondrial biogenesis regulated by PGC-1 α (37). Furthermore, PGC-1 α expression is increased during exercise, fasting, and cold exposure. Many signaling pathways induce PGC-1 α expression including p38, cAMP, MEF2C, nitric oxide, calcineurin, and calcium/calmodulin dependent kinase, allowing the integration of signals that maintain cellular energy homeostasis (38). Furthermore, PGC-1 α activity is tightly regulated posttranslationally by phosphorylation, acetylation, and arginine methylation (39-41). Importantly, PGC-1 α expression is regulated by AMP activated protein kinase (AMPK), the cellular energy sensor. Decreased ATP:ADP ratios lead to AMPK phosphorylation and subsequent upregulation of PGC-1 α (42).

Several lines of evidence demonstrate the impact of altered mitochondrial metabolism and an energy starved state in human disease causing cardiac dysfunction. Clinically, cardiac aging leads to left ventricle hypertrophy and promotes a fuel shift from fatty acid oxidation to glucose utilization (43). This defect in fuel preference is coincident

with PGC-1 α downregulation and an energy starved state. PGC-1 coactivators and downstream targets such as nuclear genes encoding mitochondrial proteins are known to diminish with aging in rodents and humans, thereby increasing the incidence of acquiring myocardial disease (14). Furthermore, the ability to increase mitochondrial biogenesis diminishes during aging and is blunted during heart failure (44).

Although there is a clear disruption in mitochondrial function and biogenesis during heart failure, further delineation of PGC-1 α signaling in a cardiac context occurred through knockout and transgenic mouse models (43, 45). PGC-1 α knockout models displayed diminished cardiac function in response to exercise (45). Furthermore Arany et al. presented the onset of an aged related cardiomyopathy with reactivation of the fetal gene program and cardiac failure in response to trans aortic constriction, validating the necessity of PGC-1 α in aging and cardiac challenge (45). Additionally, knockout PGC-1 β mice also develop accelerated heart failure in response to transaortic constriction (46). Since PGC-1 α is involved in fatty acid metabolism, Leone et al. examined cardiac fuel utilization and ATP production in PGC-1 α knockout mice (14, 43). Diminished ATP production and fatty acid oxidation rates were seen in PGC-1 α knockout mice as well as altered mitochondrial morphology. Loss of PGC-1 α can cause upregulation of PGC-1 β , and PGC-1 α/β double knockout mice were postnatal lethal (31). Overexpression of PGC-1 α in NRCMs demonstrated increased oxidative capacity and mitochondrial biogenesis (47). However, PGC-1 α overexpressing mice exhibited an increase in mitochondrial mass that resulted in heart failure (47, 48).

Whether mitochondrial dysfunction and loss of PGC-1 coactivators are the primary causes of cardiac dysfunction remains to be elucidated. However, incontrovertible evidence supports the notion that disruption in mitochondrial function or metabolism exacerbates cardiac disease etiology. Reduced expression of PGC-1 α

during heart failure is reported, in addition to increased mutation in mitochondrial DNA, due to decreased TFAM, a PGC-1 α downstream target (49, 50). Interestingly caloric restriction, a dietary regimen shown to prevent aging, causes increased PGC-1 α expression and prevents adjustments in fuel preference (51), whereas overexpression of PGC-1 α during fetal or adult stages leads to unchecked mitochondrial biogenesis and cardiomyopathy. Therefore, the therapeutic benefit of PGC-1 α overexpression needs to be titrated and fine tuned. Cumulatively, it is clear that preventing changes in PPAR signaling is important to attenuate the myocardial aging and pathological phenotype.

Pim Kinases

Pim1, a conserved serine/threonine protein kinase, confers cardioprotection in the face of pathological injury (16, 52-54). Pim kinases play major roles in cell-cycle progression, transcription, and cell survival by phosphorylating numerous constituents involved in survival cascades (54-56). The Pim gene family consists of Pim 1, 2, and 3, three distinct genes located on chromosome 6, X, and 22 respectively. Pim 1, 2, and 3 are constitutively active, have similar substrate preferences, and are ubiquitously expressed, but differ in their tissue expression (57). Pim1 is highly expressed in thymus and testis; Pim2 is abundant in the brain and thymus, whereas Pim3 is rich in the kidney (58).

Studies demonstrate Pim1 is the predominant isoform in the heart, and loss of Pim1 causes compensatory upregulation of Pim 2 and 3 (53). Pim1 is a downstream target of Akt and a key participant in exerting cardioprotective effects through Akt (53). Cardiac specific overexpression of Pim1 displayed a hyperplastic myocardium with smaller but more numerous myocytes (53). During myocardial infarction Pim1 transgenic mice exhibit decreased left ventricle infarct size and preserved cardiac function (49). Pim1 knockout mice did not display an overt cardiac phenotype, due to

the upregulation of Pim2 and Pim3. However, in response to myocardial infarction Pim1 knockout mice had increased necrosis and deterioration of hemodynamic function. Pim1 overexpression further prevents hypertrophic remodeling when subjected to TAC, preserving ventricular chamber size and blunting the activation of the fetal gene program (48). Conversely, hearts from Pim1 dominant negative mice developed spontaneous pathological hypertrophic remodeling, increased fibrosis, and deteriorated cardiac function during aging (48). Aside from protective effects upon cardiomyocytes, Pim1 expression is necessary for the c-kit cardiac stem cell (CSC) compartment (59, 60). Pim1 overexpression promotes proliferation and protection of CSCs. Furthermore, increased Pim1 expression in CSC's promotes enhanced lineage commitment prompting the formation of new cells during adoptive transfer post myocardial infarction.

Pim1 activity exerts multiple protective effects upon mitochondria and has recently been implicated in maintaining mitochondrial integrity (16). Pim1 localizes to the mitochondria during ischemia reperfusion injury and prevents cytochrome c release, Ca^{2+} induced swelling, and inner membrane induced depolarization (16). Pim1 promotes upregulation of antiapoptotic Bcl2 proteins and phosphorylates Bad to prevent initiation of the apoptotic cascade (16, 61). Furthermore, Pim kinases increase expression of mitochondrial metabolism and biogenesis regulators (c-Myc and PGC-1 α), implicating a role for Pim kinases in mitochondrial biology (55, 62). Although the expression of Pim-1 has been linked to both survival and proliferation, the exact mechanisms of this interaction are currently under investigation.

Metabolic changes are becoming increasingly relevant in determining cardiac disease outcomes in the clinic (63). Recent evidence in immortalized murine embryonic fibroblast from Pim Triple Knockout Mice (iPTKO MEFs) demonstrated lack of ATP reserves and decreased protein synthesis with iPTKO MEFs (62). Analysis of metabolic

and mitochondrial biogenesis regulators demonstrated reductions in c-Myc and PGC-1 α (62). Coupled with previous reports detailing PGC-1 α regulation of mitochondrial metabolism and biogenesis (14, 31) in addition to c-Myc dependent mitochondrial biogenesis (64), necessarily leads to the postulate that expression and activity of Pim kinases are essential for proper metabolic function and ATP production.

Accumulating evidence reveals that maintenance of mitochondrial shape, function, and metabolism are essential to cardiac function, and aberrations in mitochondrial components occurring during aging promote cardiovascular disease (8, 65, 66). Recently evidence demonstrates the ability of Pim kinase to blunt activation of the aging phenotype by antagonizing expression of cell cycle inhibitors and promoting telomere lengthening in human cardiac progenitor cells. Furthermore, since Pim1 promotes mitochondrial integrity and antagonizes acquisition of an aging phenotype, the data presented within this thesis will examine the contribution of Pim kinases to mitochondrial biology by assessing the role of Pim kinases upon mitochondrial dynamics during ischemic challenge and cardiac metabolism in response to pathological hypertrophic remodeling during aging.

Chapter 1 illuminates the effect of ischemia in neonatal rat cardiomyocytes (NRCMs) and mitochondrial dynamics. Ischemia is coincident with Drp1 localization and increased mitochondrial fission, causing a fragmented mitochondrial phenotype. Pim1 is shown to phosphorylate Drp1 and prevent mitochondrial localization, thereby maintaining areticular mitochondrial morphology. Additionally, Pim1 is shown to regulate total Drp1 protein levels. PUMA, which is necessary for Drp1 accumulation at the mitochondria, decreased with Pim1 overexpression. These data establish a role for Pim1 to maintain mitochondrial shape during ischemia thereby preventing cellular apoptosis.

Chapter 2 reports that genetic elimination of all three Pim kinases promotes hypertrophic remodeling leading to premature cardiac senescence and heart failure. Hypertrophic remodeling coincided with disrupted mitochondrial morphology and an energy-starved myocardium as evidenced by diminished levels of ATP and increased levels of phosphorylated AMPK. These results were corroborated with reduced expression of PGC-1 α and PGC-1 β , critical regulators of mitochondrial biogenesis and cellular ATP producing pathways. Additionally, c-Myc, a downstream target of Pim kinase and regulator of mitochondrial biogenesis and cardiac metabolism, was also diminished. Hypertrophic remodeling and downregulation of major regulators of mitochondrial pathways (PGC-1 α , PGC-1 β , and c-Myc) led to disruption in fatty acid oxidation and glucose metabolism. Confirming these results, overexpression of c-Myc, Pim1, or PGC-1 α rescued expression of downstream targets involved in mitochondrial biogenesis and metabolism.

CHAPTER I

Pim1 Preserves Mitochondrial Morphology by Inhibiting Dynamin-Related Protein 1 Translocation

INTRODUCTION

Ischemic heart disease is a leading cause of death in the western world. Many simultaneous cellular changes occur during cardiac ischemia and inevitably cause cell death. Mitochondria are especially sensitive to ischemic insult and trigger the initiation of apoptotic signaling (67). Recently, mitochondrial morphological dynamics have been reported to play a role in ischemia/reperfusion injury (68, 69). Mitochondria constantly undergo fusion or fission and present a reticular elongated or punctate fragmented phenotype, respectively (70, 71). Prolonged simulated ischemic injury causes extensive fragmented mitochondrial morphology associated with dynamin-related protein 1 (Drp1) translocation to the mitochondria.

In mammalian cells the pro-fission protein Drp1 cycles between the cytosol and scission events on mitochondria, where it interacts with accessory proteins to couple GTP hydrolysis and outer mitochondrial membrane fission (72). In addition to steady state activity, Drp1 is also associated with early apoptosis (70, 73-76). Cellular stress increases Drp1 shuttling which leads to fragmented mitochondrial networks, while prolonged or severe insult promotes stabilization of Drp1 to the mitochondria which increases the likelihood of progression to apoptotic cell death (73, 74, 77-79). Phosphorylation of Drp1-^{S637} prevents translocation to mitochondria whereas overexpression of Drp1^{K38A} (a dominant-negative mutation lacking GTPase activity) prevents Drp1 translocation, attenuates a fragmented mitochondrial phenotype, and decreases cell death (70, 80). Collectively, these observations suggest that Drp1 phosphorylation increases cell viability via inhibition of mitochondrial fission.

The serine/threonine kinase Pim-1 is a critical downstream effector of Akt mediated cardioprotective signaling (16, 52, 53, 60). Pim-1 is anti-apoptotic and pro-proliferative, and recent findings indicate that Pim-1 exerts beneficial effects in part by

preserving mitochondrial integrity (16). Activation of Pim-1 *in vitro* and in mouse models of hypertrophy, infarction, and ischemia / reperfusion injury enhances cardiomyocyte survival through inhibition of intrinsic mitochondrial apoptotic pathways. The possibility that Pim-1 acts upon Drp1 localization and activity has never been investigated in the context of ischemic cardiac injury. In this study Pim-1 was shown to maintain mitochondrial networks by sequestration of Drp1 from the mitochondria following ischemic injury.

METHODS

Adenoviral Constructs and Transgenic Mice

Generation of adEGFP, adEGFP-PimDN and adEGFP-PimWT adenoviral constructs, K79M kinase dead PimDN transgenic mice were reported previously (16, 52, 53).

Neonatal Rat Cardiac Myocyte (NRCM), Cell Culture and Adenoviral Infection

Neonatal rat cardiac myocytes (NRCM) maintenance and transduction were performed as previously described (16, 53). Briefly, NRCMs were maintained in DMEM 10% FBS. For adenoviral infections media on NRCMs were changed to DMEM 2% during transduction for two hours followed by refeeding with DMEM 10% FBS.

Simulated Ischemia / Reperfusion

Following adenoviral infection si and or si/R treatments were done in Glucose Free (GF) media with the following components: 1 liter of double deionized water, 3.7 grams of sodium bicarbonate, 1 vial of Glucose Free DMEM powder (Sigma D5030), 80ul of 10 mg/mL fungizone, 1% PSG, followed by sterile filter and pH of 7.4. To glucose free media 5mM of sodium cyanide and 20mM of 2-deoxy- glucose was added followed by incubation of cells for indicated times. For si/R 10mM glucose was added after si treatment for indicated times.

Plasmids and Transfections.

Drp1-S637A point mutant construct was kindly provided by L. Scorrano (Venetian Institute of Molecular Medicine, Padova, Italy). Cotransfections were done with Drp1-S637A, mCherry plasmid (Clontech) or Pim-1-mCherry. Transfections were done with Xtremegene (Roche) at a ratio of 1:3 DNA to Xtremegene in 10-cm dishes of 1.6×10^5 HeLa cells, according to manufacturer's instructions. HeLa cells were maintained in 10% FBS DMEM.

Mitochondria Morphological Analysis and Proximity Ligation Assay (PLA)

Following indicated si or si/R treatments, cells were washed twice with PBS and fixed in 4% paraformaldehyde (PFA). Cells were then stained with Tom20 (Santa Cruz 1:200). Mitochondria were imaged by confocal microscopy on a Leica TCS SP2 laser scanning spectral confocal microscope. The extent of mitochondrial fragmentation was qualified on a cell-to-cell basis and scored as reticular or fragmented. Proximity ligation assay was performed according to the manufacturers protocol (Olink Biosciences) using Drp1 antibody (1:50 BD Transduction Laboratories), Pim1 antibody (1:50 Cell Signaling Technologies), and PUMA antibody (1:50 Abcam) followed by visualization by confocal microscopy.

Subcellular Fractionation and Immunoprecipitation

Subcellular fractionation of cultured cells and isolated heart tissue was performed as described previously (16). Immunoprecipitation was performed on isolated cells in 70mM Sucrose, 190mM Mannitol, 20mM HEPES solution, .2mM EDTA solution. Cells were dounced 20 times and 10uL of lysate was taken as input followed by centrifugation at 10,000 g's for 10 mins. Supernatant was collected and cleared with IgG beads (Santa Cruz) for 2 hours and beads were removed by centrifugation for 30 seconds at 800g's. 1ug of antibody Drp1 (BD Transduction Laboratories), Pim1 (Invitrogen), PUMA (Abcam), β -actin (Santa Cruz) was incubated with sample overnight. IgG beads were added to mixture next day and incubated for 2 hours following by centrifugation of antibody IgG complex for 30 seconds at 800g's. Complex was washed twice with wash buffer (40 mM HEPES [pH 7.4], 150 mM NaCl, 2 mM EDTA, 10 mM pyrophosphate, 10 mM glycerol-phosphate, 0.3% CHAPS) followed by addition of 25 ul of 1X SDS sample buffer for immunoblot.

Cyclohexamide Inhibition

Protein half-life was determined using adenovirally infected NRCMs treated with cyclohexamide (100 µg/ml) to inhibit protein synthesis. After indicated time points NRCMs were isolated in 1X SDS sample buffer and immunoblots were performed. Proteasome inhibition was accomplished using MG-132 at a concentration of 10uM in the presence of cyclohexamide for the indicated time points followed by sample collection in sample buffer.

Ischemia / Reperfusion Surgery

I/R surgery was performed as previously described with some modifications. Briefly, sex matched FVB mice 12 -14 weeks of age were mechanically ventilated through an endotracheal cannula and were anesthetized until areflexic with 1.6% Isoflurane / oxygen mixture. Thermal regulation was maintained at 37oC with a homoeothermic blanket and probe. A left thoracotomy through the 4th intercostal space was performed to allow access to the heart and the left anterior descending artery (LAD) was reversibly ligated (slip knot) with a 7.0 silk suture tied transversely around a 1-2mm piece of PE-10 tubing 1mm below the atrial apex. The thoracic opening was closed and the mice were allowed to revive under 100% air ventilation and kept warm on a heating blanket for 50 minutes. After infarction mice were reintubated and anaesthetized as before, the thoracic cavity reopened and the slipknot loosened and the tubing removed to allow reperfusion. The thoracic cavity was securely closed and the animal allowed to recover on a warming blanket.

In vitro Kinase Assay

His-Pim-1 (wild type and kinase dead) were generated as previously described(55). Recombinant Drp1 protein was purchased from Abnova (Walnut, CA). 0.5µg Drp1 was incubated with 10 µCi γ-32P-ATP (PerkinElmer Life Sciences, Santa Clara, CA) and 0.5 µg Pim-1 kinase (wild type or kinase dead) in 30 µl kinase buffer

containing 25 mM HEPES (pH 7.5), 10 mM MgCl₂ for 20 min at room temperature. The reaction was stopped by addition of 2 × SDS sample buffer and boiling for 5 min. Samples were separated by 4-12% SDS-PAGE and Drp1 phosphorylation status by Pim-1 was visualized by autoradiography.

Cell Death Assay

NRCMs were infected with the indicated adenoviruses with and without si. Scrambled siRNA (Life technologies) and Drp1 siRNA (Life technologies) were transfected into NRCMs using HiPerfect (Qiagen) in minimal media and knockdown was achieved 48 hours later. Cell Death assay was done by propidium iodide staining (Molecular Probes) on NRCMs. Briefly, after SI treatment media was collected and NRCMs were trypsinized with .05% trypsin and cells were incubated in 1XPBS containing 10% FBS. Cells were spun down at 1800g's for 5 mins and resuspended in PI to label dead cells immediately followed by FACS analysis.

Statistical Analysis.

Statistical analysis was performed using Student's t-test. Comparison of more than two groups was performed by one-way ANOVA with Bonferroni's post hoc test or by two-way ANOVA. A P value of less than 0.05 was considered statistically significant. Error bars represent SEM. Significance indicators are *P < 0.05, **P < 0.01, and ***P < 0.001.

RESULTS

Drp1 Localizes to the Mitochondria after Simulated Ischemia

NRCMs displayed 70% reticular mitochondrial morphology in full media (Figure 1.1A). A time course of NRCMs treated with simulated ischemia (sl) demonstrated mitochondrial fragmentation occurs quickly in response to an ischemic environment, transitioning from ~ 30% of the cells displaying a fragmented phenotype to ~ 90% by 30 minutes sl (Figure 1.1A). Fragmented mitochondrial morphology has been previously associated with sl and DRP1^{K38A} was able to prevent this phenomena, but cellular localization of endogenous Drp1 during sl was not evaluated (69). Fragmented mitochondrial morphology was consistent with Drp1 mitochondrial localization (Figure 1.1B), which persisted during simulated ischemia reperfusion (sl/R; Figure 1.1C). Corroborating *in-vitro* results of Drp1 localization following ischemic injury, *in vivo* ischemia/reperfusion injury (IRI) resulted in Drp1 translocation to the mitochondria after 50 min of ischemia and 15 min reperfusion by 2.3 fold ($p<.01$; Figure 1.1D).

To confirm mitochondrial fragmentation was due to Drp1 mitochondrial compartmentalization, Drp1 was inhibited by mdivi-1, a selective pharmacological inhibitor of Drp1 activity. Incubation of NRCMs with 50 $\mu\text{mol/L}$ mdivi-1 led to the formation of enlarged, globular mitochondria, consistent with reports of Drp1 down-regulation (Figure 1.2A). Reticular mitochondria morphology was retained at a 2.5 fold higher level ($p<.01$) when NRCMs were treated with mdivi-1 during sl compared to DMSO treated controls (Figure 1.2B), confirming that mitochondrial localization of Drp1 causes mitochondrial fragmentation. Prolonged Drp1 mitochondrial association is implicated in causing cell death and simulated ischemia reperfusion (sl/R) injury in NRCMs caused a 3.54 fold increase in TUNEL positive cells (Figure 1.2C). However, NRCMs incubated with mdivi-1 prevented sl/R related cell death (Figure 1.2C). To

augment these results by showing that mdivi-1 is effective in protecting the adult heart, age and sex matched mice were subjected to 50 minutes of LAD artery ligation followed by 24 hours of reperfusion with and without mdivi-1 treatment. Total area-at-risk (AAR) was similar between control and treatment groups: 59% and 56%, respectively (Figure 1.2F). Phthalocyanine-blue dye and TTC staining indicated smaller infarct size per area-at-risk in mice injected with mdivi-1, 0.61 ± 0.04 DMSO vs. 0.43 ± 0.03 ($p < .001$) mdivi-1, showing total infarct size decreased from 36% (DMSO) to 24% in mdivi-1 treated hearts (Figures 1.2D & 1.2E).

Drp1 Phosphorylation is Regulated by Pim-1

Previous studies have shown that phosphorylation of Drp1-^{S637} reduces mitochondrial fragmentation, Drp1 accumulation at the mitochondria, and apoptotic cell death (81, 82). Since we established Pim-1 kinase preserves mitochondrial integrity and function (16), we investigated whether Pim-1 expression could affect pDrp1-^{S637} levels. Hearts isolated from PimWT mice displayed elevated levels of Drp1-^{S637} phosphorylation by 2.7 fold ($p < .01$, Figure 1.3 A&B) and total Drp1 protein levels were reduced by 50% ($p < .01$) compared to non-transgenic controls (Figure 1.3C). In contrast, Pim-1 Dominant Negative (PDN) mice displayed a 2 fold decrease ($p < .05$) in phospho-Drp1 and a 1.63 fold increase ($p < .05$) in total Drp1 protein levels, suggesting that Pim-1 may influence Drp1 phosphorylation and total protein levels (Figure 1.3 D,E, & F). Furthermore, using fractionated heart lysates from PDN mice, immunoblot analysis revealed a 2.2 fold increase ($p < .05$) of Drp1 associated at the mitochondria compared to control (Figure 1.3 G & H).

Drp1 Translocation is Regulated by Pim-1

Pim-1 preserves mitochondrial integrity, whereas mitochondria isolated from PDN mice show disrupted structural integrity upon calcium-induced swelling (16). Therefore, the effect of Pim-1 on Drp1 localization was investigated.

Total amounts of Drp1 protein decreased by 40% ($p < .05$) in Pim-1 NRCMs transduced with an EGFP-Pim-1 (adPimWT) adenovirus (Figure 1.4A). Furthermore, in NRCMs overexpressing a kinase dead Pim-1 adenovirus, EGFP-Pim-1 dominant negative (adPDN) whole cell Drp1 protein levels were increased by 1.8 fold ($p < .01$) compared to EGFP (Figure 1.4B), corroborating the *in vivo* results that loss of Pim-1 upregulates Drp1 protein levels. Differences in Drp1 localization due to Pim-1 expression and sl were assessed in cytosolic and mitochondrial subcellular fractions. Drp1 expression was decreased at baseline conditions in adPimWT NRCM cytosol whereas the mitochondrial fraction exhibited similar amounts of Drp1 (Figure 1.4C). Furthermore, Drp1 mitochondrial translocation upon sl was inhibited in adPimWT NRCMs relative to control samples (Figure 1.4C). Conversely, adPDN NRCMs displayed an increase in Drp1 expression within cytosolic and the mitochondrial fractions at baseline (Figure 1.4D). Drp1 shuttled into the mitochondria following sl treatment in both control as well as adPDN NRCMs resulting in similar Drp1 expression in the mitochondrial fraction (Figure 1.4D). Cycloheximide (CHX) chase analysis demonstrated Pim-1 does not alter Drp1 half-life (16 hours) compared to EGFP control in NRCMs, demonstrating that Pim-1 does not target Drp1 degradation (Figure 1.5). Proteasome inhibition by MG132 caused an accumulation of Drp1 at 16 hours in the presence of CHX, validating Drp1 degradation through the proteasome (Figure 1.5).

