THE PROSURVIVAL ACTIVITIES OF APOPTOSIS-INDUCING FACTOR CONVERGE UPON THE REGULATION OF MITOCHONDRIAL METABOLISM, A NOVEL CELL DEATH PATHWAY, AND THE PROGRESSION OF PROSTATE CANCER

BY

AUDREY M. LENHAUSEN

A Dissertation Submitted to the Graduate Faculty of
WAKE FOREST UNIVERSITY GRADUATE SCHOOL OF ARTS AND SCIENCES
in Partial Fulfillment of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY
Biochemistry and Molecular Biology

December 2013
Winston-Salem, North Carolina

Approved By:
John Wilkinson, Ph.D., Advisor
Steve Kridel, Ph.D., Chair
Larry Daniel, Ph.D.
Leslie Poole, Ph.D.
Yuh-Hwa Wang, Ph.D.
ACKNOWLEDGEMENTS

The first of acknowledgements typically go to a spouse for being there day in and day out, to parents for having given encouragement and inspiration throughout a lifetime, or to friends for providing the drinks and laughter necessary to make it through graduate school. While I too would like to give thanks and love to all those people in my life, my first recognition has to go to my advisor, John Wilkinson. Over the past few years I have seen and heard the horror stories of being a graduate student, and upon coming to the close of my graduate career, I have come to the realization that I have been extremely fortunate in having you as my mentor. I thank you for the scientific guidance and encouragement you have provided over the years, for teaching me how to “work smart, not hard”, and for allowing me to take a break when I needed to refuel. I have nothing but confidence in your abilities as a mentor and a scientist, and I wish you the best of luck in your future endeavors!