NRCMs transduced with adPimWT were exposed to sl followed by subcellular fractionation. Immunoblot analysis revealed a 2 fold ($p < .01$) decrease in Drp1 expression in the mitochondrial compartment (Figure 1.6A). NRCMs overexpressing a

adPDN, illustrated a 1.7 fold ($p < .05$) increase in Drp1 accumulation in the mitochondria fraction without being subjected to sl (Figure 1.6B). To substantiate that Pim-1 prevents Drp1 translocation from the cytosol, mitochondrial morphology was assessed after sl on NRCMs transduced with adEGFP or adPimWT adenovirus. Prior to simulated ischemia EGFP and PimWT NRCMs display 80% and 77% reticular mitochondrial morphology, respectively (Figure 1.6C and 1.7A). However, after 15 min of sl treatment 22% reticular mitochondria remain in EGFP NRCMs, whereas PimWT NRCMs retain 65% of their reticular morphology ($p < .01$; Figure 1.6C). At 30 mins of sl PimWT maintained 40% reticular mitochondria compared to EGFP at 11% ($P < .05$; Figure 1.6C & Figure 1.7A). adPDN NRCMs basally express Drp1 at the mitochondria and display a striking 80% fragmented phenotype compared to EGFP control, suggesting that adPDN NRCMs may be more susceptible to cell death ($p < .01$; Figure 1.6D & Figure 1.7A). Pim-1 overexpression in siDRP treated NRCMs did not increase reticular mitochondrial morphology following sl, demonstrating the necessity of Drp1 for PimWT mitochondrial effects (Figure 1.7B).

PimWT mice and adPimWT NRCMs exhibited an increase in Drp1-^{S637} phosphorylation, thereby suggesting a potential site on Drp1 for Pim-1-mediated phosphorylation (Figure 1.3B & 1.4G). Adenoviral overexpression of Drp1 in NRCMs followed by immunoprecipitation of Pim-1 displayed Pim-1 and Drp1 association before and during sl treatment (Figure 1.8A), which was confirmed by proximity ligation assay *in vitro* (Figure 1.8B). Furthermore, phosphorylation of Drp1 by Pim-1 kinase was demonstrated by *in vitro* kinase assay (Figure 1.8C). Recombinant Drp1 was used as substrate and γ -³²P-ATP incorporation was measured with recombinant active Pim-1 and recombinant kinase dead Pim-1. Mutation of serine 637 of Drp-1 to an alanine residue that cannot be phosphorylated (S637A) induces mitochondrial translocation of Drp-1 and

fragmentation, whereas mutation to phosphomimetic aspartic acid (S637D) inhibits translocation (26). Relevance of the serine 637 residue for Pim-1 mediated effects upon Drp-1 subcellular localization and mitochondrial phenotype was assessed by coexpression of Drp1-S^{637A} with either Pim-1-mCherry or mCherry alone as a control. Drp1-S^{637A} localized to mitochondria and induced fragmentation when coexpressed with mCherry control (Figure 1.9 A & B). Coexpression of Pim-1-mCherry together with Drp1-S^{637A} appeared comparable to results observed using the mCherry control, supporting the postulate that inhibition Drp-1 action by Pim-1 involves phosphorylation on serine 637 of Drp1.

Drp1 Translocation is Mediated by PUMA Following Loss of Pim

The BH3-only protein p53 upregulated modulator of apoptosis, PUMA, is reported to be required for Drp1 accumulation to the mitochondria in Hela cells (83). PLA analysis with Drp1 and endogenous PUMA in NRCMs demonstrated protein-protein interaction at basal conditions (Figure 1.10A). PLA analysis in PUMA Dominant Negative adenovirus infected (adPUMA-DN) NRCMs with Drp1 exhibited interaction between the two proteins (Figure 1.10B). This result was confirmed by immunoprecipitation of adPUMA-DN NRCMs with Drp1 before and after si, which also showed PUMA-DN and Drp1 interaction *in-vitro* (Figure 1.10C). Puma protein levels decreased by 35% in PimWT transgenic mice ($p < .05$ Figure 1.11A) and total Puma expression decreased 50% after si as shown by immunoblot, suggesting that Pim-1 reduces Drp1 localization by inhibiting PUMA ($p < .01$; Figure 1.11B). In contrast, analysis of mitochondrial fractions from adEGFP and adPDN NRCMs showed a 2.2 fold increase in PUMA expression at the mitochondria mediated by PDN ($p < .05$; Figure 1.11C). Collectively, these results support the premise that inhibiting PUMA may rescue PDN-induced Drp1 localization to mitochondria and blunt fragmented mitochondrial

morphology. In order to test this hypothesis, NRCMs were infected with adPDN alone or dually infected with adPDN and adPUMA-DN. Immunoblot analysis following cell fractionation displayed a 1.7 fold increase in Drp1 mitochondrial translocation ($p < .05$) compared to EGFP control, however, overexpression of PUMA-DN significantly attenuates this accumulation by 2.4 fold ($p < .01$; Figure 1.11D). Protein analysis of whole cell lysates confirmed no change in total Drp1 protein levels attributing the effect of PUMA-DN solely upon localization of Drp1 (Figure 1.11E). To support PUMA-DN inhibition of Drp1 translocation, mitochondrial morphology was also evaluated after PUMA-DN rescue of the PDN associated fragmented mitochondrial phenotype. Addition of PUMA-DN to NRCMs with PDN promoted a 60% reticular mitochondrial morphology, compared to adPDN alone (Figure 1.11F). Extended Drp1 localization to the mitochondria can cause cell death, therefore we assessed if PumaDN could prevent apoptosis following SI. NRCMs infected with adPDN and subjected to 30 min SI resulted in 27% cell death compared to control ($p < .05$), whereas NRCMs dually infected with adPDN and adPumaDN displayed only 13% cell death ($p < .001$; Figure 1.11G).

DISCUSSION

Mitochondria constantly undergo the process of fission and fusion sustaining a homeostatic balance within the cell. Why mitochondria undergo fusion and fission is not well understood, but it is hypothesized fission and fusion allow for complementation of mitochondrial mutations and elimination of dysfunctional mitochondria, thereby preserving functional mitochondrial populations (84). Mitochondrial fission and fusion classically involves the participation of four individual proteins: Drp1, Mfn1, Mfn2, Fis1 and Opa1. Fusion involves the GTPase Opa1, Mfn 1, and Mfn2; whereas Fis1 and Drp1 promote mitochondrial fission(68).

Disruption of mitochondrial morphological dynamics sensitizes cells to apoptotic cell death(75, 85, 86) and current studies have reported disturbances within the balance of these two processes can implicate the outcome of cardiovascular disease and neurodegenerative disease(82). In this study we show that ischemic challenge in myocytes promotes translocation of Drp1 to the mitochondria resulting in a fragmented mitochondrial phenotype (Figure 1.1 and 1.2) and that Pim-1 kinase prevents these changes. Drp1 has been known to be associated with mitochondrial fission, and its persistent sequestration at the mitochondria is associated with cell death. The mechanism by which Drp1 causes fission is not completely understood.

In the past, Pim-1 has been shown to have multiple protective effects at the mitochondria (16, 61, 87) and a relationship between Pim-1 and mitochondrial morphologic dynamics has not been examined previously. Pim-1 overexpression prevented Drp1 accumulation at mitochondria and decreased total Drp1 levels (Figure 1.6A, 1.4A & 1.4C). Conversely, the PDN mutant significantly increased total protein levels and mitochondrial Drp1 localization at baseline, but Drp1 translocation following si

did not increase relative to control samples (Figure 1.6B, 1.4B & 1.4D). Collectively, these findings support the postulate that Pim-1 inhibition of Drp1 translocation is the predominant mechanism of Drp1 regulation by Pim-1. Drp1 translocation was significantly reduced by Pim-1 overexpression during SI and Drp1 accumulation at mitochondria is promoted by PDN under basal condition. Since mitochondria already display a fragmented phenotype during SI prior to reperfusion, small increases of mitochondrial Drp1 promote fragmented mitochondrial morphology.

Protein-Protein interaction between Pim-1 and Drp1 occurs under both basal and ischemic conditions (Figure 1.8A), consistent with prior reports of Pim-1 binding to and stabilizing proteins (81) that raise the possibility that Pim-1 promotes Drp1 cytosolic sequestration by association. Drp1 mitochondrial shuttling is inhibited by phosphorylation at S⁶³⁷, with Drp1-S⁶³⁷ dephosphorylation modulated by calcineurin (26, 88). Dephosphorylation of Drp1-S⁶³⁷ prevents cytosolic sequestration of Drp1 and promotes mitochondrial fission, consistent with our observation of increased levels of phospho-Drp1 in Pim-1 overexpressing transgenic hearts. Although Drp1-S⁶³⁷ is a phosphorylation target for Pim-1, additional candidate residues on Drp1 are also likely phosphorylated to alter Drp1 function and localization, which will be assessed in future studies.

Drp1 mediated fission events primarily occur through multiple protein-protein interactions that form multimeric complexes (89). It was recently reported that Puma is necessary for Drp1 to accumulate at the mitochondria; however a direct interaction between Puma and Drp1 remained unexplored (83). Our findings also suggest a pivotal role for Puma and Drp1 mitochondrial localization. Puma is an upstream activator of the apoptotic cell death pathway through the mitochondria and mediates the cell death response during ischemic injury (90). Mechanistically, Puma's proapoptotic activity is

linked to sequestration of Bcl-2 and Bcl-xL that causes a displacement of Bax and/or Bak and activating the proapoptotic functions of these proteins (91, 92). A loss of Pim kinase activity promoted an increase of Drp1 at the mitochondria and a greater number of fragmented mitochondria, however, addition of PumaDN was able to rescue fragmented mitochondrial phenotype. This suggests that Puma may play a role during Drp1 scission events and the BH3 domain of PUMA may be necessary for the proper functioning of either Drp1 assembly or GTPase activity.

Several lines of evidence support the cardioprotective activity of Pim-1 including enhanced regeneration of the myocardium after myocardial infarction and preservation of mitochondrial structure during cardiac challenge. In this study we demonstrate an additional beneficial effect of Pim-1 on preventing mitochondrial fission through Drp1 cytosolic sequestration. Interference with mitochondrial structural dynamics can prevent apoptotic cell death as the intrinsic pathway of apoptosis occurs at the mitochondria. Collective findings of cardiac overexpression of Pim offer an excellent solution to utilize Pim-1 as a therapeutic agent for the treatment and intervention of cardiac cell death.

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FIGURES

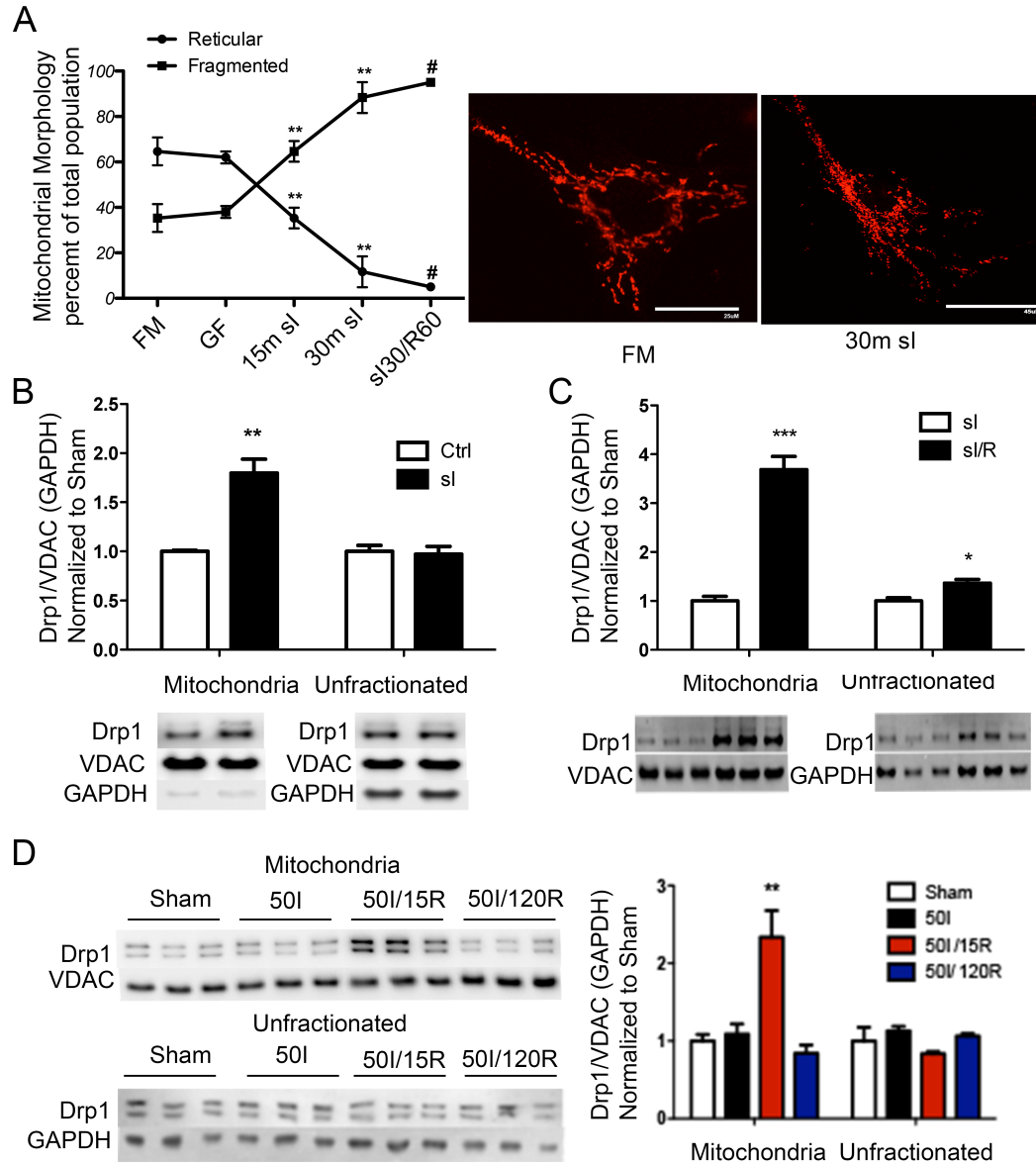


Figure 1.1: Drp1 Localizes to the mitochondria after sl in vitro.

(A) Tom20-labeled NRCMs showing the transition from reticular to punctate mitochondria phenotypes after either sl or sl/R insults. Quantification of mitochondrial phenotype in NRCMs subjected to sl or sl/R. FM, full media; GF, glucose-free media; *, significant compared with 30 min GF control; #, not significant versus sl 30. (B) Immunoblot of Drp1 after 30 min of sl versus control, full media M199 with 10% FBS NRCMs. Ctrl, control; **, significant compared with control. (C) Immunoblot of Drp1 localization in NRCMs after sl/R (30 min/30 min) versus 30 min sl. (D) Immunoblot of Drp1 localization in adult murine hearts subjected to a sham LAD ligation, or 50 min LAD ligation, or 50 min/15 (120) min LAD ligation/reperfusion. **, significant compared with sham. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

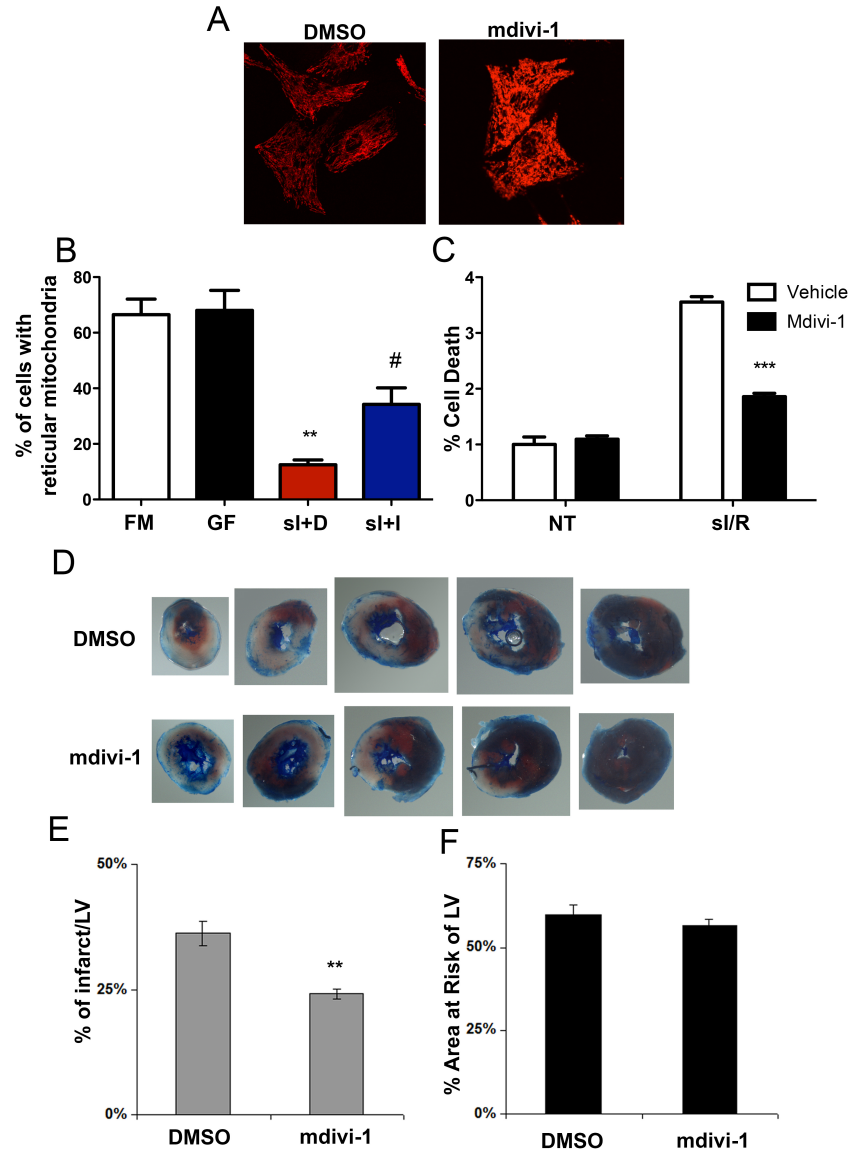


Figure 1.2: Drp1 inhibition by mdivi-1.

(A) Representative confocal image of NRCMs treated with 50 μ M mdivi-1 labeled with Tom20. (B) Analysis of mitochondrial morphology in NRCMs treated with full-media, glucose-free, sl for 30 min with mdivi-1 or DMSO control. +D, DMSO; FM, full-media; GF, glucose-free; +I, mdivi-1; **, significant compared with FM; #, significant compared with sl+D. (C) TUNEL analysis between mdivi-1- and DMSO-treated cells after sl/R (30 min/20 h). mdivi-1 and DMSO were present during all times of treatment. ***, significant compared to vehicle sl/R. (D) Mice were injected i.p. with mdivi-1 or DMSO vehicle and subjected to LAD ligation-induced I/R (50 min/24 h). The hearts were excised, labeled with phthalocyanine-blue dye, and sectioned. Incubating in 1% TTC was used to identify viable tissue and phthalo-blue to delineate the area at risk. Representative images and a graph of 1-mm heart sections stained with TTC and phthalocyanine-blue are shown. (E) Measurement of infarct size in mdivi-1- and DMSO-treated groups. **, significant compared with DMSO controls. (F) No significant difference in area at risk was noted between the DMSO and mdivi-1 groups. #P < 0.05; *P < 0.05; **P < 0.01; ***P < 0.001.

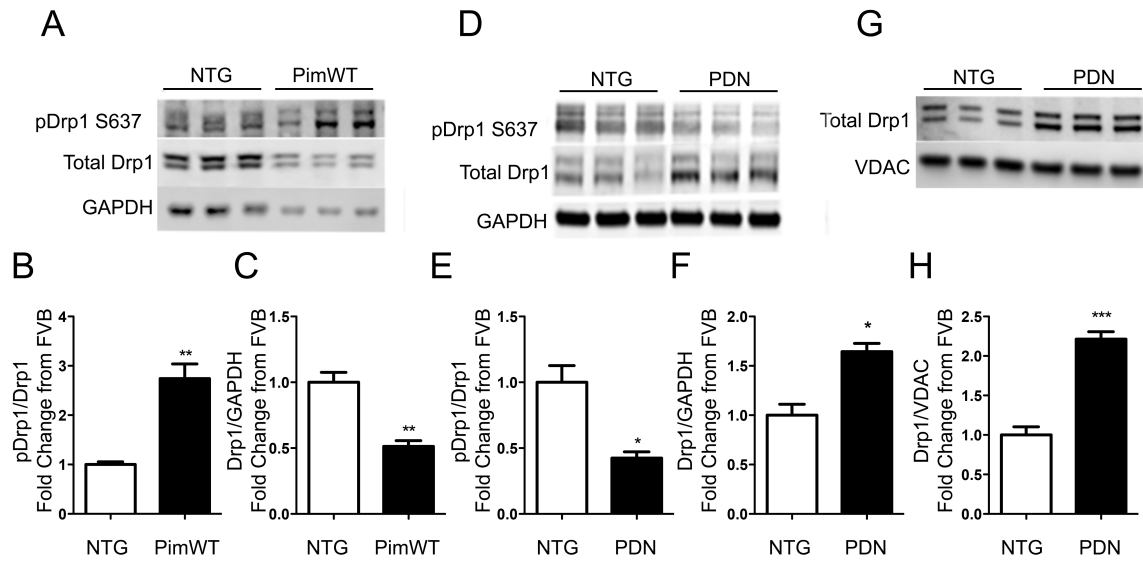


Figure 1.3: Drp1 Phosphorylation is regulated by Pim-1.

(A–F) Immunoblot and quantification of pDrp1S637 and total Drp1 in murine PimWT and PDN whole-heart lysates. (G–H) Immunoblot of Drp1 localization in isolated mitochondria from PimDN (PDN) and FVB nontransgenic (NTG) control hearts harvested from age- and sex-matched mice. *P < 0.05; **P < 0.01; ***P < 0.001.

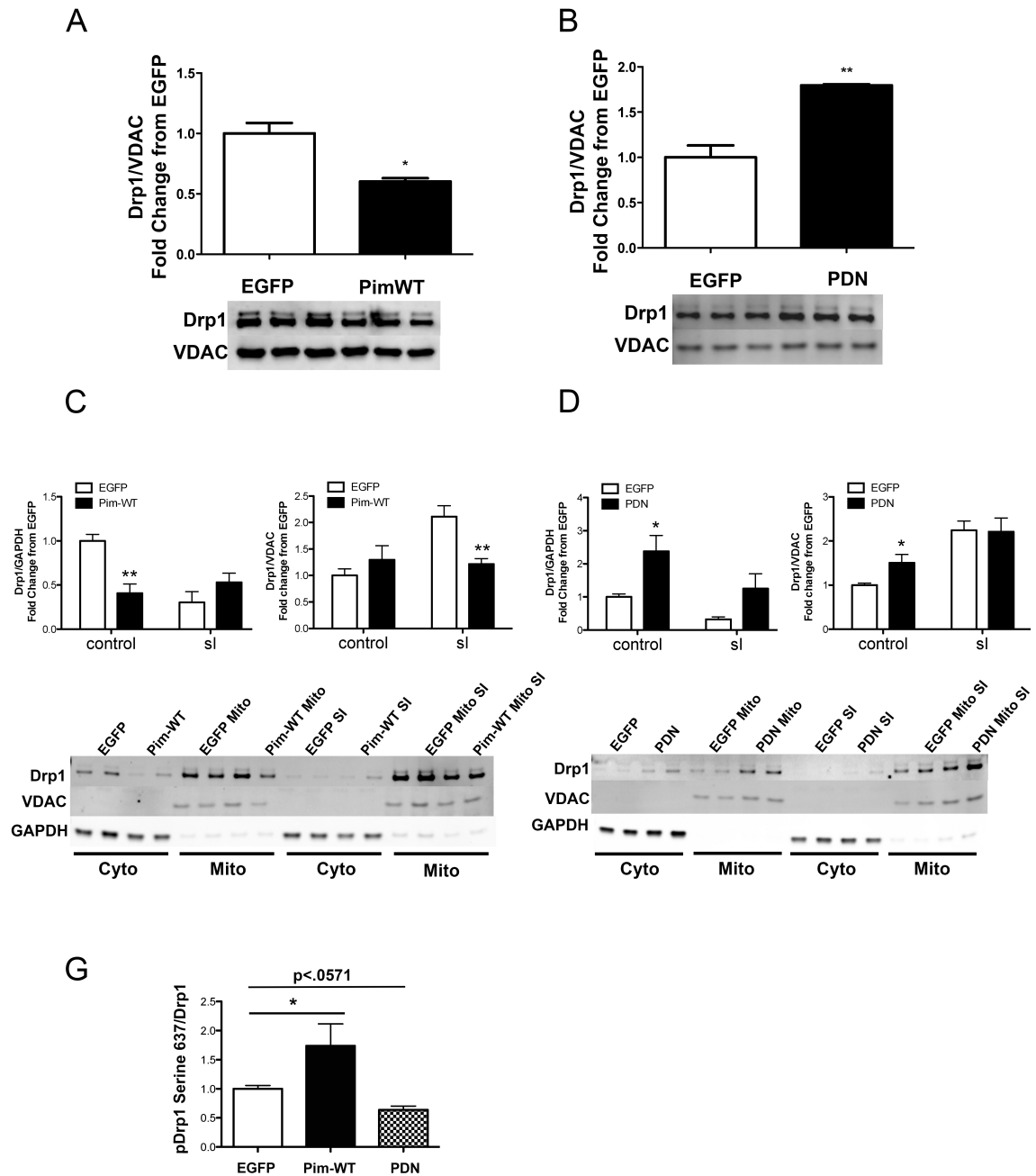


Figure 1.4: Pim-1 mediates Drp1 localization and protein levels.

(A) Immunoblot and analysis of total Drp1 expression against total VDAC in NRCMs infected with adEGFP or EGFP-Pim-1 (adPimWT) adenovirus and subjected to si. (B) Total Drp1 protein expression normalized to total VDAC levels in NRCMs infected with adEGFP or adPDN adenovirus. *, significant compared with EGFP. (C and D) Drp1 localization in adEGFP- and adPim-WT- or adEGFP- and adPDN-infected, fractionated NRCMs after si. (E) Phosphorylation status of Drp1-S637 in adEGFP-, adPim-WT-, and adPDN-infected NRCMs. *, is significant compared with EGFP control. *P < 0.05, **P < 0.01.

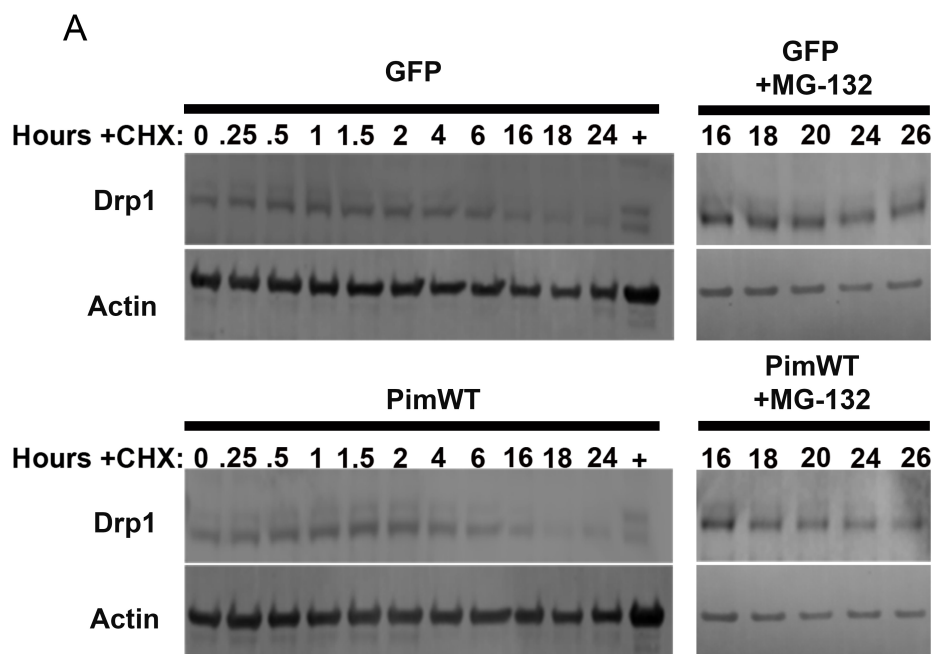


Figure 1.5: Drp1 half-life is not altered with Pim-1.

(A) Cycloheximide chase was performed on adEGFP- and adPimWT-infected NRCMs followed by analysis of proteosomal degradation of Drp1 with MG132. CHX, cycloheximide.

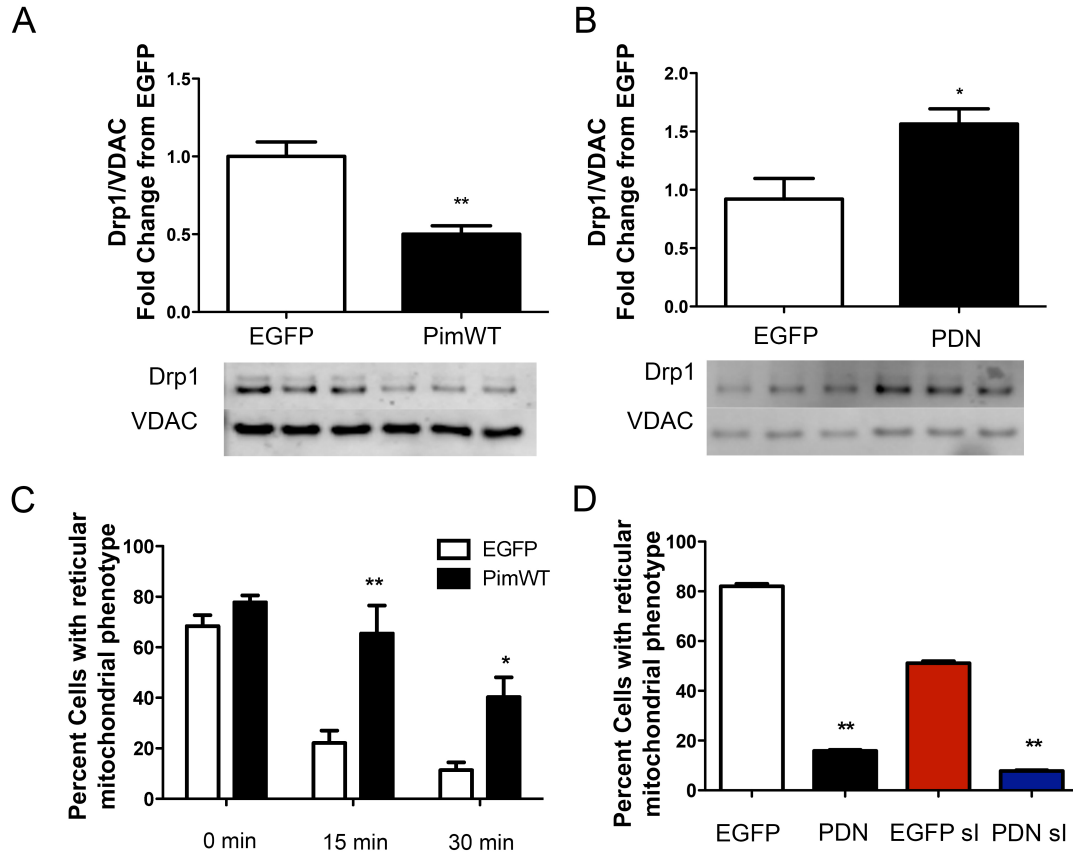


Figure 1.6: Pim-1 overexpression affects Drp1 Localization in NRCMs.

(A) Immunoblot of Drp1 localization in NRCMs transduced with adEGFP control virus or adEGFP-PimWT after sl. (B) Protein analysis of Drp1 compartmentalization in adEGFP- or adPDN-transduced NRCMs after fractionation. (C) Analysis and quantification of mitochondrial morphology in NRCMs infected with adPimWT or adEGFP subjected to up to 30 min of sl. *, significant to EGFP by two-way ANOVA (D) Quantification of adPDN and adEGFP NRCMs maintaining reticular mitochondria before and after 30 min of sl. **, significant compared with EGFP control. * $P < 0.05$; ** $P < 0.01$.

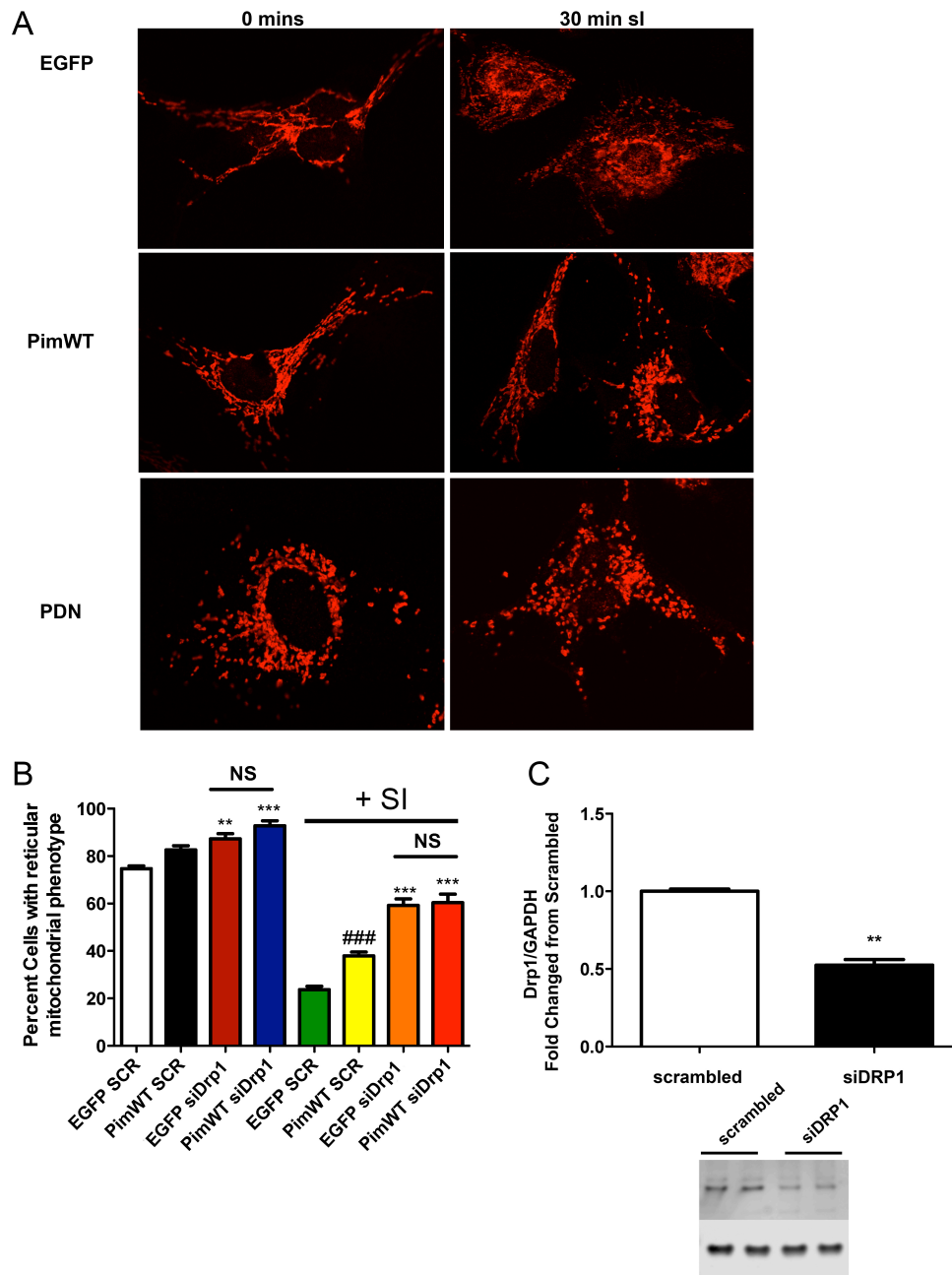


Figure 1.7: Pim-1 maintains reticular mitochondrial morphology in response to si.

(A) Confocal images displaying mitochondrial morphology of NRCMs transduced with adEGFP, adPimWT, and adPDN adenovirus and subjected to 30 min si. (B) Quantitation of reticular mitochondrial morphology in NRCMs with siRNA against Drp1 and rescued with PimWT and or EGFP control adenovirus. *, significant compared with respective scrambled control; #, significant compared with EGFP scrambled (SCR) with si. All si conditions had significantly less reticular mitochondria compared with their respective controls. (C) Immunoblot confirming down-regulation of Drp1 followed by siRNA Drp1. **P < 0.01; ###P < 0.001; ***P < 0.001.

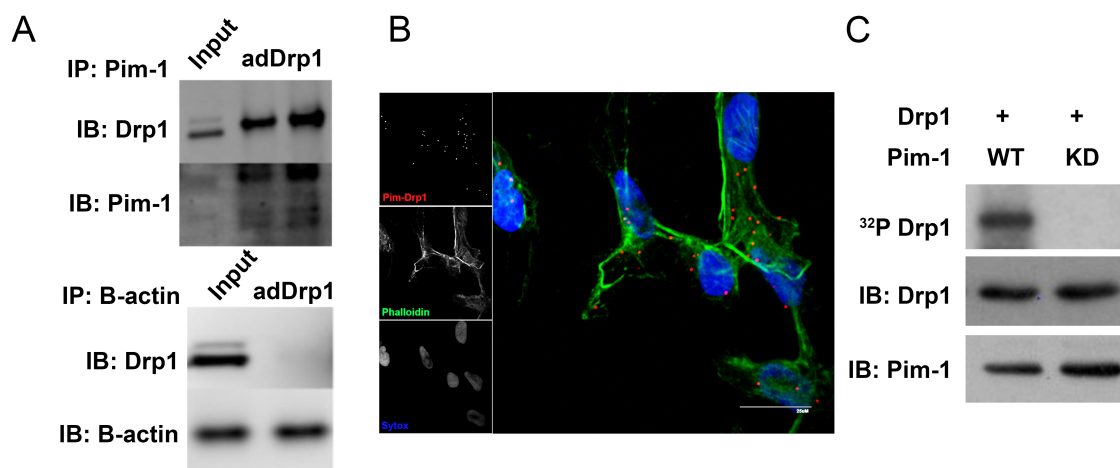


Figure 1.8: Pim-1 Directly Phosphorylates Drp1.

(A) Immunoprecipitation of Drp1-infected NRCMs with Pim-1 followed by immunoblot of Drp1 and Pim-1. (B) Proximity ligation assay with Pim-1 and Drp1 in cultured HeLa cells. Each red dot represents a single protein–protein interaction. (C) Phosphorylation of Drp1 by Pim-1 kinase in vitro. Recombinant Drp1 was used as substrate in an in vitro kinase assay that measures γ -³²p-ATP incorporation. His-Pim-1 (wild-type, kinase dead) was expressed in *Escherichia coli* and affinity-purified for use in the kinase assay. Phosphorylated proteins were separated by SDS/PAGE and visualized by autoradiography.

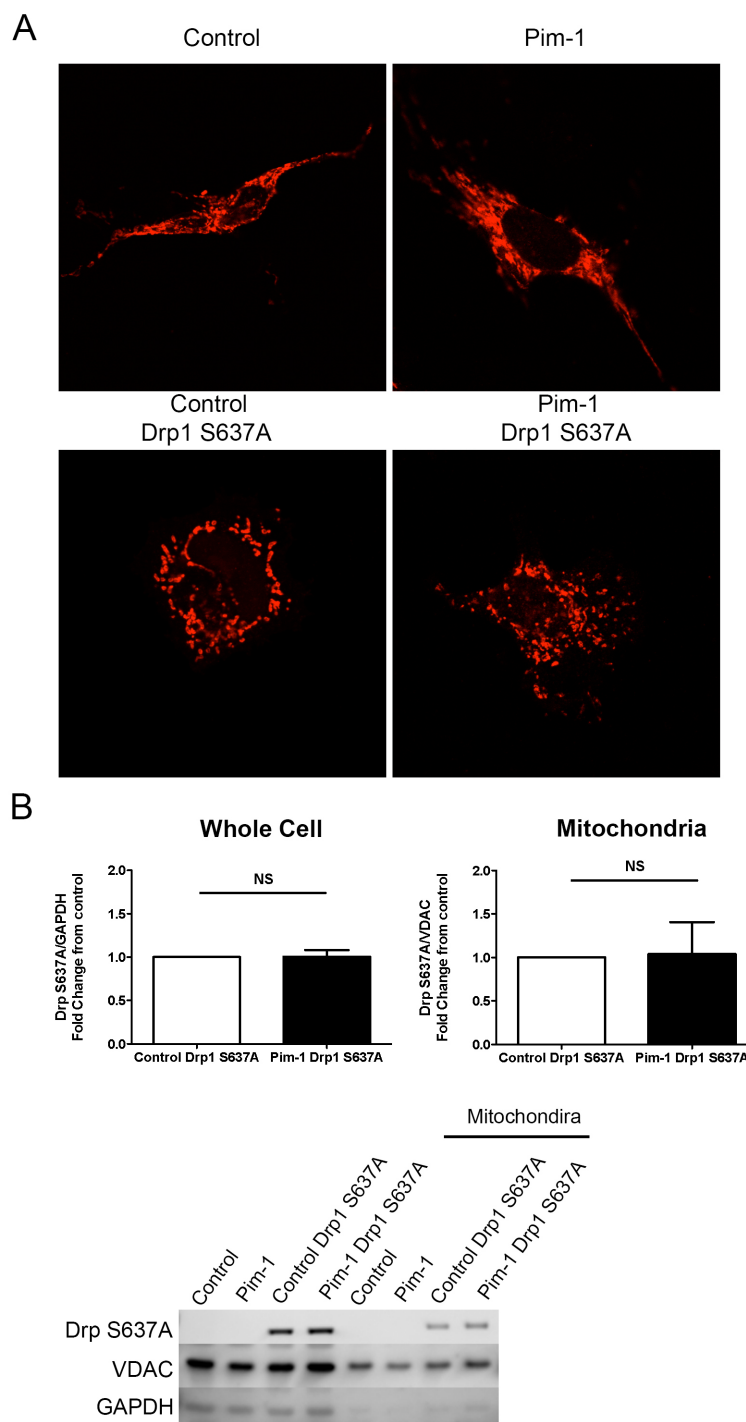


Figure 1.9: Pim-1 does not rescue Drp1-S637A-mediated mitochondrial fragmentation and localization.

(A) Confocal images displaying mitochondrial morphology of cardiac fibroblast transfected with control plasmid expressing mCherry, Drp1-S637A, or Pim-1-mCherry plasmid. (B) Immunoblot demonstrating Drp1-S637A localization with and without Pim-1-mCherry.

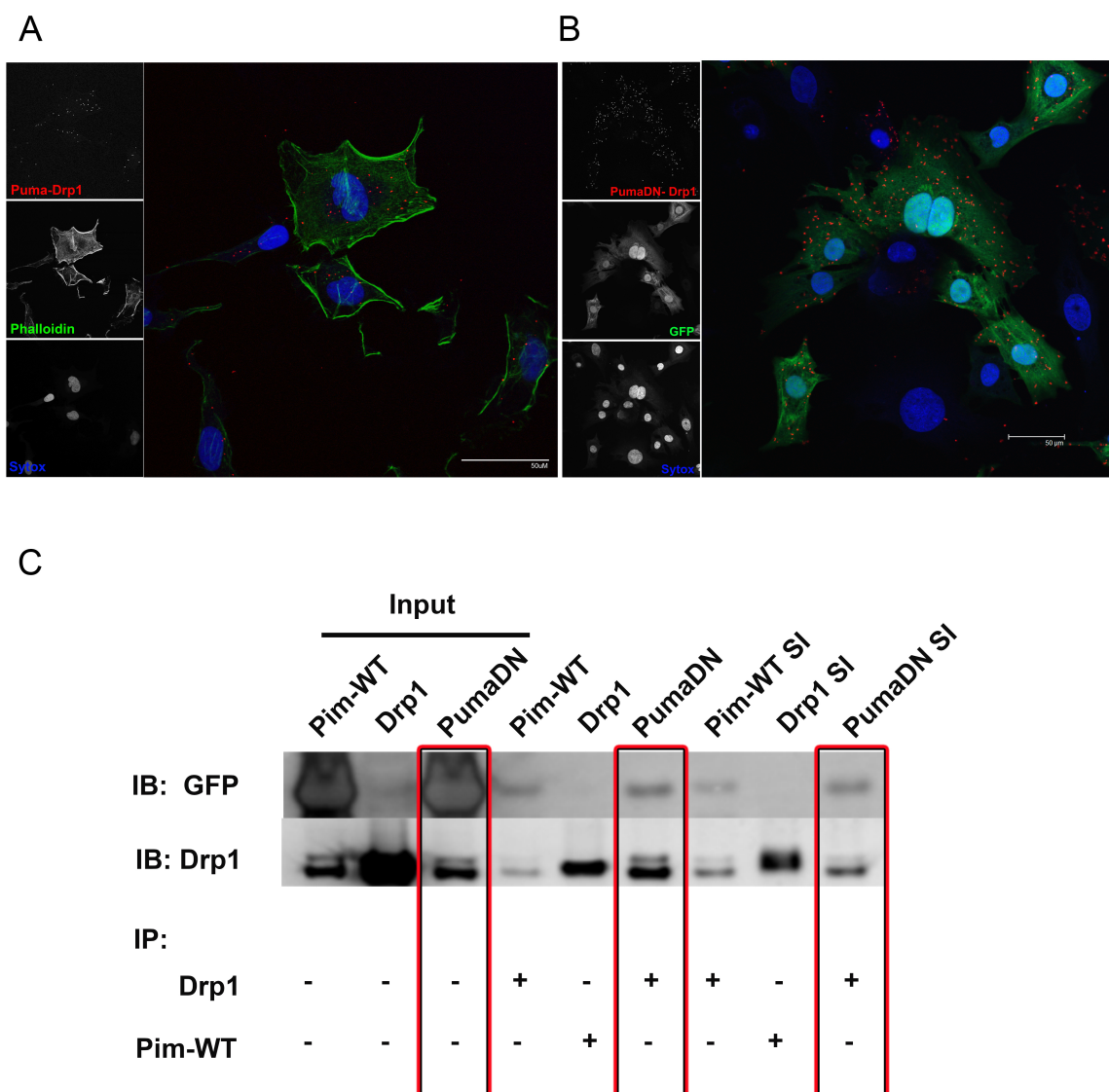


Figure 1.10: Drp1 binds to PUMA in vitro.

(A) Proximity ligation assay demonstrating endogenous interaction of PUMA and Drp1 in NRCMs. (B) Proximity ligation assay was performed with adPumaDN NRCMs. Red dots display a single interaction between Drp1 and PumaDN under baseline conditions (C) NRCMs were infected with adPimWT, adDrp1, or adPumaDN, as indicated (Upper). Drp1 and PimWT were immunoprecipitated with specific antibodies indicated beneath the immunoblot. Immunoprecipitated complexes were stained for GFP to detect adPimWT and adPumaDN or Drp1, as indicated (Left). Red boxes highlight evidence for PumaDN and Drp1 interaction.