I also would like to thank my mother for my personality and optimism, my aunt for her support and encouragement, my friends for not abandoning me, my boyfriend for making me laugh everyday, and Amanda Wilkinson for making lab less mundane. Your love, support, encouragement and conversation have all contributed to my success as a graduate student.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>ii</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>vi</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ix</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>I. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>II. The impact of AIF upon regulation of the mitochondrial proteome</td>
<td>14</td>
</tr>
<tr>
<td>III. AIF in the regulation of a novel, PGAM5-mediated cell death</td>
<td>42</td>
</tr>
<tr>
<td>pathway displaying characteristics of mitophagy</td>
<td></td>
</tr>
<tr>
<td>IV. Development of an in vivo tissue recombination approach to study</td>
<td>82</td>
</tr>
<tr>
<td>the temporal impact of AIF upon the progression of prostate cancer</td>
<td></td>
</tr>
<tr>
<td>V. SUMMARY (conclusions)</td>
<td>95</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>104</td>
</tr>
<tr>
<td>CURRICULUM VITAE</td>
<td>116</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Novel AIF binding partners identified by TAP screen</td>
<td>26</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>FIGURE 1: Major forms and splice variants of AIF.</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>24</td>
</tr>
<tr>
<td>FIGURE 2: TAP screen.</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>27</td>
</tr>
<tr>
<td>FIGURE 3: Lentiviral knockdown of AIF in MEF, MDA, and HeLa cell lines.</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>29</td>
</tr>
<tr>
<td>FIGURE 4: Establishing the role of AIF in mitochondrial protein import.</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>32</td>
</tr>
<tr>
<td>FIGURE 5: RT-PCR of nuclear- and mitochondrial-encoded mRNA in LNCAP and PC3 cell lines.</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>34</td>
</tr>
<tr>
<td>FIGURE 6: Mitochondrial protein synthesis in AIF proficient and deficient HeLa cells.</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>36</td>
</tr>
<tr>
<td>FIGURE 7: AIF does not play a role in mitochondrial protein acetylation.</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>53</td>
</tr>
<tr>
<td>FIGURE 8: PGAM5S and PGAM5L associate with AIF.</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>55</td>
</tr>
<tr>
<td>FIGURE 9: PGAM5S and PGAM5L promote intrinsic cell death.</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>57</td>
</tr>
<tr>
<td>FIGURE 10: Labeling and extraction of PGAM5S and PGAM5L.</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>59</td>
</tr>
<tr>
<td>FIGURE 11: PGAM5S and PGAM5L promote caspase-independent cell death.</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>60</td>
</tr>
<tr>
<td>FIGURE 12: AIF is not required for PGAM5S and PGAM5L to promote cell death.</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>62</td>
</tr>
<tr>
<td>FIGURE 13: AIF inhibits caspase activation following PGAM5 upregulation.</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>63</td>
</tr>
<tr>
<td>FIGURE 14: The upregulation PGAM5S and PGAM5L does not activate ASK1-mediated MAPK pathway.</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>65</td>
</tr>
<tr>
<td>FIGURE 15: PGAM5L phosphatase activity is not relevant to cell death function.</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>67</td>
</tr>
<tr>
<td>FIGURE 16: PGAM5S phosphatase activity remains undetected.</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>68</td>
</tr>
<tr>
<td>FIGURE 17: A novel interaction between ASK1 and AIF.</td>
<td></td>
</tr>
</tbody>
</table>
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>Point mutations disrupt PGAM$_S$ and PGAM$_L$ interaction with Keap1 and Bcl-X$_L$</td>
</tr>
<tr>
<td>19</td>
<td>PGAM$_S$ and PGAM$_L$ interaction with Keap1 and Bcl-XL is unrelated to cell death activity</td>
</tr>
<tr>
<td>20</td>
<td>Transmission electron microscopy (TEM) images of healthy mitochondria</td>
</tr>
<tr>
<td>21</td>
<td>PGAM$_S$ promotes fragmented mitochondria and abnormal cristae formation</td>
</tr>
<tr>
<td>22</td>
<td>PGAM$_S$ promotes aggregation of mitochondria, abnormal cristae formation and disruption of OMM</td>
</tr>
<tr>
<td>23</td>
<td>PGAM$_L$ promotes aggregation of mitochondria and abnormal cristae formation</td>
</tr>
<tr>
<td>24</td>
<td>Characterization of AIF deficient mouse prostatic epithelial stem cells</td>
</tr>
</tbody>
</table>
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACD</td>
<td>Autophagic Cell Death</td>
</tr>
<tr>
<td>AIF</td>
<td>Apoptosis-Inducing Factor</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic Lateral Sclerosis</td>
</tr>
<tr>
<td>ASK1</td>
<td>Apoptosis-Signal regulating Kinase 1</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>Cyto c</td>
<td>Cytochrome c</td>
</tr>
<tr>
<td>DISC</td>
<td>Death-Inducing Signaling Complex</td>
</tr>
<tr>
<td>EF-Tu</td>
<td>Elongation Factor Tu</td>
</tr>
<tr>
<td>ES</td>
<td>Embryonic Stem</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron Transport Chain</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin Adenine Dinucleotide</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-Associated protein with Death Domain</td>
</tr>
<tr>
<td>GEMM</td>
<td>Genetically Engineered Mouse Model</td>
</tr>
<tr>
<td>Hq</td>
<td>Harlequin mice</td>
</tr>
<tr>
<td>Hsp70</td>
<td>Heat Shock Protein 70 (mt)</td>
</tr>
<tr>
<td>IAP</td>
<td>Inhibitor of Apoptosis Protein</td>
</tr>
<tr>
<td>IMM</td>
<td>Inner Mitochondrial Membrane</td>
</tr>
<tr>
<td>IMS</td>
<td>Inner Mitochondrial Space</td>
</tr>
<tr>
<td>MLS</td>
<td>Mitochondrial Localization Signal</td>
</tr>
<tr>
<td>MMP</td>
<td>Mitochondrial Membrane Permeabilization</td>
</tr>
<tr>
<td>MPSC</td>
<td>Mouse Prostate Stem Cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Long Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide Adenine Dinucleotide</td>
</tr>
<tr>
<td>OMM</td>
<td>Outer Mitochondrial Membrane</td>
</tr>
<tr>
<td>PCa</td>
<td>Prostate Cancer</td>
</tr>
<tr>
<td>PGAM5</td>
<td>Phosphoglycerate Mutase family member 5</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>PINK1</td>
<td>PTEN-Induced protein Kinase 1</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and Tensin homolog</td>
</tr>
<tr>
<td>PTM</td>
<td>Post-translational Modification</td>
</tr>
<tr>
<td>RIP1</td>
<td>Receptor-Interacting Protein kinase 1</td>
</tr>
<tr>
<td>RIP3</td>
<td>Receptor-Interacting Protein kinase 3</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SMAC</td>
<td>Second-Mitochondrial Activator of Caspases</td>
</tr>
<tr>
<td>TAP</td>
<td>Tandem-Affinity Purification</td>
</tr>
<tr>
<td>TFAM</td>
<td>Mitochondrial Transcription Factor A</td>
</tr>
<tr>
<td>TIM</td>
<td>Translocase of the Inner Membrane</td>
</tr>
<tr>
<td>TMRM</td>
<td>Tetramethylrhodamine Methyl Ester</td>
</tr>
<tr>
<td>TOM</td>
<td>Translocase of the Outer Membrane</td>
</tr>
</tbody>
</table>
APPROVED
Audrey M. Lenhausen