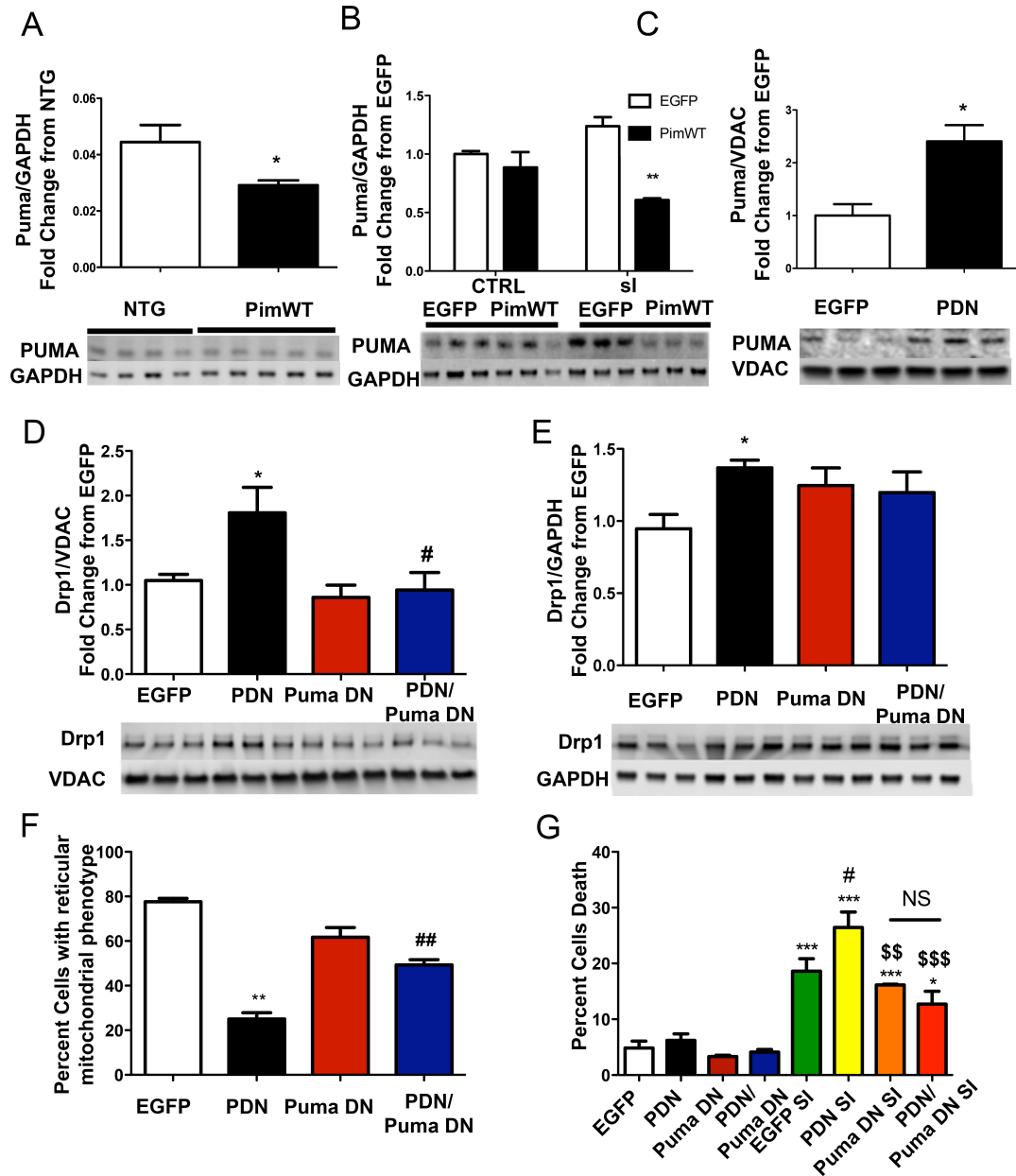


Figure 1.11: PUMA mediates the effect of Pim-1 on Drp1 translocation.

(A) Immunoblot of PUMA in nontransgenic (NTG) and PimWT transgenic mice. Immunoblot of NRCM lysates infected with either (B) adPimWT and adEGFP or (C) adPDN and adEGFP adenovirus for PUMA. (D) Immunoblot of Drp1 protein levels at the mitochondria after rescue with adPumaDN. *, significant compared with EGFP control; #, significant compared with adPDN. (E) Drp1 protein expression in total NRCM lysates after infection with adEGFP, adPDN, adPumaDN, and adPDN + adPumaDN. (F) Morphological assessment of reticular mitochondria in the aforementioned groups by Tom20 staining. **, significant compared with EGFP; ##, significant compared with PDN. (G) Cell death analysis with propidium iodide of NRCMs subjected to simulated ischemia. *, significant compared with respective baseline control; #, significant compared with EGFP si; \$, significant compared with PDN si. #P < 0.05; *P < 0.05; ##P < 0.01; \$\$\$P < 0.01; **P < 0.01; \$\$\$P < 0.001; ***P < 0.001.

SUMMARY POINTS

- Ischemic injury prompts a disruption in mitochondrial dynamics promoting fission over fusion resulting in fragmented and punctated mitochondria.
- Drp1 translocation from the cytosol to the mitochondria during ischemia causes an increase in mitochondrial fission altering mitochondrial morphology.
- Transgenic mice overexpressing Pim-1 kinase display decreased Drp1 protein and increased phosphorylation at serine 637 on Drp1.
- NRCMs overexpressing Pim-1 and subjected to ischemic challenge display decreased Drp1 in the mitochondrial compartment and retention of reticular mitochondrial morphology associated with survival.
- Pim-1 directly interacts and phosphorylates Drp1 promoting cytosolic sequestration.
- Pim-1 prevents the expression of pro-apoptotic PUMA necessary for Drp1 mitochondrial translocation.

CHAPTER II

Metabolic Dysfunction Consistent With Premature Aging Results From Deletion of Pim Kinases

INTRODUCTION

Cardiac senescence and the aging phenotype are characterized by a multitude of changes on the cellular and organ level that occur over the lifetime of an organism. In contrast to rapid expansion of nascent cardiomyocytes taking place in postnatal development, *de novo* generation of myocytes in response to aging is very limited (4-6). The onset of ventricular hypertrophy at the cellular and organ level is a hallmark of cardiac aging, which compensates for losses in cellular density and concomitant diminution of functional hemodynamic output. The consequence of pathological cardiac hypertrophy is eventual alterations in mitochondrial metabolism and energy homeostasis promoting glucose utilization over fatty acid oxidation, exacerbating disease etiology (14, 31, 45). Preservation of mitochondrial integrity and function antagonizes aging, as myocardial senescence is associated, in part, with decreased mitochondrial content and altered metabolic function (66, 93, 94).

Transcriptional coregulators PPAR γ coactivator-1 (PGC-1) α and β serve as critical regulators of mitochondrial biogenesis and cellular ATP producing pathways (43, 45, 95, 96). PGC-1 α and PGC-1 β coactivate downstream transcription factors involved in mitochondrial biogenesis such as ERR α , NRF-1, and TFAM. PGC-1 α is enriched and highly inducible in the heart. However, ablation of PGC-1 α leads to compensatory upregulation of PGC-1 β (31, 45). PGC-1 coactivators regulate the mitochondrial fatty acid oxidation (FAO) pathway, which serves as the primary supply for bioenergetic fuel in the healthy adult heart. Heart failure and hypertrophy prompt reprogramming of fuel utilization to rely predominantly on glucose metabolism similar to the fetal heart (14). Downregulation of PGC-1 signaling and the cognate downstream target PPAR α contributes to the fuel shift toward fetal metabolism in the hypertrophied heart presenting as metabolic dysfunction (31, 46). Furthermore, transgenic mice with single knockdown

of PGC-1 α or PGC-1 β demonstrate age-dependent contractile dysfunction and impaired mitochondrial function, whereas mice lacking PGC-1 α and PGC-1 β die shortly after birth from heart failure (31, 45, 97).

Pim-1, a conserved serine/threonine protein kinase, exerts multiple protective effects upon mitochondria and has recently been implicated in affecting metabolism through PGC-1 α (16, 55, 62). Additionally, Pim-1 stabilizes and phosphorylates c-Myc, a known regulator of mitochondrial metabolism and biogenesis (55, 64). Pim-1 also impacts upon mitochondrial dynamics through phosphorylation and cytosolic sequestration of Drp1 (98). The Pim gene family consists of Pim-1, -2, and -3; three different genes transcribed from alternative start sites. All three Pim family members are constitutively active, exhibit similar substrate preferences, and differ primarily in tissue expression (65, 99). Pim-1, the predominant isoform in the heart, can be genetically deleted in mice prompting compensatory upregulation of Pim-2 and -3 (53, 100).

Pim-1 is highly expressed in postnatal hearts but diminishes precipitously during postnatal development (53). Recently, our group documented the remarkable ability of Pim-1 overexpression to “rejuvenate” aged human cardiac stem cells by decreasing senescent markers, promoting proliferation, and survival (101). Taken together, these studies implicate Pim kinases in the maintenance and preservation of a “youthful” cellular phenotype. Since Pim-1 promotes mitochondrial integrity and antagonizes acquisition of an aging phenotype, the relationship between Pim kinase, mitochondrial biogenesis, cardiac senescence, and metabolism resulting from loss of Pim kinase activity are investigated in this report.

METHODS

Mice and Echocardiography

The Pim Triple KnockOut (PTKO) mouse model is previously described in Mikkers et al (58). Briefly, Pim1 knockout mice were generated initially by homologous recombination using the targeting vector pGTP810 in FVB mice. Using Pim1 knockout mice PTKO mice were generated by deleting exons 1,2,and 3 for Pim2 with a PGK-*Php* cassette. Pim3 exons 3,4,5 and 6 were deleted using a promoterless IRES- β Geo cassette (58). Echocardiography was performed under mild isoflurane sedation (.5 - 1.5%) using a Vevo 770 High resolution system with wildtype FVB and PTKO mice (58). Cardiac function was analyzed in the parasternal long axis view tracking the endocardium with the supplied analysis software to obtain left ventricle inner diameter during diastole (LVIDd), left ventricle anterior wall thickness during diastole (LVAWd), ejection fraction (EF), and heart rate (HR).

Transmission Electron Microscopy and Mitochondrial Area

Mitochondria were examined by transmission electron microscopy. Animals were euthanized by cervical dislocation before dissection. The heart was excised and submerged immediately in 2% glutaraldehyde in 0.1M cacodylate buffer with 1% sucrose. Cardiac tissue was cut with a razor blade into 1 mm² pieces and incubated in fixative solution on ice for 10 minutes. All of the following steps, through the 100% ethanol dehydration step, were performed on ice. After incubation, each sample received a fresh replacement of fixative solution and was fixed for 1 hour. Following fixation, the samples were washed three times (10 minutes each wash) in 0.1M cacodylate buffer with 1% sucrose. The samples were then postfixed with 1% osmium tetroxide in 0.1M cacodylate buffer with 1% sucrose for 1 hour in the dark and washed three times (10 minutes each wash) in water. The samples were dehydrated through a

graded series of ethanol solutions from 30% to 50%, 70%, 85%, 95% and 100% (10 minutes each). Two successive 10 minute incubations in 100% ethanol were performed before the samples were transferred into 100% acetone at room temperature followed by an additional 10 minute incubation step in 100% acetone. Tissue samples were infiltrated with 1:2 EPON/acetone resin followed by 2:1 EPON/acetone resin for 8 hours each. Infiltration with pure EPON was completed overnight. Tissue samples were transferred into a flat embedding mold in pure EPON and polymerized at 60°C for 48 hours. Thin sections (60nm thick) from the embedded samples were cut, collected on 100 mesh copper grids, and stained with uranyl acetate and lead citrate. Images were captured using a FEI Tecnai 12 transmission electron microscope operated at 120kV. Images were acquired at 11000X. Measurements of mitochondrial area per section were calculated as follows using ImageJ software: mitochondrial area was measured followed by normalization to area of section. Sample size was n=3 for each group analyzed.

Immunohistochemistry and Cell Counts

Heart sections were deparaffinized followed by antigen retrieval in citrate (10 mM, pH 6.0). One-hour block in TNB buffer was followed by incubation in primary antibody overnight. Primary antibodies and concentration used are listed (Table 2.1). Three washes to remove unbound primary antibody in Tris/NaCl were followed by secondary antibody incubation for 1.5 hours at room temperature. Specimens were mounted with Vectashield (Vector Laboratories). Topro to label nuclei was added in the last wash step after incubation with secondary antibodies at 1:5000 for 20 minutes. Images were obtained on a Leica DMRE confocal microscope. Area assessments for myocyte size were performed using the outlining tool from ImageJ after images of cardiac specimens stained with wheat germ agglutinin. Sections were stained with sarcomeric actin to visualize cardiomyocytes and myocardial area. Infarct size was

calculated as proportion of infarcted area relative to total area of the respective section with at least 6 high power fields were analyzed per heart for non-transgenic (NTG) or Pim Triple KnockOut (PTKO) mouse heart samples. Image acquisition and size measurements were performed under sample-blinded conditions.

Masson Trichrome staining was performed according to the manufacturers protocol (Trichomes Accustain (Masson), Sigma–Aldrich) and micrographs were acquired using Leica DMRE confocal microscope.

Sample preparation, Immunoblotting, RT-PCR, Mitochondrial DNA Content, and Microarray Analysis

Immunoblotting was performed using protein samples separated on 4-12% NuPAGE Novex Bis-Tris Gel (Invitrogen) by electrophoresis. Protein contents of gels were then transferred onto a polyvinylidene fluoride (PVDF) membrane, blocked in iBLOCK (Life Technologies), and exposed to primary antibodies over night. Alkaline phosphatase (AP), horseradish peroxidase (HRP), Fitc-, Cy5, or Cy3-conjugated IgGs (Jackson ImmunoResearch) were used as secondary antibodies. Fluorescence signal was detected and quantified by using a Typhoon 9400 fluorescence scanner together with ImageQuant 5.0 software (Amersham Biosciences).

mRNA was isolated using the Quick RNA Mini Prep kit (Zymo Research) according to the manufacturer protocol. cDNA was transcribed using the cDNA preparation kit (Biora). For RT-PCR sybr green (Biorad) was employed using the manufacturer protocol. Data were analyzed with the $\Delta\Delta C(t)$ method. A complete list of primers used for qPCR is provided (Table 2.2). Mitochondrial DNA (mtDNA) copy number was quantified by qPCR from isolated total DNA derived from heart tissue of PTKO mice (n=4) and wildtype mice (n=4) according to the manufacturer protocol of NucleoSpin tissue kit (Macherey-Nagel). 50 ng of total heart DNA was used and each

sample was measured in duplicates. Two different primer pairs were used to quantify and confirm relative mtDNA copy number: COXI and Cytochrome b (mitochondrial) and β -Globin/H-19 for genomic DNA. All sequences are listed in Table 2.2. Data obtained by qPCR were analyzed by the $\Delta\Delta CT$ method.

Microarray analysis was performed according to the manufacturer protocol using PPAR signaling array from Bar Harbor technologies (catalog number: 00198247) on CFX Connect thermal cycler (Bio-Rad).

ATP Assay, Citrate Synthase Assay

Lysates for ATP assay and citrate synthase assay were obtained from NTG and PTKO. Briefly, mouse hearts were extracted after cervical dislocation and immediately snap frozen in liquid nitrogen. Hearts were then homogenized in isolation buffer: 70mM Sucrose, 190mM Mannitol, 20mM HEPES solution, .2mM EDTA solution. Homogenized lysates were then employed in an ATP assay (Invitrogen) and citrate synthase assay (Promega) according to the manufacturer protocol.

Cell Culture and Viral Transduction

Immortalized murine embryonic fibroblast from wildtype (iWT MEFs) and PTKO (iPTKO MEFs) were provided by Dr. Andrew S Kraft (Medical University of South Carolina, Charleston) and were cultured as previously described (15). Briefly, immortalized MEFs were maintained in DMEM (Invitrogen 11965-092) containing 10% fetal bovine serum and 1% penicillin-streptomycin-glutamine (PSG). Adenovirus EGFP was created and expanded as previously described (22, 26). The PGC-1 α adenovirus is identical to that used in Lehman et al. 2000 JCI (27). Adenovirus c-Myc was purchased from Vector Biolabs (Philadelphia, PA Catalog# 1285) and expanded. For adenoviral transduction iWT MEFs and iPTKO MEFs were infected in 2% fetal bovine serum DMEM for two hours followed by two wash steps of phosphate buffered saline and incubation in

10% DMEM and collected 24 hours later for analysis. A 50 multiplicity of infection was used for adenovirus EGFP, c-Myc, and 200 for PGC-1 α .

Mitochondrial respiration assay

Mitochondrial isolation procedures were followed as previously published (28). Hearts were removed while still contracting from mice anesthetized with ketamine/xylazine (50mg/kg and 10mg/kg, respectively) via intraperitoneal injection. Individual mouse hearts were rapidly minced in ice-cold isolation buffer (100 mmol/L KCl, 50 mmol/L MOPS pH 7.4, 1 mmol/L EGTA, 5 mmol/L MgSO₄, 1 mmol/L ATP, 0.2% essentially fatty acid free BSA). Henceforth, all steps were performed at 0°C on wet ice. Tissue was homogenized in isolation buffer with a Polytron tissue grinder at 11,000 RPM for 2.5 s, followed by 3 strokes at 500 RPM with a Potter-Elvehjem PTFE tissue grinder. The homogenate was centrifuged at 600×g twice for 5 min and the supernatant was saved. Mitochondria were pelleted from the supernatant by centrifugation at 3,000×g twice, and the pellet was rinsed with isolation buffer. The final mitochondrial pellet was resuspended in 100 μ l of resuspension buffer (220 mmol/L mannitol, 70 mmol/L sucrose, 2 mmol/L Tris base, and 20 mmol/L HEPES pH 7.4). Protein concentration was determined by Bradford assay using BSA standards. Oxygen consumption measurements were performed with an Oxygraph Clark type electrode (Hansatech Instruments) in respiration buffer (10 mmol/L MgCl₂, 100 mmol/L KCl, 50 mmol/L MOPS pH 7.0, 1 mmol/L EGTA, 5 mmol/L KH₂PO₄, 0.2% essentially fatty acid free BSA). 200 μ g of mitochondria were added to a final volume of 1 ml respiration buffer at 30°C. Complex I activity was measured using 2 mmol/L pyruvate and 2 mmol/L malate as substrates. Complex II activity was measured using 5 μ mol/L rotenone with 5 mmol/L succinate as substrate.

Telomere Measurements

Telomeres were measured in NTG and PTKO hearts using quantitative fluorescence in-situ hybridization (Q-FISH) or multiplex quantitative PCR method as previously described (100) (102). Telomere length was labeled and analyzed by quantitative in situ hybridization (QFISH) using DAKO's PNA probe (K5325) and Leica confocal microscopy. Results were obtained by altering manufacturer's protocol as follows. Slides were deparaffinized, rehydrated and treated with 3.7% Formaldehyde for 2 minutes, then subsequently washed with TBS. Next, followed antigen retrieval using 10mM Citrate pH 6.0 for 15 minutes. Slides were washed again with TBS and underwent proteolytic treatment using Proteinase K (Dako, S3004) diluted 1:10 in TBS, for 8 minutes. Preceding another wash, slides were dehydrated in cold ethanol. PNA probe was applied to dried sections, coverslipped using a 2mm round coverslip, and incubated on a 85°C hot plate for 5 minutes then allowed to hybridize at 37°C overnight. The following day, slides were immersed in Rinse Solution to remove coverslips and washed with 70% Formamide pH 7.2. Next, slides were washed in pre-heated Wash Solution at 65°C and quenched with 3% H₂O₂ for 20 minutes at room temperature. Slides were then washed, incubated with Sheep anti-FITC/HRP 1:200 in TNB for 1 hour at room temperature, washed again, amplified with Tyr/FITC 1:50 for 10 minutes, then dehydrated in cold ethanol. Lastly, slides were counter-stained and coverslipped using Topro 3 1:200 in Vectashield. Telomere signal in each nucleus was acquired using Leica software and divided by nuclear size followed by normalization to NTG controls. Additionally, signals were scanned using identical setting to control experimental variation.

Telomere length was measured by real time PCR using a modified monochrome multiplex quantitative PCR method previously described (102). Albumin and the telomere template were amplified at the same time to account for the differences in DNA

concentration per well and sample. To account for difference in reaction samples, 12 replicates of each sample were prepared using 10ng of DNA, 1X syber green, albumin, and telomere primers, bringing the reaction volume to 15uL. The thermal cycle protocol used is as follows, stage 1 (2 cycles of 15 min at 95°C, 15 seconds at 94°C and 15 seconds at 49°C), stage 2 (15 seconds at 94°C, 10 seconds at 62°C and 15 seconds at 73°C with plate acquisition), stage 3 (32 cycles of 15 seconds at 94°C, 10 seconds at 62°C, 15 seconds at 73°C with signal acquisition, 10 seconds at 84°C, 15 seconds at 87°C with signal acquisition), and stage 4 (1 cycles of 0.05 seconds at 65°C with signal acquisition).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5.0 (Graphpad Software). Statistical analysis was performed using Student's t-test. Echocardiography time course analysis was assessed by two-way ANOVA with Bonferroni's post hoc test. A P value of less than 0.05 was considered statistically significant. Error bars represent SEM. Significance indicators are *P < 0.05, **P < 0.01, and ***P < 0.001.

RESULTS

During physiological aging the expression of Pim kinases are regulated in a cardiac context. Pim1 expression is downregulated by 53% starting at 100 days and remains 50% decreased at 324 days compared to 3 days during physiological cardiac aging ($p<.01$ Figure 2.1A). Pim2 expression does not change with cardiac aging (Figure 2.1A). Pim3 expression is upregulated at 14d by 2.2 fold and increases at 100 days and 324 days by 2.6 and 3.9 fold, respectively compared to 3 days ($p<.01$ Figure 2.1A). During myocardial infarction Pim1 expression increases in the infarct area at Day 1 by 2.59 fold and gradually diminishes over time by 40% within the infarct region (Figure 2.1B). Pim2 expression increases in the remote region at Day 1 by 2.62 fold and also diminishes over time (Figure 2.1C). Pim3 expression starts to decrease at 1 day following infarction in both the remote and infarct region and by two weeks is decreased by 78% in infarct and remote region (Figure 2.1D). Increased Pim expression early during myocardial infarction is consistent with a prosurvival role for Pim genes.

Pathological hypertrophic remodeling during cardiac aging is well documented (103, 104), as is the ability of Pim-1 to antagonize myocardial hypertrophy in response to pressure overload (52). Expression of Pim1, 2, and 3 kinases decrease by 62%, 81%, and 76% at 2 weeks, respectively in response to transaortic constriction (Figure 2.1E). Consistent with hypertrophic remodeling, PGC-1 α expression is diminished with TAC by 59%, 72%, 88%, and 77% at 1 day, 4 day, 7 day, and 2 weeks (Figure 2.1F). Therefore, blunting of cardiac hypertrophy previously reported following Pim-1 overexpression (52, 54) was compared to the effect of Pim kinase deletion in PTKO mice. Cell size area was increased by 1.4 fold in PTKO mice at one month ($p<.001$ Figure 2.2 A & B). Increases were significant for *Nppa* (atrial natriuretic peptide; 19 fold ($p<.05$), *Nppb*

(brain natriuretic peptide; 2 fold ($p<.05$), *Acta1* (α -skeletal actin; 4 fold ($p<.01$), and *Myh7* (β -myosin heavy chain; 43 fold ($p<.05$) following Pim deletion consistent with reactivation of the fetal gene program (Figure 2.2 C-F). Phosphorylated ribosomal S6 and phosphorylated 4EBP1 were diminished by 53% and 52% respectively, indicating decreased mTOR activity ($p<.001$ and $p<.05$) Figure 2.3 A&B). However, consistent with hypertrophic remodeling phosphorylated Akt was increased by 1.4 fold and RCAN1.4 expression was unregulated by 4.4 fold in PTKO mice, a downstream readout for NFAT transcriptional activation in pathological hypertrophy ($p<.001$ and $p<.05$ Figure 2.3 C&D).

Body weight and heart weight were decreased by 22% ($p<0.001$) and 27% in PTKO mice, respectively ($p<0.001$; Figure 2.4 A & B). However, heart weight to body weight ratio was unchanged between PTKO and wild-type mice (Figure 2.4 C). Smaller left ventricle interior diameter during diastole was revealed by longitudinal echocardiography from one to six months of age in PTKO mice ($p<0.001$ Figure 2.5 A). Furthermore, left ventricle anterior wall thickness during diastole demonstrated a strong trend toward increased wall thickness at one month and became significant at three months of age, showing an increase of 20% which persisted at six months ($p<0.05$ Figure 2.5 B). A 30% reduction in ejection fraction was evident at six months in PTKO mice, indicating premature cardiac failure ($p<0.001$ Figure 2.5 C). Heart rate was maintained at consistent levels to ensure comparability between individual measurements (Figure 2.5 D). Consistent with cardiac hypertrophy and heart failure markers of fibrosis collagen 1 α 1 and collagen 3 α 1 were upregulated 1.6 fold and 2.5 fold, respectively ($p<.01$ Figure 2.5 E&F). Furthermore, enlargement of the left ventricle in PTKO mice was evident by Massons trichrome staining (Figure 2.5 G&H).

Collectively, these findings demonstrate hypertrophic remodeling in PTKO mice and progression toward failure at six months of age.

Markers of Senescence are Elevated with Loss of Pim Kinases.

Senescent markers characterization was performed to determine the aging phenotype in PTKO mice. p16 expression was evident throughout the myocardium of PTKO mice at three months, indicating irreversible cellular senescence (Figure 2.6 A). Increased p16 expression was confirmed by immunoblot in PTKO mice relative to non-transgenic controls (Figure 2.6 B). p16 upregulation was validated by a concurrent reduction in p16 transcriptional repressors Id1 and Id2 by 60% and 64% in PTKO cardiac lysates respectively ($p < 0.05$, $p < .01$ Figure 2.7 A & B). Moreover, increased Ets-1 level (p16 transcriptional activator, 2.4 fold) also supports p16 induction ($p < .01$; Figure 2.7 C). p53 showed a 2.5-fold increase in PTKO mice at three months ($p < 0.01$ Figure 2.6 C) consistent with reduction of MDM2 (p53 ubiquitin ligase MDM2; 57% lower ($p < 0.01$; Figure 2.6 D). Telomere mean fluorescence intensity was diminished by 41.5% in PTKO as measured by quantitative fluorescence in-situ hybridization (Q-FISH) ($p < .001$; Figure 2.6 E & F). Additionally, telomere length was decreased by 40% in PTKO mice by qPCR analysis ($p < .01$; Figure 2.6 G). The collective phenotypic profile observed is consistent with expectations for premature cardiac senescence and predisposition toward heart failure in PTKO mice.

Knockdown of Pim 1, 2, and 3 in neonatal rat cardiomyocytes (NRCMs) stimulated an increase in p53 by 1.6 fold ($p < .05$), whereas p16 was unchanged (Figure 2.8 A-D). Furthermore, NRCMs treated with physiological (insulin) or pathological (phenylephrine) growth stimulus promoted an increase in p53 with loss of Pim 1, 2, and 3 by 2 fold ($p < .001$) and 1.9 fold ($p < .01$) respectively (Figure 2.8 A&B). p16 gene

expression was unchanged compared to respective scrambled controls during stress (Figure 2.8 C&D). Upregulation of *Nppa* by 1.4 fold ($p<.05$), *Nppb* by 1.4 fold ($p<.05$), and *Myh7* by 2.5 fold was also noticed in NRCMs knocked down for Pim 1, 2, and 3, whereas alpha skeletal actin displayed a strong increasing trend (Figure 2.8 C). Validation of siRNA efficiency of resulted in 80%, 80%, and 67% decrease in gene expression of Pim1, 2, and 3 respectively ($p<.001$ Figure 2.8 D,E, and F). These data further endorse the premature cardiac aging phenotype of PTKO mice.

Mitochondrial Morphological and Functional Aberrations Occur with Loss of Pim.

Pim1 protects mitochondrial integrity and preserves mitochondrial morphology (16, 98). Mitochondrial vacuoles and disrupted cristae structures were evident by electron microscopy in hearts of PTKO mice at one month after birth (Figure 2.9 A). Additionally, mitochondrial area and mitochondrial area per section were decreased by 11% and 10% in PTKO mouse hearts, respectively ($p<0.05$; Figure 2.9 A). Validating these results, voltage-dependent anion channel (VDAC) expression was reduced by 16% in cardiac lysates of PTKO mice indicating a reduction in mitochondrial mass ($p<0.05$; Figure 2.9 B).

Loss of mitochondrial function is consistent with a reduction in energy reserve as revealed by analysis of ATP levels (Figure 2.9 C). Corroborating these results, an increase in the ratio of phosphorylated AMPK to total AMPK by 4 fold was noted in PTKO hearts by immunoblot ($p<0.01$; Figure 2.9 D). Collectively, these results reveal alterations in mitochondrial morphology and functional capacity leading to energy deficiency resulting from loss of Pim kinases.

Metabolic Regulators are Decreased with Deletion of Pim Kinases

Pim kinase deletion impairs expression of PGC-1 α (62), with loss of PGC-1 coactivators promoting alteration of mitochondrial morphology (31). Therefore, the impact of Pim kinase deletion upon expression of PGC-1 coactivators and downstream metabolic regulators was assessed. PGC-1 α and PGC-1 β expression were decreased by 42% and 82%, respectively, in PTKO cardiac lysates at one month ($p<0.05$; Figure 2.10A). Furthermore, downstream transcriptional targets of PGC-1 coactivators including *Erra* (estrogen-related receptor alpha), *Nrf1* (nuclear respiratory factor 1), and *Tfam* (mitochondrial transcription factor A) were also decreased respectively by 44% ($p<0.05$), 83% ($p<0.01$), and 81% ($p<0.01$) in PTKO mice (Figure 2.10 A). Peroxisome proliferative activated receptor gamma coactivator-related protein (*PPRC1*) was also diminished by 73% with Pim loss, evidently revealing lack of compensatory upregulation for related proteins ($p<0.05$; Figure 2.12 A). Furthermore, decrease in PGC-1 α (30%) and PGC-1 β (20%) were confirmed at the protein level by immunoblot, corroborating gene expression analysis of PGC-1 coactivators ($p<0.05$; Figure 2.10 B & C).

Mitochondrial biogenesis is also dependent upon c-Myc expression that is a downstream target of Pim kinase (55, 64). c-Myc protein levels were diminished by 36% in PTKO mice ($p<0.01$; Figure 2.10 D). The impact of decreased regulators of mitochondrial biogenesis is apparent by measurement of mitochondrial DNA content as a correlate for mitochondrial number that was reduced by 50% in PTKO mice, consistent with decreased expression of PPAR γ coactivators with Pim deletion ($p<0.05$; Figure 2.10 E & F).

Decreased expression of PGC-1 coactivators alters the expression profile of cardiac metabolic genes (14, 45, 94), so representative genes involved in fatty acid oxidation, glycolysis, and oxidative phosphorylation were assessed. Fatty acid oxidation

related enzymes located in the inner mitochondrial membrane were decreased including *Acate3* (acyl coenzyme A thioesterase 3; 14.9 fold ($p < 0.001$), *Acadv1* (acyl coenzyme A long chain; 6.9 fold ($p < 0.001$)), and *Acadm* (FAO-acetyl CoenzymeA dehydrogenase; 1.7 fold ($p < 0.01$)) (Figure 2.11 A). In contrast, transporters of fatty acids such as *Cpt1b* (carnitine palmitoyl transferase) and *Cpt2* (carnitine palmitoyl transferase 2) were increased by 3.3 fold ($p < 0.001$) and 2.3 fold ($p < 0.01$), respectively, consistent with previous literature demonstrating upregulation of fatty acid transporters with prolonged AMPK activation (Figure 2.11 A) (105). The rate-limiting step of glycolysis involves *Hk2* (hexokinase 2) that was reduced by 15.3 fold ($p < 0.001$ Figure 2.11 B) in PTKO hearts. Conversely, downstream targets involved in the glycolytic pathway: *Pfk* (phosphofructokinase), *Pdha1* (pyruvate dehydrogenase E1 α 1), *Pfkfb2* (6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2) were highly upregulated by 3 fold ($p < 0.001$), 3.6 fold ($p < 0.001$), and 3.3 fold ($p < 0.001$) (Figure 2.11 B). Furthermore, pyruvate respiration was increased whereas succinate respiration was unaltered ($p < 0.05$; Figure 2.12 B & C) indicating a shift away from fatty acid oxidation and toward glucose metabolism similar to PGC-1 α/β double knockouts (31). Genes involved in lipid sterol biosynthesis were also dramatically reduced in cardiac tissue of PTKO mice indicating disruption in the PPAR signaling circuit ($p < 0.05$; Figure 2.12 D). mRNA analysis for genes involved in oxidative phosphorylation, *NDUFA* (NADH dehydrogenase 1 α subcomplex subunit 9), *NDUFV* (NADH dehydrogenase ubiquinone flavoprotein 1), and *ldh2* (isocitrate dehydrogenase 2), revealed upregulation of 3.8 fold ($p < 0.001$), 2.2 fold ($p < 0.001$), and 2.9 fold ($p < 0.001$) respectively (Figure 2.11 C). However, *Cytc* (cytochrome C) a target of PGC-1 α was decreased by 8 fold ($p < 0.001$ Figure 2.11 C). Microarray analysis of PPAR signaling genes further confirmed altered expression of genes involved in the PPAR signaling pathway (Figure 2.13). To assess global changes

a microarray analysis of PPAR signaling genes involved in fatty acid oxidation, transport, and lipid sterol biosynthesis was performed, confirming altered expression of genes involved in the PPAR signaling pathway (Online Fig VII). 62 PPAR-related signaling genes were detected in cardiac tissue, 32 showed a downregulation, 21 genes remained unchanged, and 8 genes were induced. Three out of the four genes included in the targeted screen (Acadm, PGC-1 α , Cpt1b, and Cpt2) demonstrated similar results to our targeted approach. Acadm and PGC-1 α were downregulated, whereas Cpt1b was upregulated. Cpt2 was upregulated in our screen and was unchanged in the microarray, this discrepancy might be due to differences in primer pairs as Cpt2 is predicted to have 5 alternatively spliced transcripts (NCBI: AceView). Taken together, these results demonstrate disruption of metabolic processes with Pim ablation mimicking the phenotype of PGC-1 α/β double knockouts (31).

c-Myc Rescues Expression of Metabolic Regulators

The metabolic phenotype of PTKO mice correlates with loss of c-Myc expression secondary to Pim kinase ablation, so the participation of c-Myc was assessed by a rescue experiment involving overexpression of PGC-1 α , c-Myc, and Pim1 using immortalized murine embryonic fibroblast from wildtype (iWT MEFs) and PTKO (iPTKO MEFs) mice. Metabolic deficiencies in the iPTKO MEFs were confirmed to be comparable to those of PTKO cardiac lysates. Expression of c-Myc and PGC-1 α were diminished by 82% and 55% (respectively) in iPTKO MEFs ($p < 0.05$; Figure 2.14 A) similar to PTKO mouse hearts. Phosphorylated AMPK: Total AMPK ratio was increased in iPTKO MEFs by 2.3 fold consistent with decreased cellular energy stores ($p < 0.05$; Figure 2.14 B). Similarly, alterations of metabolic regulators and reduction in fatty acid oxidation enzymes were comparable between iPTKO MEFs and PTKO heart lysates

(Figure 2.14 C). Since the iPTKO MEFs possess analogous metabolic derangements to PTKO heart samples, molecular restorations were attempted by overexpression of either c-Myc, PGC-1 α , and Pim1. Overexpression of EGFP, Pim1-EGFP, PGC-1 α -EGFP, and c-Myc-EGFP adenovirus was confirmed in iPTKO MEFs (Figure 2.14 D). c-Myc overexpression in iPTKO MEFs increased expression of metabolic regulators *PGC-1 α* (1.7 fold; $p < 0.05$), *PGC-1 β* (1.6 fold; $p < 0.05$), *Erra* (1.4 fold; $p < 0.05$), *Nrf1* (1.2 fold; $p < 0.05$), and *Avadv1* (1.2 fold; $p < 0.05$) (Figure 2.15 A). Pim1 overexpression increased expression of metabolic regulators *PGC-1 α* (1.6 fold; $p < 0.05$), *PGC-1 β* (2.2 fold; $p < 0.05$), *Erra* (1.5 fold; $p < 0.05$), *Nrf1* (1.3 fold; $p < 0.05$), and *Avadv1* (1.5 fold; $p < 0.05$) (Figure 2.15 B). PGC-1 α overexpression in iPTKO MEFs restored gene expression of the PGC-1 α downstream targets: *Erra* and *Acadv1* by 2.3 fold and 1.8 fold respectively ($p < 0.001$; Figure 2.15 C). Consequential to increased metabolic regulator expression, the ratio of phosphorylated AMPK to total AMPK decreased by 34% ($p < .01$) with c-Myc, 35% with Pim1, and 41% ($p < .01$) with PGC-1 α overexpression, indicating improvement in cellular energy stores following interventions to overexpress c-Myc, Pim1, or PGC-1 α (Figure 2.15 D,E, and F). In summary, the phenotype resulting from loss of Pim kinases can be rescued with overexpression of c-Myc, Pim1, or PGC-1 α .

DISCUSSION

Cardiac aging is characterized by pathological hypertrophic remodeling that is a primary indicator of and confirmed risk factor for heart failure (104). Pathological hypertrophy and progression toward heart failure were evident at six months in PTKO mice (Figure 2.2 and 2.5) consistent with previous published studies demonstrating antagonism of myocardial hypertrophy by Pim1 (52). Increased AKT phosphorylation and RCAN1.4 expression, a readout for activated NFAT, was evident with loss of Pim, which serve as molecular underpinnings for hypertrophic signaling (Figure 2.3) and remodeling. Additionally, fetal gene reprogramming typical for hypertrophic stimulation is blunted by Pim1 overexpression (52). Promotion of hypertrophic effects at the molecular, cellular, and organ level in PTKO hearts relatively early in life (Figure 2.2) and enhanced senescence marker expression (Figure 2.6) is consistent with Pim kinase deletion manifesting with features of an accelerated aging phenotype.

Pathological hypertrophic remodeling causes an energetic fuel shift from fatty acid oxidation to glucose metabolism (14). Cellular metabolic changes are potent regulators of the aging phenotype and PTKO mice demonstrate an up-regulation of glycolytic enzymes and diminished fatty acid oxidation enzymes, exhibiting metabolic alterations (Figure 2.11). Mitochondrial metabolism and energetics converge upon the PPAR signaling circuit and the influence of Pim kinases upon PGC-1 α is established (45, 62). Now for the first time to our knowledge, the connection between downregulation of PGC-1 α and β and downstream targets impacted by Pim kinase deletion has been revealed in the heart by our results (Figure 2.10). Furthermore, PGC-1 α is important and necessary to maintain a pool of healthy mitochondria, as PGC-1 α regulates mitochondrial biogenesis (47). Disruption in mitochondrial biogenesis occurs during

aging causing accumulation of unhealthy mitochondria and potential increases in toxic metabolic byproducts that potentially accelerate cellular aging (106). Diminished PPAR signaling occurs with aging in human and murine animal models, supporting our contention that disrupted metabolism and diminished mitochondrial biogenesis through loss of PGC-1 α in PTKO mice contributes to premature aging (50, 107).

Downstream of Pim kinases is c-Myc, a potent regulator of mitochondrial biogenesis and PGC-1 β (64, 108, 109). Diminished expression of c-Myc was evident in PTKO hearts and iPTKO MEFs (Figure 2.10 and 2.14). Acute overexpression of c-Myc in iPTKO MEFs promoted upregulation of PGC-1 α and PGC-1 β in addition to downstream regulators of mitochondrial biogenesis. c-Myc activation during pathological cardiac injury is shown to promote glucose metabolism by down regulating PPAR γ coactivators (64). In comparison, the observed loss of c-Myc still prompted enhanced glycolytic enzyme expression in PTKO mice, suggesting alternative mechanism(s) for promotion of glucose metabolism in PTKO mice. The loss of Pim kinase consequently promotes diminished expression of c-Myc along with PPAR γ coactivators presenting as cellular metabolic alterations consistent with acquisition of an aging phenotype.

Mitochondria play a central role in energy metabolism, with mitochondrial morphology intimately tied to mitochondrial function (106). Pim kinases protect mitochondrial integrity by up-regulating anti-apoptotic Bcl-2 family members (Bcl-2 and Bcl-XL) and phosphorylating BAD on serine 112 (16, 54). Furthermore, Pim1 regulates mitochondrial morphological dynamics by regulating Drp1 localization and phosphorylation (98). Imbalance in mitochondrial fission sensitizes mitochondria to cell death in addition to promoting formation of smaller mitochondria as seen in PTKO hearts (Figure 4A). Pim deletion also prompted altered mitochondrial morphology and disarray

of myofibrils (Figure 2.9). Mitochondrial morphology alterations can increase apoptotic sensitivity to cell death and propensity to permeabilize in response to stress. Disruption of mitochondrial dynamics occurring with Pim loss promotes instability in mitochondrial DNA (98, 110) and reduction in mitochondrial DNA content precedes pathological hypertrophy (111). Consistent with these assertions, PTKO mice display diminished levels of mitochondrial DNA content prior to heart failure (Figure 2.10 E & F) that can serve as an additional predisposing factor for premature aging at the mitochondrial level with loss of Pim.

Telomere attrition disrupts mitochondrial function through a molecular mechanism involving p53 mediated cellular senescence leading to a p53 dependent repression of PGC-1 α (112). Telomere length decrease in PTKO mice (Figure 2.6 E-G) is a plausible inductive mechanism for upregulation of p53 activity and subsequent blunting of PGC-1 α . Conversely, increased telomere length resulting from Pim1 kinase overexpression is associated with decreased expression of senescent markers including p53 and increased cell cycling in human cardiac progenitor cells (101). Comparable beneficial effects of telomere length maintenance are also observed following Pim1 kinase overexpression in murine cardiac progenitor cells (100). Furthermore, PTKO mice at 4 weeks of age exhibit an accelerated aging phenotype evident by telomere attrition of 40% by qPCR and FISH (Figure 2.6 E-G). This dramatic decrease would be comparable to the relative age of 30 months in a normal mouse (6). Increased cellular turnover during physiological aging leads to a reduction in telomere length with each cell division (113-115). Although Pim1 deletion blunts cellular proliferation that may help slow telomeric shortening, any modest beneficial effect is apparently overwhelmed by depression of TERT activity that compromises preservation of telomere length (100), leading to telomeric shortening in PTKO mice consistent with accelerated physiological

aging. Therefore, the impact of Pim kinase upon telomere biology highlights an important potential mechanism affecting mitochondrial function and causing cellular senescence that is the subject of ongoing investigation.

Pim kinases promote proliferation by regulating cell cycle proteins such as p21, p27, and cdc25A through phosphorylation (116-118). Furthermore, Pim phosphorylation and stabilization of c-Myc enhances cell cycle entry (55), leading to the plausible idea that Pim kinases antagonize the aging phenotype and favor ongoing mitotic activity by blunting the impact of cell cycle inhibitors. Indeed, downregulation of cell cycle inhibitors p16 and p53 leading to increased proliferation occurs in aged and senescent human cardiac progenitor cells overexpressing Pim1 kinase (101). Conversely, in the PTKO mice the increased expression of p16 and p53 support a role for the Pim kinase family in preserving phenotypic characteristics of cellular “youthfulness” (Figure 2.6 and 2.8).

The cardioprotective activity of Pim1 through the preservation of mitochondrial structure and morphology is well established primarily based upon the overexpression of Pim1 (16, 98). However, the converse of this experimental approach implies that the loss of Pim kinase would promote deterioration of mitochondrial structure and function. Such diminution of Pim kinase occurs naturally with organismal aging, but ours is the first assessment of germline deletion for all three Pim kinase family members upon myocardial biology. Degenerative changes in mitochondria such as altered structural integrity, shifts in metabolic fuel selectivity, and decreased ATP production are hallmarks of cellular aging and senescence (119). Clearly, select essential features of the aging and senescent phenotype are recapitulated with accelerated timing in the PTKO line. Now a link can be placed to connect Pim kinases to aging and metabolic changes through the PPAR signaling circuit. Important to note as a limitation of this study is the

PTKO mouse models are global knockouts and not cardiac-specific. Therefore, *in vivo* findings although consistent with expectations remain correlative in nature, so these findings were buttressed by mechanistic molecular signaling *in vitro* studies with iPTKO MEFs. At present, the circumstantial evidence points toward alterations in mitochondrial biogenesis and metabolism coincident with premature cardiac aging of PTKO hearts. Looking forward to an interventional strategy, prevention of metabolic derangements is important to antagonize the cardiac aging phenotype. So too, delineating underlying causes of aging are important to develop therapeutic interventions to treat aging myopathy in the elderly population. The PTKO mouse model could serve in this capacity as an excellent model system to study cardiac aging and senescence. Collectively, this study gives credence to the possibility of utilizing Pim1 as a therapeutic agent for treatment of and intervention in the pathogenesis of cardiac aging.

This concludes Chapter II, thank you to my co-authors: Mathias H. Konstandin, Bevan Johnson, Andrea De La Torre, Mirko Voelkers, Jacqueline Emathingier, Haruhiro Toko, Brett Collins, Lucy Ormachea, Kaitlen Samse, Dieter A. Kubli, Andrew S. Kraft, Asa B. Gustafsson, Daniel P. Kelly, and Mark A. Sussman.

FIGURES

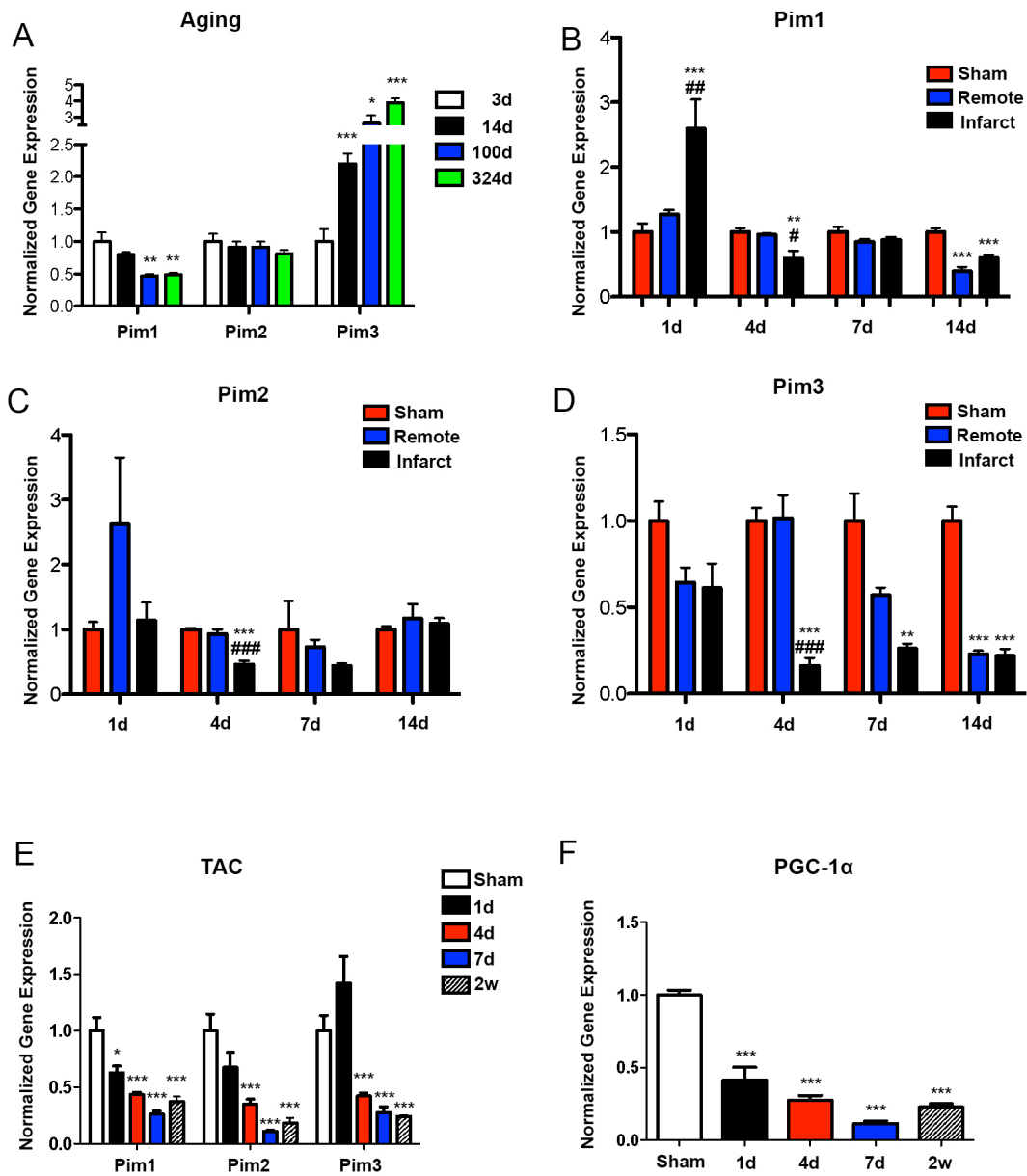


Figure 2.1: Expression of Pim kinases during aging and transaortic constriction.

A) Gene expression analysis of Pim 1,2, and 3 during physiological cardiac aging. B-D) Pim1, Pim2, and Pim3 gene expression respectively post myocardial infarction. E) Time course analysis of Pim kinase gene expression post transaortic constriction. F) PGC-1α gene expression post transaortic constriction. *, **, *** is significant compared to 3day (Figure 1A) or Sham. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. #, ##, ### is significant compared to remote. # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$

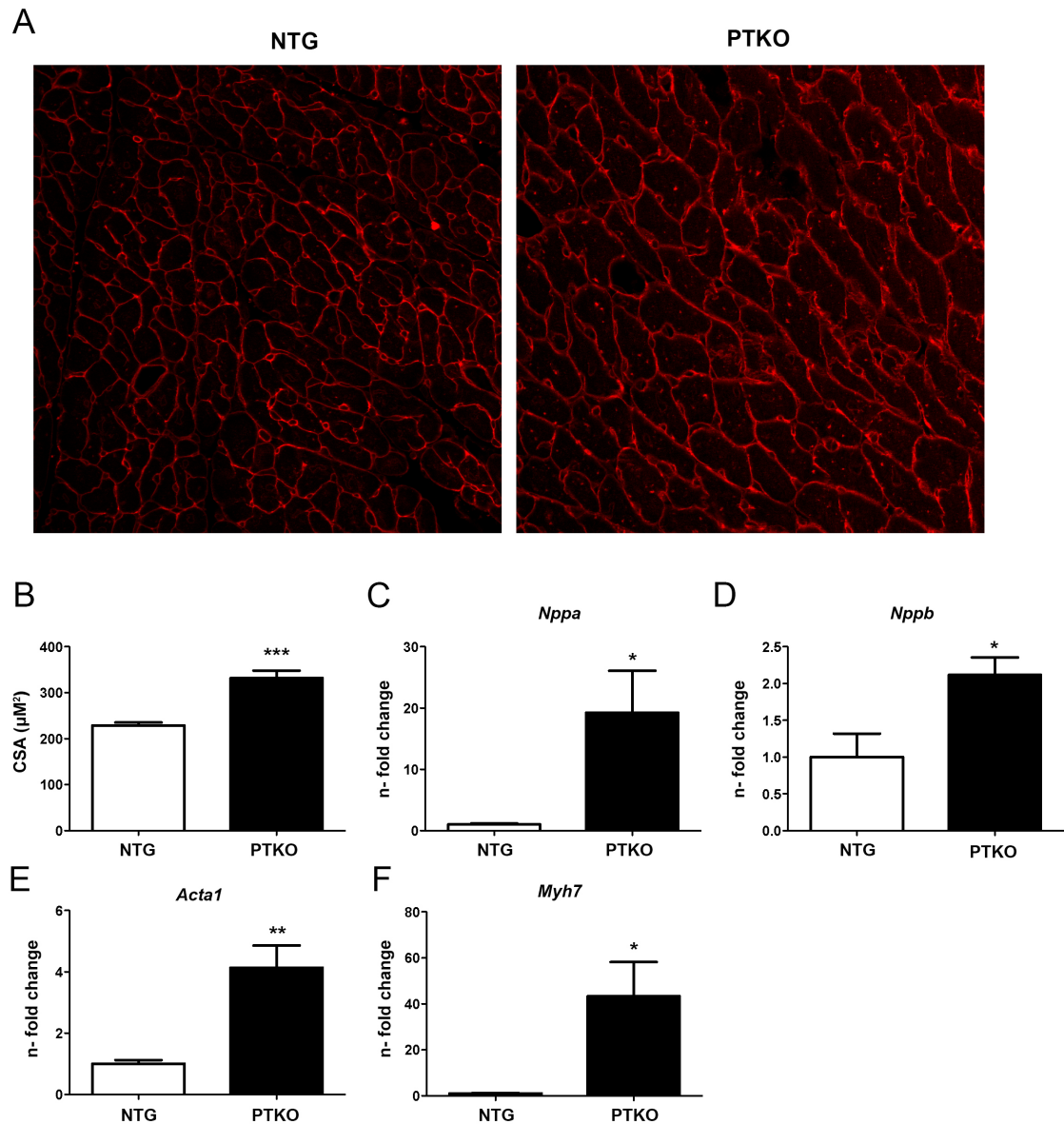


Figure 2.2: Characteristics of cardiac hypertrophy in PTKO mice.

A) Wheat germ agglutinin staining in cardiac sections demonstrating cell surface area of Non-Transgenic (NTG) and Pim Triple Knockout (PTKO) mice at 1 month. B) Quantification of cell size area (CSA) in heart tissue sections at one month (n=4 NTG and PTKO mice). C-F) mRNA levels of fetal cardiac gene markers: *Nppa* (atrial natriuretic peptide), *Nppb* (brain natriuretic peptide), α SKA (alpha skeletal actin), and β -MHC (beta myosin heavy chain) respectively. *, **, *** is significant compared to NTG mice. *P < 0.05; **P < 0.01; ***P < 0.001.

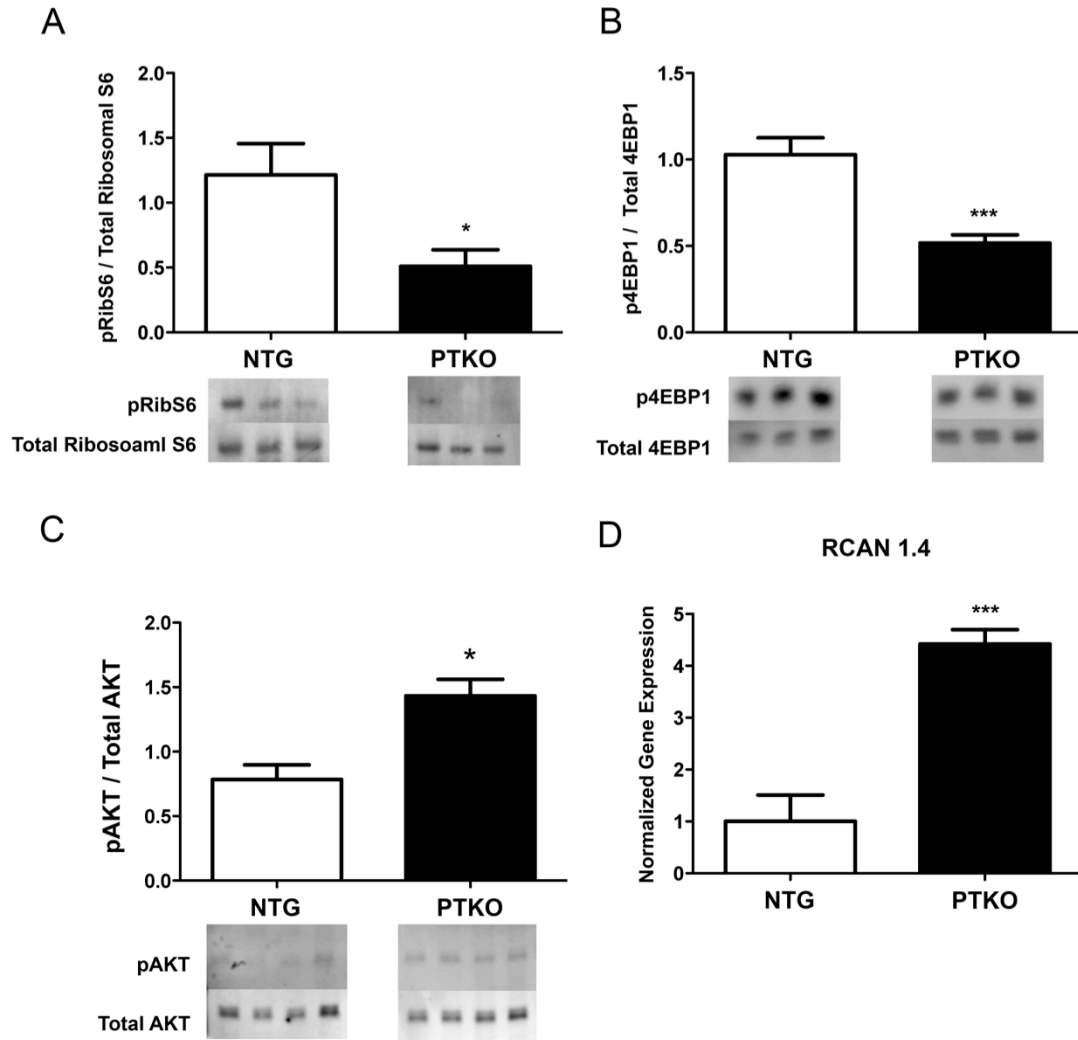


Figure 2.3: Hypertrophic pathways in PTKO mice.

A-B) Immunoblot of mTORC1 targets phosphorylated and total ribosomal S6 and phosphorylated and total 4EBP1 with loss of Pim. C) Phosphorylated Akt levels in PTKO mice assessed by immunoblot. D) Gene expression of RCAN1.4 in PTKO mice. n=6 NTG and n=6 PTKO. *, **, *** is significant compared to NTG. *p < 0.05; **p < 0.01; ***p < 0.001.

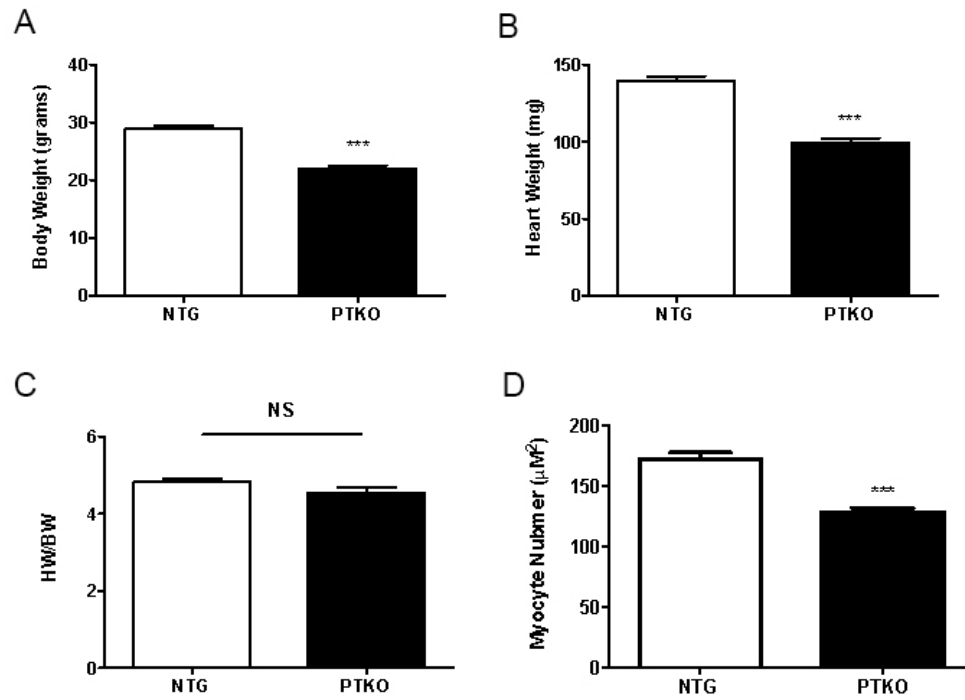


Figure 2.4: Biometric parameters in PTKO mice.

A-C) Body weight, heart weight, and heart weight to body weight ratio in NTG and PTKO mice at six months respectively. $n=8$ NTG and $n=9$ PTKO. D) Myocyte number in PTKO hearts assessed by confocal microscopy. $n=4$ NTG and $n=4$ PTKO. *** is significant compared to NTG mice at six months. *** $p < 0.001$.

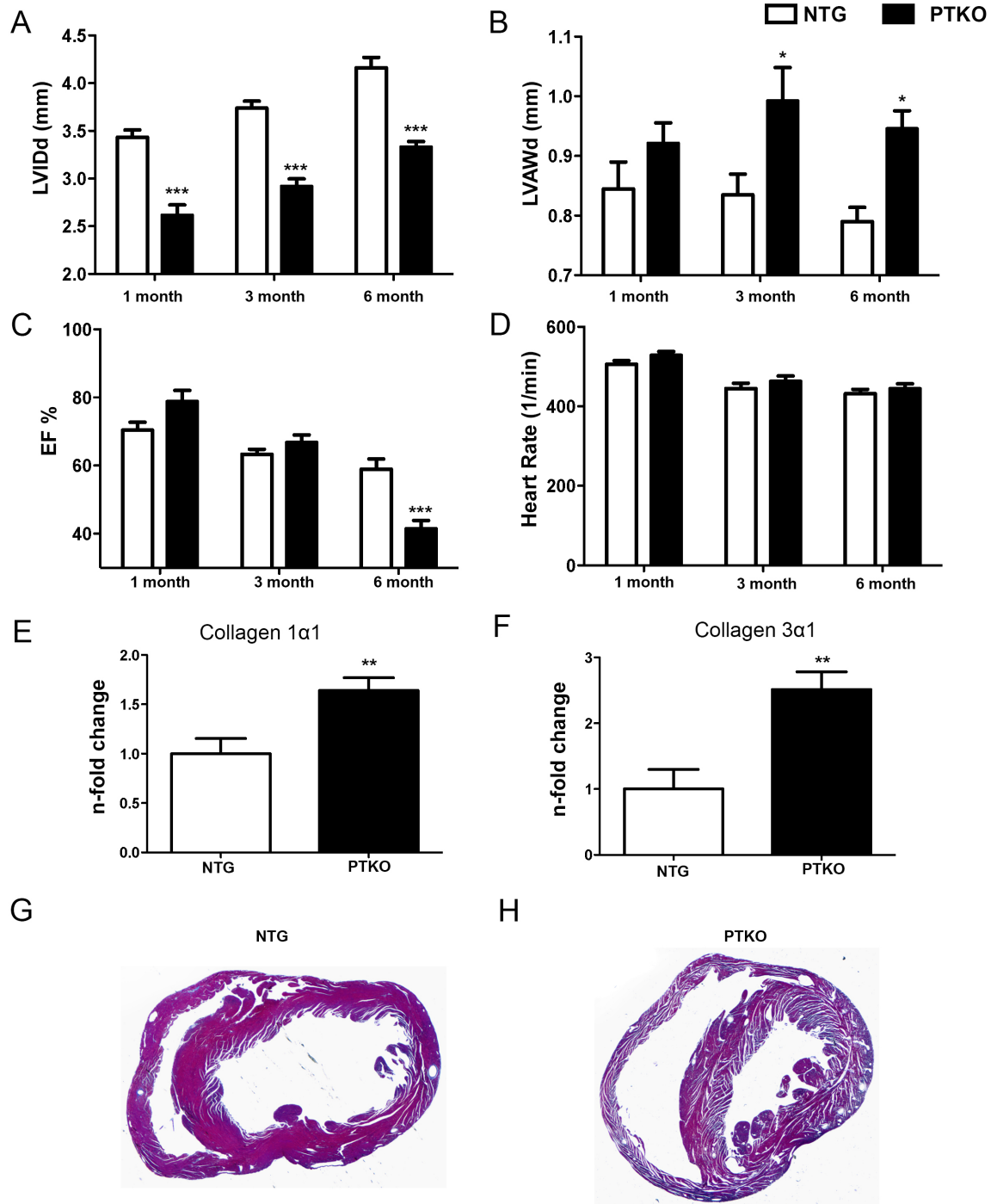


Figure 2.5: Premature cardiac failure at six months with Pim deletion.

A) Left ventricle inner diameter during diastole (LVIDd) in millimeters. B) Left ventricle anterior wall thickness during diastole (LVAWd) in millimeters. C) Percent ejection fraction. D) Average heart rate during echocardiography measurements. E-F) Gene expression analysis of collagen 1α1 and collagen 3α1 in cardiac tissue. G and H) Low magnification image of NTG and PTKO hearts with trichrome staining. n=8 NTG and n=9 PTKO. *, **, *** is significant compared to NTG mice at each individual time point. *P < 0.05; **P < 0.01; ***P < 0.001.

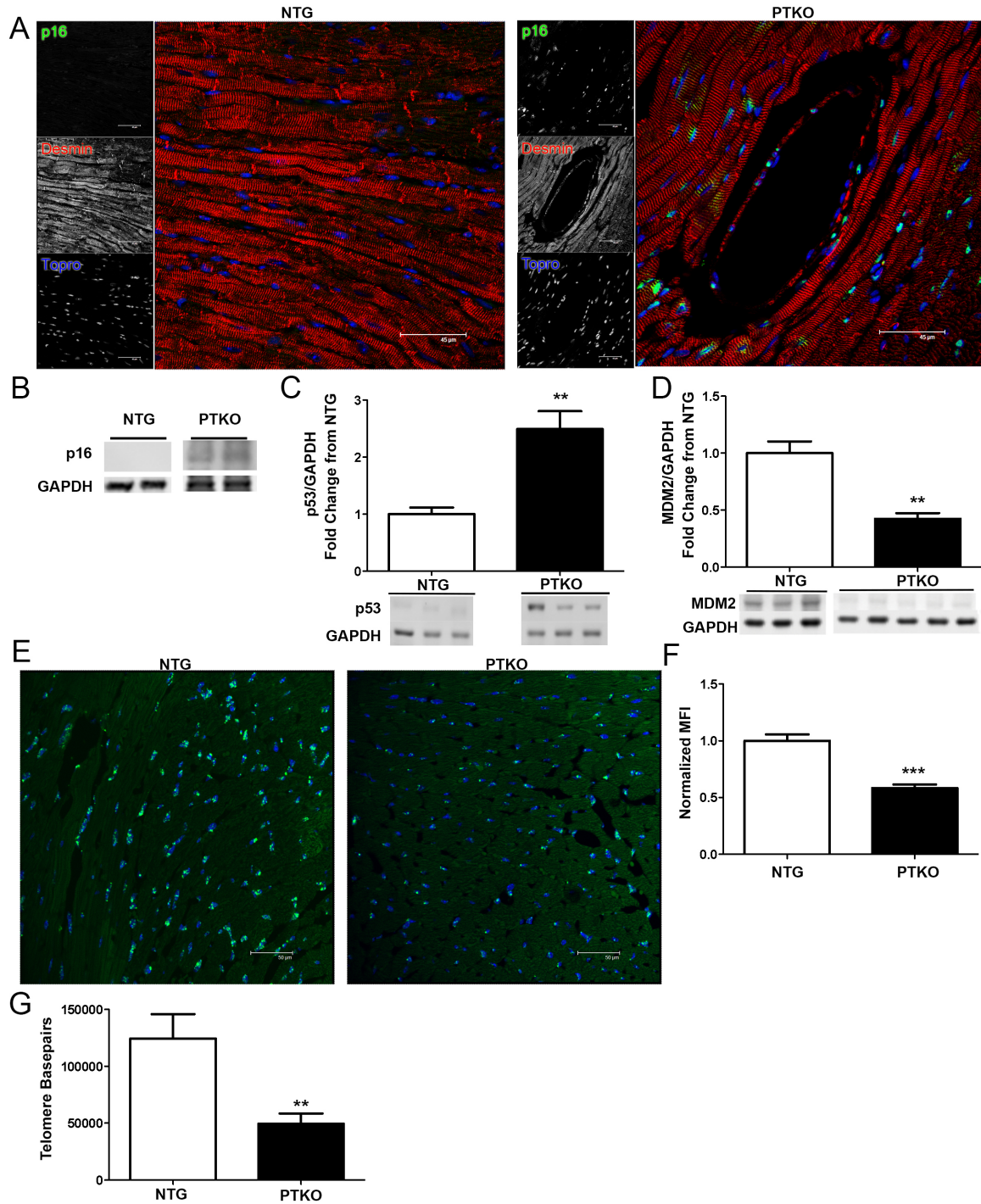


Figure 2.6: Cellular senescence in PTKO mice.

A) Confocal images of p16 staining in NTG and PTKO mouse hearts. B-D) Protein analysis of p16, p53, and MDM2 respectively in total cardiac lysates n=3 NTG and n=3 PTKO mice at 3 months. E & F) Representative Q-FISH images and quantification normalized to mean fluorescence intensity (MFI) in NTG (n=3) and PTKO (n=3) mice at 3 months. G) q-PCR measurements of telomere lengths in NTG (n=6) and PTKO (n=6) at one month. *, **, *** is significant compared to NTG mice. *P < 0.05; **P < 0.01; ***P < 0.001.

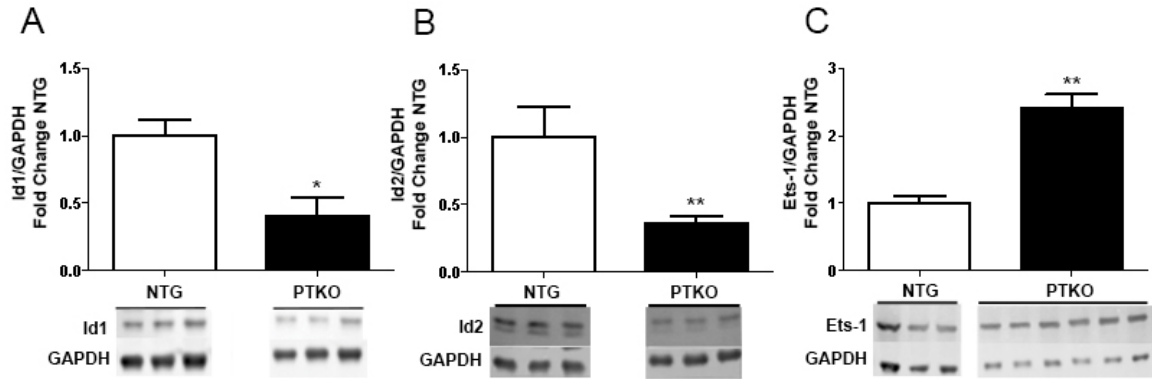


Figure 2.7: Pim deletion activates p16 pathway.

A & B) Immunoblot of p16 transcriptional repressors Id1 and Id2 in NTG (n=3) and PTKO (n=3) mice. C) Protein analysis of p16 transcriptional activator Ets-1 in NTG (n=3) and PTKO (n=4) cardiac lysates. * and ** is significant compared to NTG mice. *P < 0.05 and **P < 0.01.

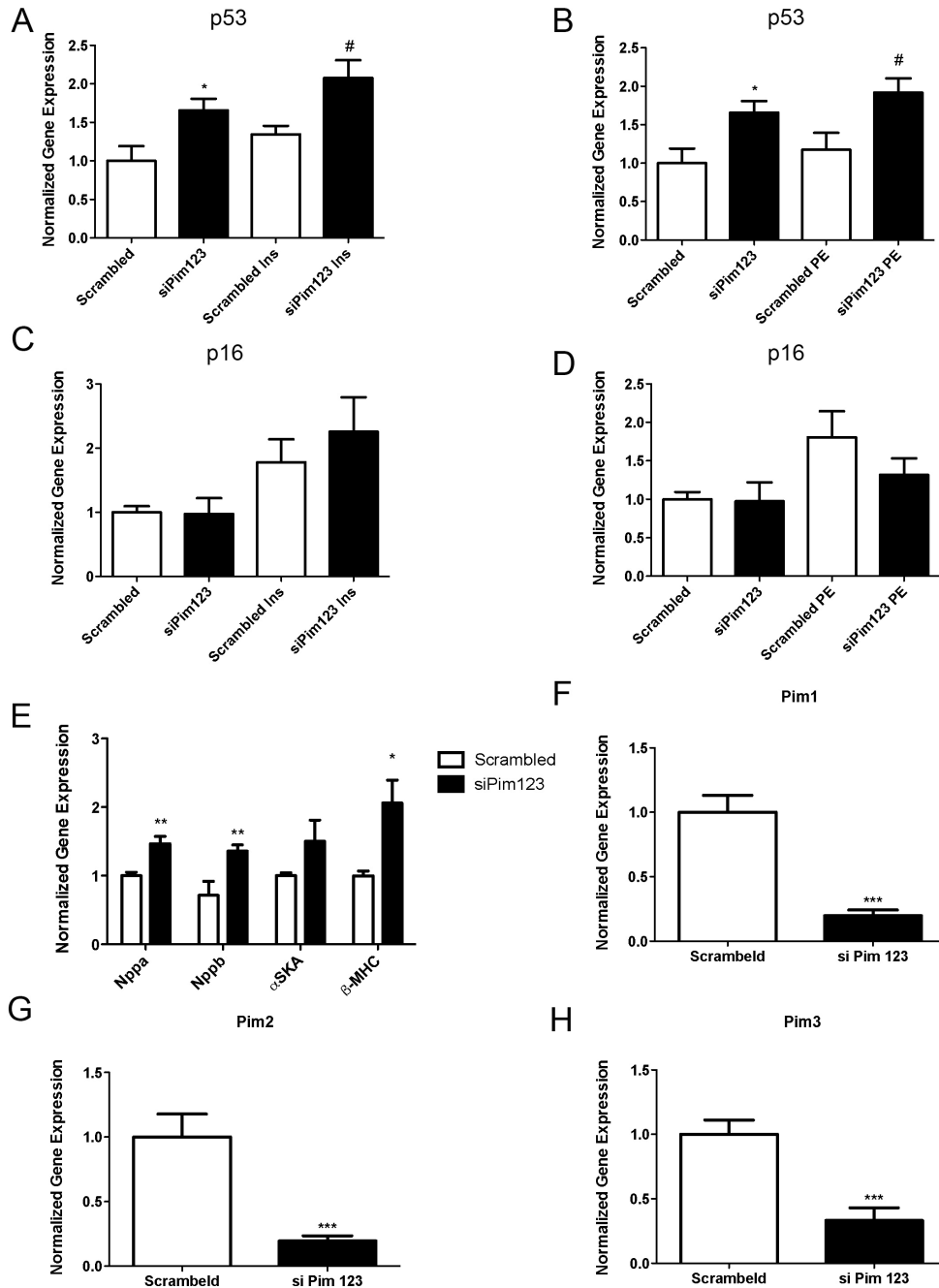


Figure 2.8: Increased senescence occurs in NRCMs with Pim kinase knockdown.

A&B) Gene expression of p53 in NRCMs with siRNA to Pim123 followed by insulin or phenylephrine stimulation respectively (n=4). C&D) mRNA analysis of p16 following Pim123 knockdown and growth stimulation (n=4). E) Analysis of fetal gene program in NRCMs with Pim kinase knockdown (n=4). F-H) Validation of siRNA efficiency to Pim kinases in NRCMs by quantitative PCR. *p < 0.05, **p < 0.01, and *** p<.001 when compared to scrambled alone. # p<.05 when compared to scrambled PE.

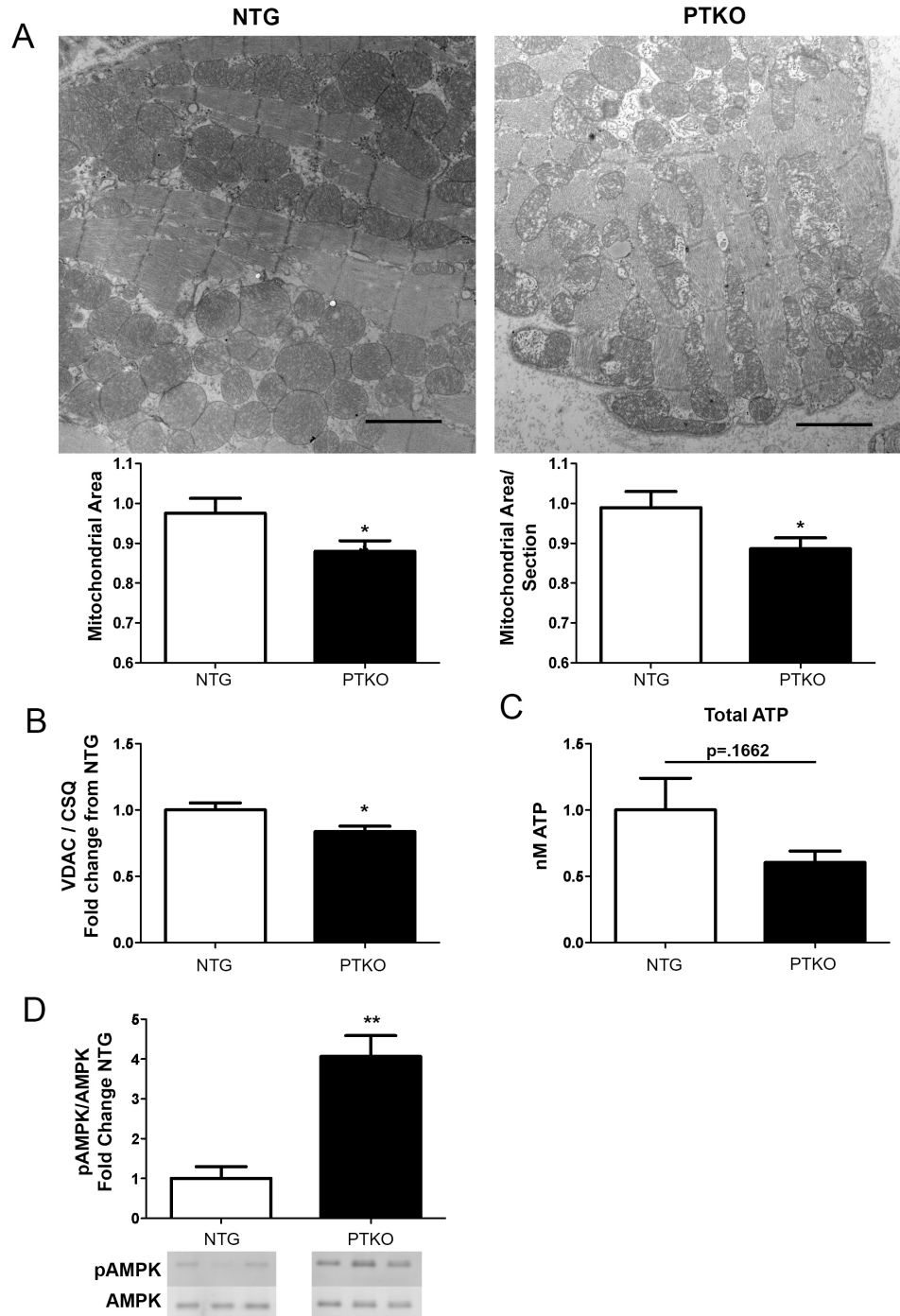


Figure 2.9: Mitochondrial morphological and functional aberrations with loss of Pim.

A) Electron microscopy scans of NTG (n=3) and PTKO (n=3) hearts at one month and quantification of mitochondrial area and mitochondrial area per section. B) Protein analysis of VDAC in NTG (n=4) and PTKO (n=4) mouse hearts. C) Total ATP levels in NTG (n=13) and PTKO (n=10) heart lysates. D) Immunoblot for pAMPK and total AMPK and analysis of pAMPK : AMPK ratio in cardiac samples of NTG (n=6) and PTKO (n=6) mice. * and ** is significant compared to NTG mice. *P < 0.05 and **P < 0.01.

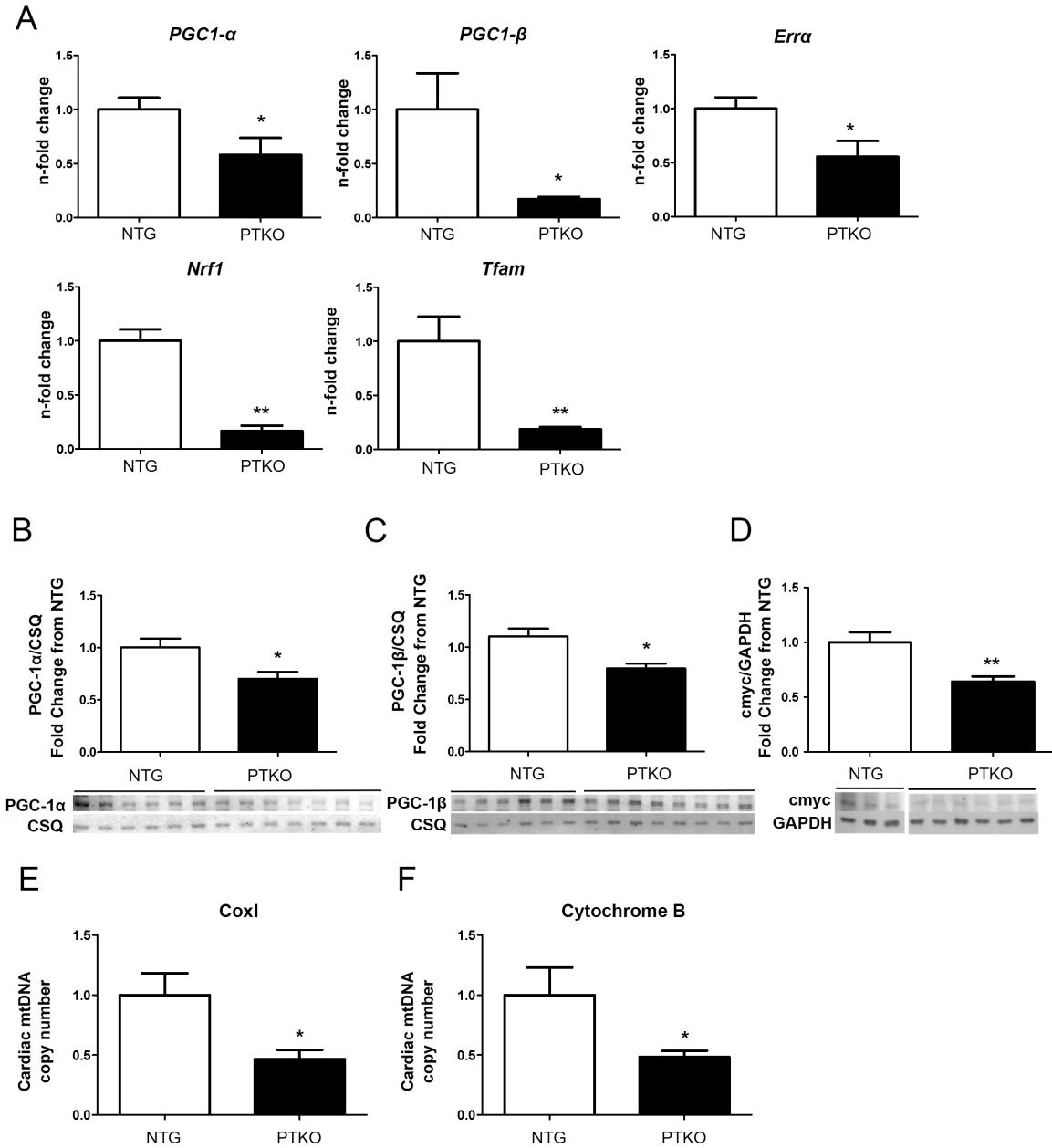


Figure 2.10: PPAR γ coactivators and downstream targets are decreased with Pim deletion.

A) mRNA levels of PGC-1 α , PGC-1 β , Err α , NRF1, and TFAM in 1 month NTG (n=6) and PTKO (n=6) hearts. B-D) Protein analysis of PGC-1 α , PGC-1 β , and c-Myc respectively in cardiac samples; NTG (n=6) and PTKO (n=7) hearts. E-F) Mitochondrial DNA analysis of Cox1 and Cytochrome B normalized to β -globin; NTG (n=4) and PTKO (n=4). * and ** is significant compared to NTG mice. *P < 0.05 and **P < 0.01.

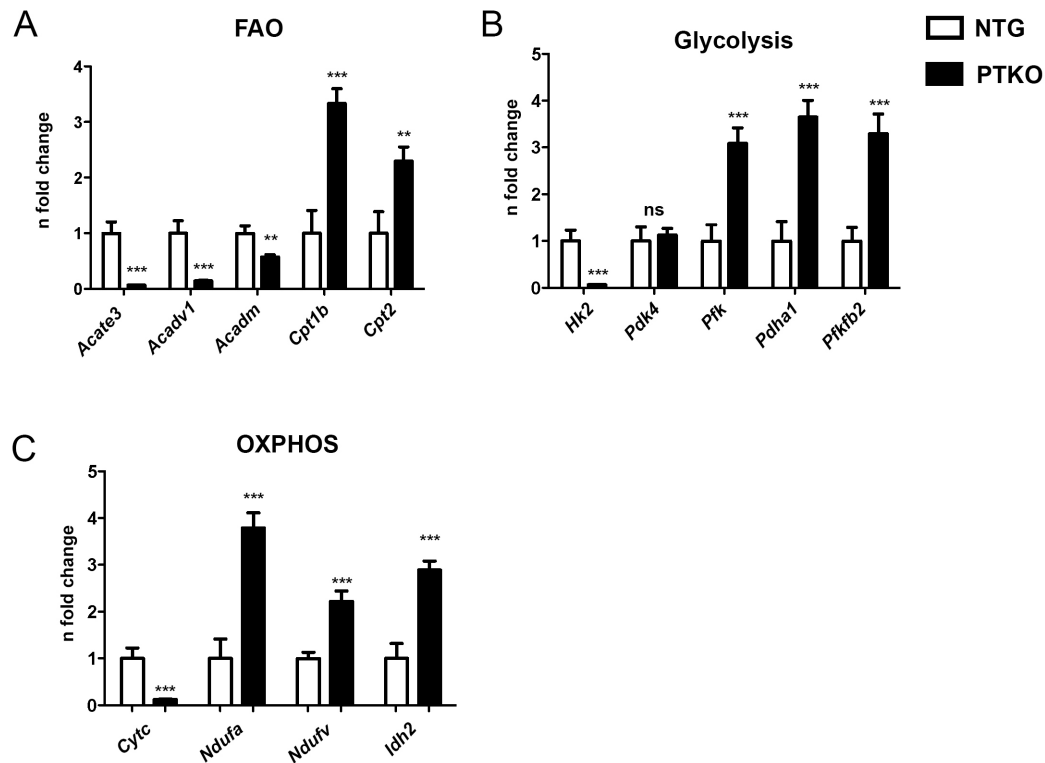


Figure 2.11: Alterations in metabolic gene expression profile ensue with loss of Pim kinases. mRNA levels of A) Fatty acid oxidation genes (Enzymes: Acate3, acyl coenzyme A thioesterase 3, mitochondrial; Acadv1, acyl coenzyme A long chain; Acadm, FAO-acetyl CoenzymeA dehydrogenase. Transporters: Cpt1b, carnitine palmitoyl transferase; Cpt2, carnitine palmitoyl transferase 2. B) Glycolysis genes (Hk2, hexokinase 2; Pdk4, pyruvate dehydrogenase kinase 4; Pfk, phosphofructokinase; PDHA1, pyruvate dehydrogenase E1 alpha 1; PFKFB2, (6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2). C) Oxidative Phosphorylation (OXPHOS) (Cytc, cytochrome c; Ndufa, NADH dehydrogenase 1 alpha subcomplex subunit 1; Ndufv, NADH dehydrogenase ubiquinone flavoprotein 1); Idh2, isocitrate dehydrogenase 2. n=6 NTG and n=6 PTKO. *, **, *** is significant compared to NTG mice. *P < 0.05; **P < 0.01; ***P < 0.001.

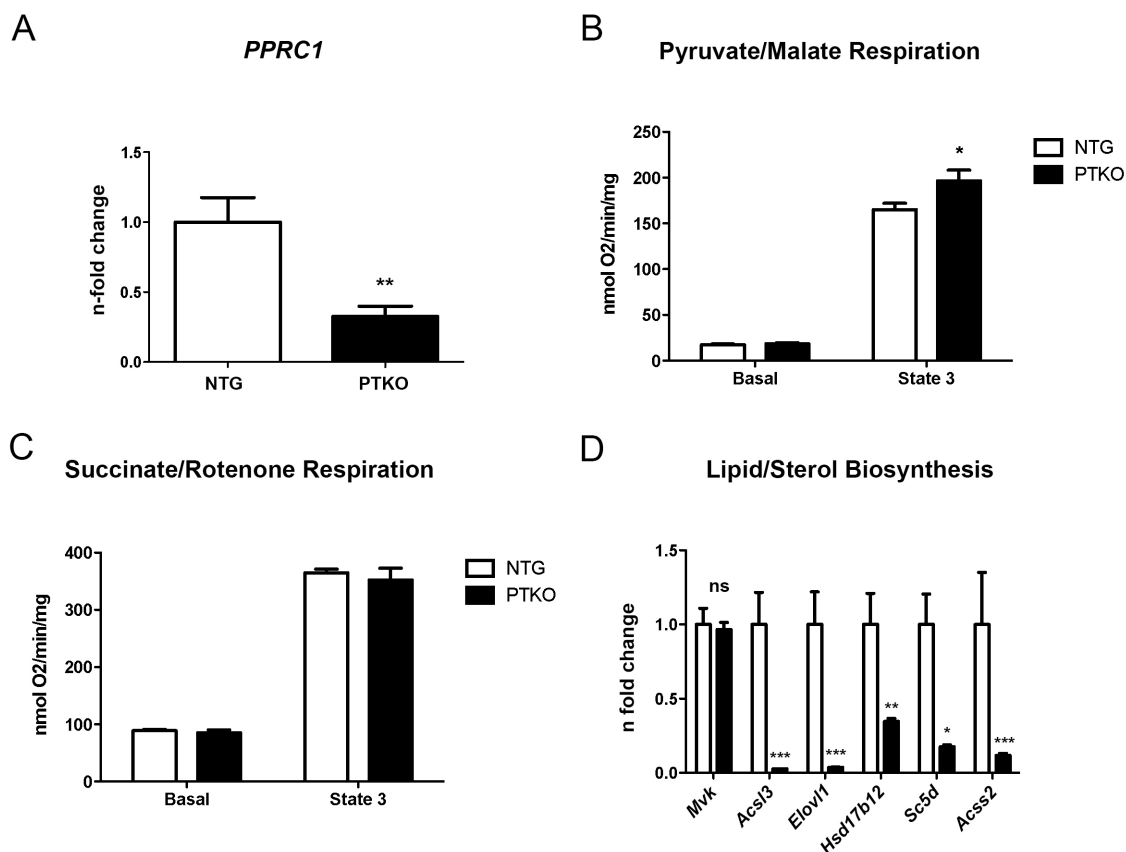


Figure 2.12: Functional mitochondrial alterations and reduced PPAR signaling in PTKO mice.

A and B) Mitochondrial respiration assay using glutamate and succinate as substrates in NTG (n=4) and PTKO (n=4) one month isolated mitochondria. C) mRNA levels of *PPRC1* in NTG (n=6) and PTKO mice (n=6) at one month D) mRNA analysis of genes downstream of PPAR signaling involved in lipid sterol biosynthesis in NTG (n=6) and PTKO (n=6) one month hearts. *, **, *** is significant compared to NTG. *P < 0.05; **P < 0.01; ***P < 0.001.

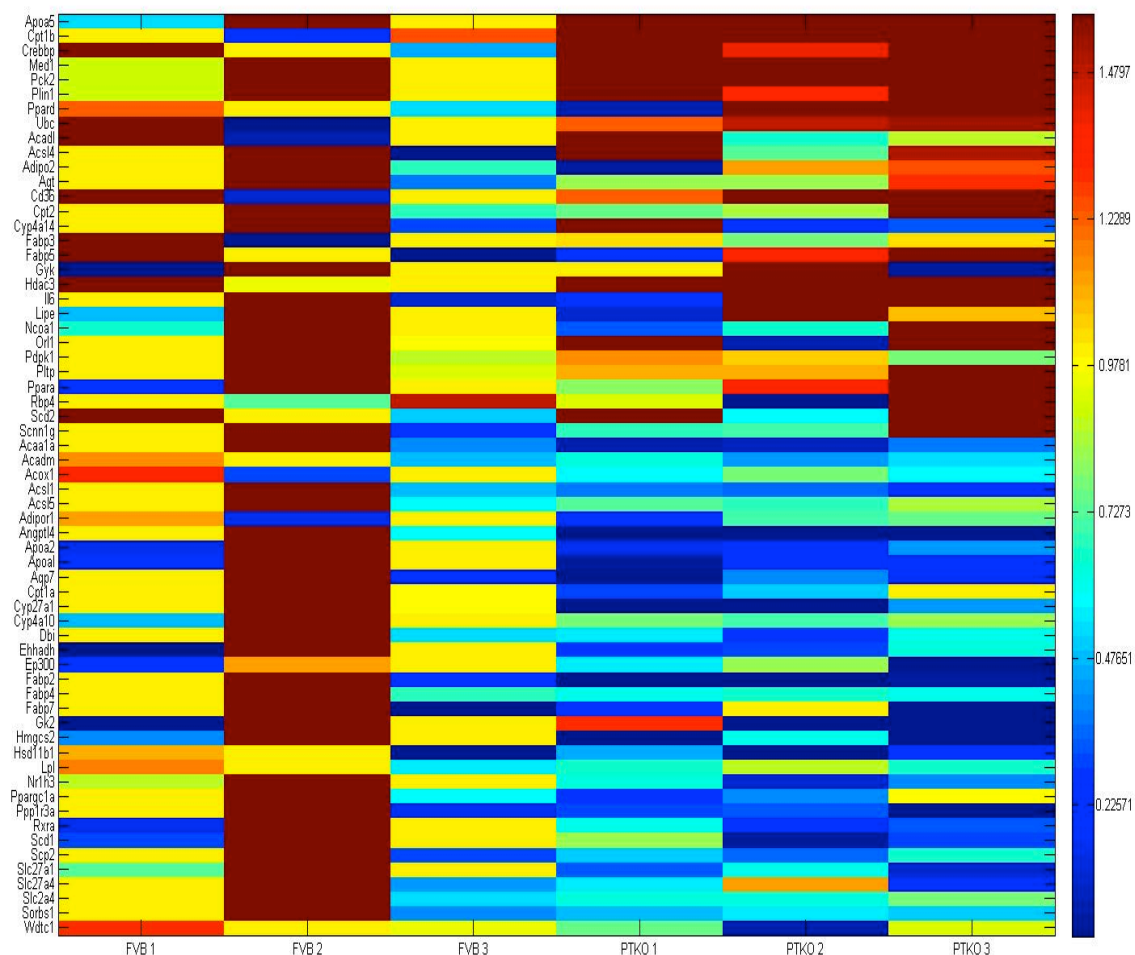


Figure 2.13: Microarray analysis of genes involved in PPAR signaling.

Microarray analysis of cardiac mRNA from NTG and PTKO mice at 1 month. 62 PPAR-related signaling genes were detected in cardiac tissue, 32 showed a downregulation, 21 genes remained unchanged, and 8 genes were induced. Three out of the four genes included in the targeted screen (Acadm, PGC-1 α , Cpt1b, and Cpt2) demonstrated similar results to our targeted approach. Acadm and PGC-1 α showed decreased gene expression, Cpt1b exhibited increased expression. Genes included in the microarray panel are involved in fatty acid oxidation, fatty acid transport, cholesterol synthesis and lipid/sterol biosynthesis.

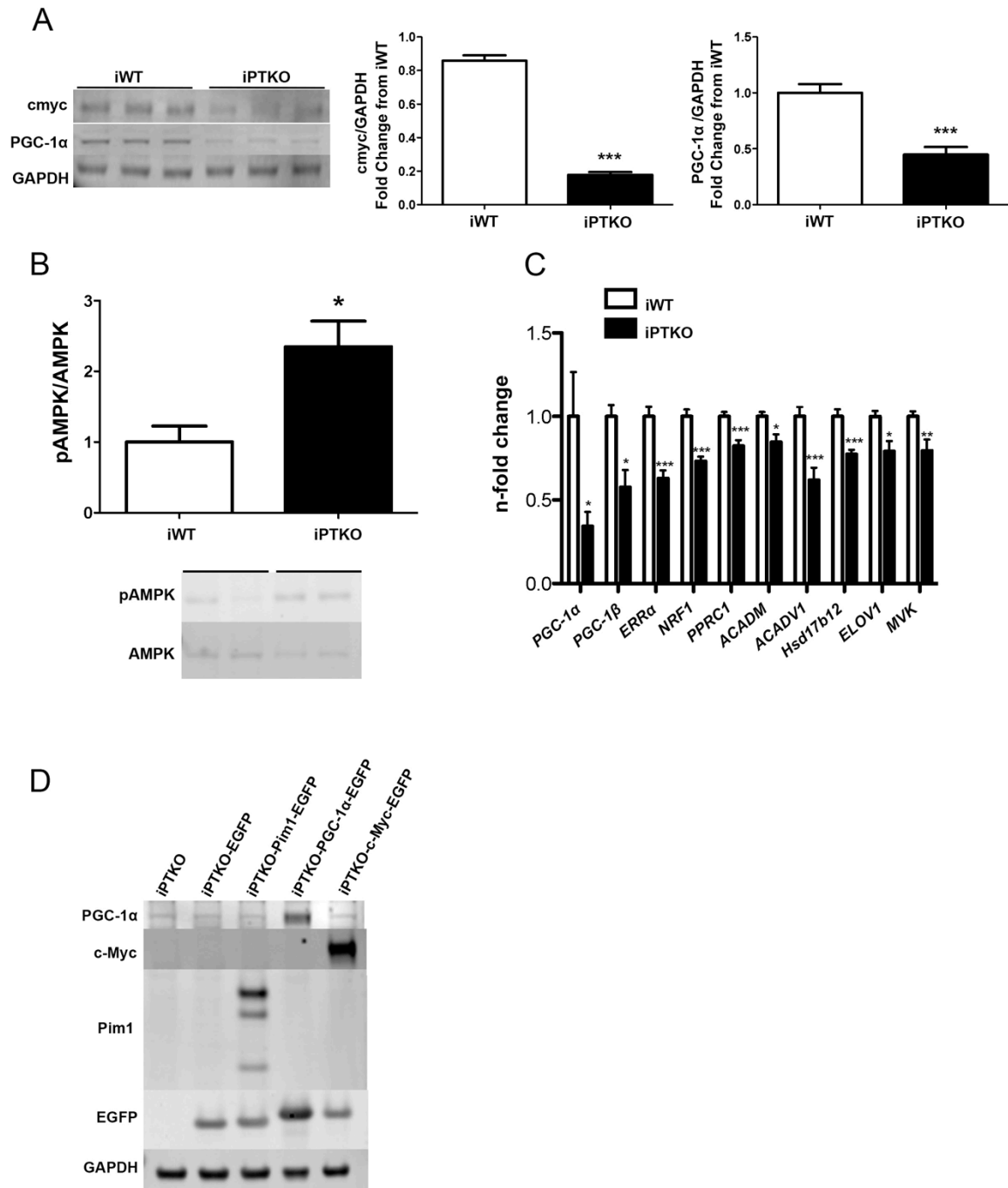


Figure 2.14: iPTKO MEFs demonstrate similar metabolic dysfunction as PTKO mice.

A) Immunoblot of c-Myc and PGC-1α in iWT MEFs and iPTKO MEFs (n=3) B) Phosphorylation levels of AMPK and quantification of pAMPK : AMPK in iPTKO MEFs compared to iWT MEFs (n=3). C) q-PCR of genes involved in the PPAR signaling circuit (n=3). D) Protein analysis of adenoviral overexpression of PGC-1α, c-Myc, Pim1, and EGFP. *, **, *** is significant compared to iWT MEFs. *p < 0.05; **p < 0.01; ***p < 0.001.

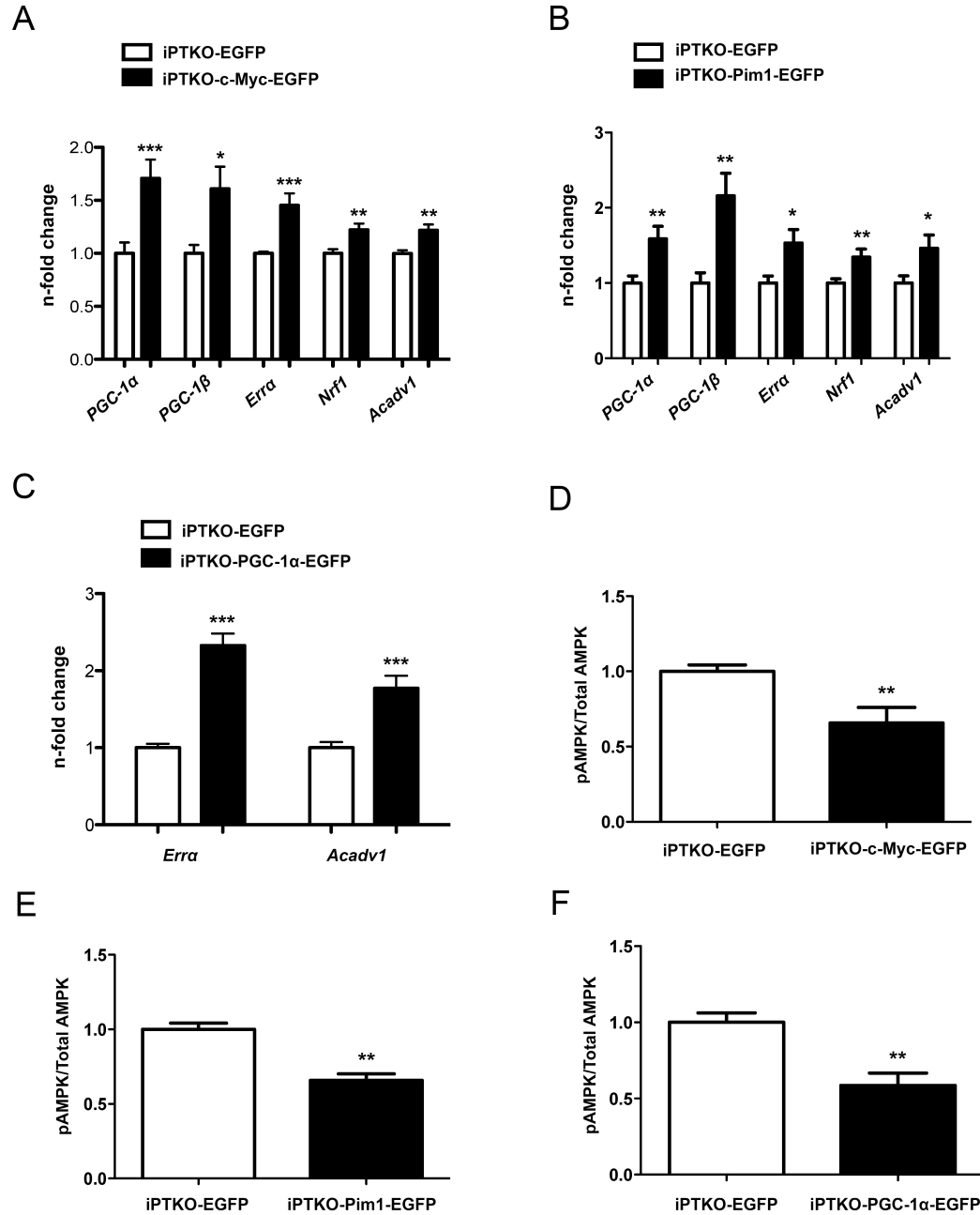


Figure 2.15: Rescue of metabolic genes with c-Myc, Pim1, and PGC-1α overexpression. Overexpression of c-Myc, Pim1, and PGC-1α adenovirus respectively in iPTKO MEFs followed by: A-C) qPCR of PPAR coactivators and downstream targets. D-F) Quantification of pAMPK : Total AMPK ratio. n=3 individual experiments and *, **, *** is significant compared to iPTKO MEFs infected with EGFP adenovirus mice. *P < 0.05; **P < 0.01; ***P < 0.001.

SUMMARY POINTS

- PTKO mice demonstrate hypertrophic remodeling and activation of the fetal gene program, which eventually leads to premature heart failure.
- Cellular senescence precedes heart failure as evidenced by increased expression of p16, p53, and telomere attrition in PTKO mice.
- Altered mitochondrial morphology and an energy starved phenotype occur with loss of Pim kinases
- Diminished expression of regulators of mitochondrial biogenesis and metabolism (PGC-1 α , PGC-1 β , and c-Myc) coincided with reduced mitochondrial DNA content.
- Disrupted PPAR signaling decreased enzymes involved in fatty acid oxidation and increased enzymes regulating glucose metabolism.
- Metabolic changes and energy homeostasis was restored with overexpression of c-Myc and PGC-1 α in immortalized Pim Triple Knockout MEFs.

Table 2.1: List of Antibodies

Application	Antibody	Dilution	Amplification	Company
Immunoblot	PGC-1 α	1:500	no	Calbiochem ST-1204
Immunoblot	PGC-1 β	1:500	no	R and D systems AF5656
Immunoblot	c-Myc	1:500	no	CST 9402
Immunoblot	p16	1:250	no	Santa Cruz sc-1661
Immunoblot	MDM2	1:500	no	Life Tech 337100
Immunoblot	P53	1:500	no	CST 9282
Immunoblot	Id1	1:250	no	Santa Cruz sc488
Immunoblot	Id2	1:250	no	Santa Cruz sc489
Immunoblot	Ets-1	1:250	no	Santa Cruz sc350
Immunoblot	pAMPK	1:500	no	CST 2535
Immunoblot	AMPK	1:500	no	CST 2532
IHC	p16	1:50	no	Santa Cruz sc-1661
IHC	Desmin	1:200	no	Abcam 15200
IHC	Wheat Germ Agglutinin	1:200	no	Life Tech W32464

**Table 2.2: List of Mouse Primers
5' to 3'**

PGC-1 α	For primer	TCAGGAGCTGGATGGCTTGGGA
Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha	Rev primer	ACCAACCAGGGCAGCACACTCTA
PGC-1 β	For primer	CTTTGCGGCACGGCAGGACT
Peroxisome proliferator-activated receptor-gamma coactivator 1 beta	Rev primer	TGGCCTGGGCTGAGCTTGGT
Err α	For primer	CAAGAGCATCCCAGGCTT
Estrogen-related receptor alpha	Rev primer	GCACTTCCATCCACACACTC
NRF1	For primer	GAACTGCCAACCACAGTCAC
Nuclear respiratory factor 1	Rev primer	TTTGTTCACCTCTCCATCA
TFAM	For primer	AGGCTTGGAAAAATCTGTCTC
Mitochondrial transcription factor A	Rev primer	TGCTCTTCCCAAGACTTCATT
Acate3	For primer	CAGAAGCCTGTTGAAGTTGGT
acyl-Coenzyme A thioesterase 3, mitochondrial	Rev primer	GACTTGAAATGTCGCTGTCC
Acadv1	For primer	ATCTCTGCCCAGCGACTTT
Aceyl coenzyme A long chain	Rev primer	TTCTGGCTTGTCCAGAACTG

**Table 2.2: List of Mouse Primers
5' to 3'**

Acadm FAO-acetyl CoenzymeA dehydrogenase	For primer	GGAAATGATCAACAAAAAAGAAGTATTT ATGGCCGCCACATCAGA
Cpt1b Carnatine palmitoyl transferase	Rev primer For primer	TCTAGGCAATGCCGTTTAC GAGCACATGGGCACCATAC
Cpt2 Carnatine palmitoyl transferase 2	Rev primer For primer	AGTATCTGCAGCACAGCATCGTA GGCTTCTGTGCACTGAGGTATCT
Hk2 Hexokinase 2	Rev primer For primer	TGCTACAGGTCCGAGCCA ATGCTGTCGTACACGTGC
Pfk Phosphofructokinase	Rev primer For primer	CGTTGAGGTAGGAATACTTCTGCA ACCTCTCCGAAAGGAGTGGA
Pdk4 Pyruvate dehydrogenase kinase 4	Rev primer For primer	CCGCTGTCCATGAAGCA GCAGAAAAGCAAAGGACGTT
Idh2 Isocitrate dehydrogenase 2	Rev primer For primer	CCCTATTGCCAGCATCTTTG TGTCCAGGAAGTCTGTGGTG
PDHA1 Pyruvate dehydrogenase E1 alpha 1	Rev primer For primer	GGGACGTCTGTTGAGAGAGC TGTGTCCATGGTAGCGGTAA
PFKFB2 6-phosphofructo-2-kinase/fructose-2,6- biphosphatase 2	Rev primer For primer	CGGGAATGGATCTACACTGG GGAGAGCAAAGTGAGGGATG
Cytc Cytochrome C	Rev primer For primer	ACCAAATCTCCACGGTCTGTT GGATTCTCAAATACTCCATCAG
Ndufa NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 9 (Ndufa9)	Rev primer For primer	ATCCCTTACCCTTTGCCACT CCGTAGCACCTCAATGGACT
	Rev primer	

**Table 2.2: List of Mouse Primers
5' to 3'**

Ndufv NADH dehydrogenase (ubiquinone) flavoprotein 1 (Ndufv1)	For primer	TGTGAGACCGTGCTAATGGA CATCTCCCTTCACAAATCGG
	Rev primer	
PPRC1 Peroxisome proliferator-activated receptor gamma coactivator-related protein 1	For primer	AATACCTGGCCGAATGACTC AGTTGTCACCTTGGACACGA
	Rev primer	
Mvk mevalonate kinase	For primer	GGGACGATGTCTTCCTTGAA GAACTTGGTCAGCCTGCTTC
	Rev primer	
Acsl3 Long-chain-fatty-acid—CoA ligase 3	For primer	CCAGCAGCTGCGTCAGGGTC TAAGACCCGCGGGCTCCGAC
	Rev primer	
Elov1 Fatty acid elongase 1	For primer	GCTCCAGGAGGAATGGGCTCC CACAGGGCCAAGGGCAGACA
	Rev primer	
Hsd17b12 hydroxysteroid (17-beta) dehydrogenase 12	For primer	GGGCCTTCCAGGTGTGGTGC GTGCCACCTGTAACAACCTGCCCA
	Rev primer	
Sc5d sterol-C5-desaturase	For primer	CCAAATGGCTGGATTTCATCT GTCCACAGGGTGAAAAGCAT
	Rev primer	
Acss2 acyl-CoA synthetase short-chain family member 2.	For primer	CTGTGGAGGAGCCACGGGAGTT TGGAGGAATGGGCCAGGGCAT
	Rev primer	
Nppa (ANP) Atrial natriuretic peptide	For primer	TGGGTCTTGTTAGGGCTCAAACCT TGAAACTCAAGGGACACCCATCGT
	Rev primer	
Nppb (BNP) Brain natriuretic peptide	For primer	AATGGCCCAGAGACAGCTCTTGAA CTTGTGCCCCAAAGCAGCTTGAGAT
	Rev primer	
α SKA alpha skeletal actin	For primer	CGCCAGCCTCTGAAACTAGA AGCCGTTGTACACACAAGA
	Rev primer	

**Table 2.2: List of Mouse Primers
5' to 3'**

Myh7, β -MHC	For	GAGCCTTGGATTCTCAAACG
Beta Myosin Heavy Chain	primer	
	Rev	GTGGCTCCGAGAAAGGAAG
	primer	
Primers for quantification of Mitochondrial DNA		
CoxI (mitochondrial)	For	CTGAGCGGGAATAGTGGGTA
	primer	
	Rev	TGGGGCTCCGATTATTAGTG
	primer	
Cytochrome B (mitochondrial)	For	ATTCCTTCATGTCGGACGAG
	primer	
	Rev	ACTGAGAAGCCCCCTCAAAT
	primer	
H19 (nuclear)	For	GTCCACGAGACCAATGACTG
	primer	
	Rev	GTACCCACCTGTCGTCC
	primer	
B1 globin (nuclear)	For	GCACCTGACTGATGCTGAGAA
	primer	
	Rev	TTCATCGGCGTTACCTTTCC
	primer	

CONCLUSION OF THE DISSERTATION

Heart disease is an overall term for many pathologies associated with detrimental changes of the myocardium including ischemic heart disease and pathological hypertrophy. The heart is dependent on mitochondrial function and integrity for cellular homeostasis and cardiac performance. Mitochondrial dysfunction occurs with aging and pathological challenge, conditioning the heart toward acquiring cardiovascular disease. However, it is controversial whether mitochondrial functional decline is the casual or secondary mechanism leading to heart disease. Nonetheless, numerous reports validate salutary effects mitigating mitochondrial bioenergetics to protect against pathological cardiac injury, emphasizing the importance of maintaining mitochondrial function and metabolism to prevent age related changes (11, 120).

As heart failure progresses with age it is important to consider proteins regulating signaling pathways associated with decreased activity during aging as these signaling pathways are phenotypic of a young or youthful state. Expression and activity of Pim kinases diminish during aging and are reactivated after injury (52, 53). Furthermore, overexpression of Pim promotes a hyperplastic heart with an increased number of myocytes, whereas loss of Pim exacerbates ischemic injury and promotes hypertrophic pathological remodeling (53). The Pim kinase signaling cascade is namely involved in survival and proliferation (59, 62), however the influence of Pim kinases on mitochondrial proteins and integrity provides clues of potential beneficial effects upon disease pathology through preserving the mitochondrial compartment (16, 65).

Mitochondrial dynamics maintains a fine balance between fission and fusion processes and contributes to ischemic heart disease when deregulated (15, 121). Alterations in mitochondrial morphology eventually lead to a disruption in mitochondrial function, further aggravating ischemic injury (8). As such, our data demonstrates the

translocation of Drp1 from the cytosol to the mitochondria during ischemic injury promoting excess mitochondrial fission (98). This evidence leads to the postulate that Drp1 translocation and fission are important facets effecting cardiac survival. Pim1 directly binds and phosphorylates Drp1 and phosphorylation of serine 637 promotes Drp1 cytosolic sequestration (Figure 1.8) (26). Introduction of Drp1 S637A and Pim1 overexpression did not rescue increased mitochondrial fission following ischemia, providing evidence of Pim1 phosphorylation occurring at serine 637 on Drp1 (Figure 1.9). Interestingly, Pim1 also demonstrated regulation of total Drp1 protein levels, although the mechanism for this is not completely understood from our data (Figure 1.3 C&F; Figure 1.4 A&B). However, from our data we can conclude Pim1 does not promote degradation of Drp1 and degradation of Drp1 occurs through the proteasome (Figure 1.5). Therefore, Pim1 regulation of total Drp1 may occur through transcriptional repression or selective translational control.

Pim kinases prevent cellular apoptosis mainly by promoting the upregulation of mitochondrial protective proteins Bcl-XL and Bcl-2 in addition to inhibiting the activity of proapoptotic proteins Bad and Bax (16, 53). Pim overexpression prevents the translocation of Bax and Bak to the mitochondria inhibiting mitochondrial outer membrane permeabilization (16). Decreased PUMA protein levels are seen with overexpression of Pim1 during ischemia, providing further insight into the repression of the apoptotic cascade by Pim1 (Figure 1.11). PUMA protein expression at baseline is equivalent to control with Pim1 overexpression; however PDN NRCMs display increased mitochondrial PUMA at baseline (Figure 1.11 B). These data indicate the complex regulation of PUMA by Pim1, which may include increased protein turnover, or activation of a repressor preventing PUMA upregulation during ischemic challenge.

Pim kinases impact mitochondrial biology through the regulation of metabolism and ATP synthesis by Pim3 (62). Our data confirmed this link by demonstrating the loss of PGC-1 α and PGC-1 β , regulators of mitochondrial biogenesis and metabolism, in cardiac tissue of PTKO mice (Figure 2.6). Loss of PGC-1 α coincided with reduced activation of the mitochondrial biogenesis program, essentially ablating the ability to produce additional mitochondria as evidenced by decreased mitochondrial DNA content (Figure 2.6).

Loss of Pim kinases leads to cardiac hypertrophy and heart failure (Figure 2.1 and 2.2) (52). Properly functioning adult cardiomyocytes utilize fatty acid oxidation to produce cellular energy, however pathological cardiac hypertrophy promotes a metabolic fuel shift away from fatty acid oxidation toward glucose utilization, and metabolic changes are shown as potent regulators of the aging phenotype (14, 43). Pathological hypertrophy correlates with loss of PGC-1 α , exacerbating a metabolic shift to a glycolytic program over fatty acid oxidation as shown in Figure 2.7. Collectively, reductions in mitochondrial biogenesis and metabolism induce an energy-starved state with loss of Pim kinases. However, not clear with the data presented here is the sequence of events leading to cardiac failure. As both loss of PGC-1 α and pathological hypertrophic remodeling promote the development of cardiomyopathy independently, hypertrophic remodeling also leads to decrease in PGC-1 α .

c-Myc, a target of Pim kinases, further bolsters the notion that Pim kinases ability to protect against adverse mitochondrial changes during aging (55, 64). c-Myc promotes mitochondrial biogenesis and maintains metabolic processes. In addition, c-Myc expression and activity are enhanced by Pim kinase. c-Myc activity is also necessary for cell cycle progression, and loss of Myc diminishes proliferative potential, possibly leading

to cellular senescence. Figure 2.4 confirms cellular senescence in PTKO mice with upregulation of p16, p53 and shortened telomere lengths.

Interestingly, an established link between mitochondrial dynamics and the PGC-1 α signaling circuit, together with data presented here demonstrating regulation of mitochondrial dynamics and metabolism by Pim kinases, suggest a central role for Pim kinases in mitochondrial signaling (122). PGC-1 α and its downstream target gene product, estrogen-related receptor- α (ERR α), stimulate the expression of Mfn2, and reduction in Mfn2 increased Drp1 and mitochondrial fission (25). Furthermore, loss of Mfn2 is shown to decrease oxygen consumption and the expression of oxidative phosphorylation proteins (123, 124). These data also provide an interesting facet of investigating Pim kinase effect upon mitochondrial fusion upregulation to antagonize fission during ischemia.

Aging is becoming increasingly relevant as a clinical indicator of cardiovascular disease. Therefore it becomes necessary to understand the underlying mechanisms of aging. Pim kinases expression is regulated during aging and myocardial pathologies (Figure 2.1). Pim1 expression, the main cardiac isoform, diminished with aging while Pim2 expression changed very little and Pim3 was upregulated (Figure 2.1). During myocardial infarction Pim kinase expression was initially upregulated followed by downregulation shortly thereafter (Figure 2.1). Conversely, trans aortic constriction led to decreased expression of Pim1,2, and 3 (Figure 2.1). Collectively, these data imply that diminished Pim may be a precipitating factor in heart disease. The mechanisms by which Pim kinases protein and activity levels diminish are unknown. However, it can be speculated that Pim kinase protein levels are short lived (57) and during cardiac insult energy expensive mechanisms such as protein synthesis are shut down in order to use ATP to maintain only basic cellular function.

Surmounting evidence elucidate that cardiac disease including myocardial infarction (125), hypertrophy (14, 45), and ischemia (15) is exacerbated by mitochondrial functional derangements. Therefore, molecular interventional strategies to prevent the deterioration of mitochondrial function and consequently averting heart failure are clinically necessary. Reexamination of the data in this thesis essentially leads to the conclusion that Pim signaling is integral to maintain mitochondrial dynamics and metabolism to prevent cardiac aging and disease. Coupled with reports demonstrating the ability of Pim1 to rejuvenate the aging phenotype by decreasing cell cycle inhibitors and increasing telomere lengths, bodes well for the argument to utilize Pim kinases as a therapeutic tool to prevent mitochondrial alternations coincident with cardiac aging (101).

To extend the findings within this thesis, future studies should determine a relatively safe and effective method of activating endogenous or delivering exogenous Pim kinase. Activation of endogenous expression of Pim kinases provides a promising therapeutic intervention. Employing a Pim1 reporter construct for high throughput screening of small molecules could potentially lead to drug targets that activate Pim1 expression. Furthermore, growth factors such as interleukins, leukemia inhibitory factor (LIF), and epidermal growth factor (EGF) that activate Pim expression through the Jack/STAT could be employed to activate endogenous Pim expression (54, 57). However, one limitation to small molecule and growth factor stimulation is a whole body stimulation of Pim kinases instead of a cardiac specific overexpression. In addition to activation of endogenous Pim expression, direct protein delivery systems are being explored as an efficient and effective therapeutic tool. Transcription and translation into protein are not rapid processes and direct protein delivery is a valid option. Fusion of Pim to peptides, which readily cross the membrane barrier, displaying functional Pim kinase activity have been established in the hematopoietic system (126). The trans

activator of transcription (TAT) domain of HIV contains a protein transduction domain and can be fused to Pim for cellular expression (126). Purified TAT Pim can subsequently be delivered to the myocardium. One relatively new concept is a cardiac specific peptide that can be attached to Pim as a protein following delivery to the myocardium by intravenous injection, achieving rapid expression (127). Furthermore, inducible vector expression systems are also being investigated and could potentially be a promising tool for delivering Pim kinases. Targeted viral vectors are also under investigation to deliver Pim in the heart. Adeno associated virus 9 and 6 vectors have cardiac tropism allowing cardiac specific expression (128). Although numerous options are available to overexpress Pim kinases in the heart, a challenging hurdle is regulated and targeted cardiac expression, which will need to be addressed in future studies.

A cure for cardiovascular disease still remains elusive, however several lines of evidence demonstrate the importance of maintaining mitochondrial function to antagonize aging and subsequent heart failure (12, 45, 65). Previous published data demonstrates the prosurvival effects of Pim overexpression in a cardiac context, however exact mechanism of protection remain to be determined (52, 53, 100). The data presented here provide convincing evidence of Pim kinase ability to antagonize insults to the mitochondrial compartment. Our results provide a platform for future studies to investigate pathways regulating mechanisms of mitochondrial regulation by Pim kinases that may be able to further advance the concept of employing Pim as a therapeutic tool.

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