THE PROSURVIVAL ACTIVITIES OF APOPTOSIS-INDUCING FACTOR CONVERGE UPON THE REGULATION OF MITOCHONDRIAL METABOLISM, A NOVEL CELL DEATH PATHWAY, AND THE PROGRESSION OF PROSTATE CANCER

Dissertation under the direction of
John C. Wilkinson, Ph.D., Assistant Professor of Biochemistry

Apoptosis-inducing factor (AIF) is a mitochondrial NADH-oxidase originally discovered for its ability to trigger apoptosis through chromatin condensation and DNA cleavage. Although AIF is recognized for its apoptotic function, AIF-mediated cell death has emerged as an atypical cell death pathway; and accumulating evidence indicates AIF to play a more significant role in cell survival by maintaining mitochondrial homeostasis. A loss in AIF protein leads to a variety of mitochondria-specific alterations, including defects in mitochondrial structure, alteration to mitochondrial fission and fusion dynamics, and decreases to the protein complement of the electron transport chain resulting in diminished oxidative phosphorylation. Along with the ability to promote healthy mitochondrial function, AIF has demonstrated a supportive role in cancer cell growth which may be connected to its ability to regulate mitochondrial metabolism. The mechanisms governing how AIF impacts various mitochondrial features are unknown, and a concomitant role for deregulated AIF activity in human disease, in particular cancer, is poorly defined.

In this study, we reveal novel AIF binding partners that have led to the investigation of the role of AIF in various mitochondrial processes ranging from protein import to the acetylation of mitochondrial proteins. A substantial decrease in transcript levels of mitochondrial-encoded genes is seen following the loss of AIF in the prostate cancer cell line, PC3, correlating with the
decrease in protein expression shown in previous studies. This implicates the regulation of mitochondrial transcription as the means by which AIF is able to impact mitochondrial metabolism, potentially to the benefit of cancer cell growth and metastasis. To further examine the significance of AIF in prostate cancer progression, we have developed and characterized AIF deficient prostate stem cells for use in an innovative tissue regeneration mouse model that will be utilized to define the temporal role of AIF in the development of prostate cancer.

In exploring the interaction between AIF and the newly discovered binding partner, phosphoglycerate mutase family member 5 (PGAM5), we have uncovered a novel cell death pathway in which AIF plays a protective role by attenuating caspase activation that occurs as a consequence of upregulating PGAM5. Transmission electron microscopy reveals aberrant mitochondrial characteristics following PGAM5 expression indicative of enhanced mitochondrial fission and mitophagy, suggesting the protective actions of AIF may be connected to a potential role in regulating mitochondrial fusion and/or stabilizing mitochondrial structure.

Overall, the data presented in these studies help to further highlight AIF as a pro-survival molecule that serves a critical role in controlling normal mitochondrial biogenesis and function, and helps to establish the capacity of AIF to mediate diseases such as prostate cancer.
CHAPTER 1
INTRODUCTION

Cell death is a fundamental biological process necessary for the development and maintenance of multicellular systems. As humans, we not only rely on this process to shape our organs and develop our nervous system at the very beginning of our life cycle; but we continue to remain dependent upon cell death to maintain homeostasis throughout our entire lifetime by destroying foreign pathogens and removing damaged or unwanted cells [1-4]. The full significance of cell death to the maintenance of our well-being is illuminated by the serious consequences that arise from dysregulation of this process. Insufficient cell death signaling can lead to autoimmunity, persistent infection, or cancer, while excessive signaling can promote neurodegeneration, cardiovascular disease, and tissue damage [5-10]. With implications in such a wide range of diseases, cell death has emerged as a principal field in biomedical research; creating an ever expanding knowledge of the various types of cell death.

The current paradigm designates apoptosis, necrosis and autophagic cell death (ACD) as the three major pathways employed in the removal of cells. These distinct forms of cell death are classified according to morphological appearance, enzymological criteria, and immunological response. Apoptosis is the most studied and well-defined form of cell death, and is generally considered to be the predominant pathway. It is commonly facilitated by a family of cysteine proteases, known as caspases. Humans have twelve caspases divided into either an inflammatory or apoptotic subfamily, with the apoptotic caspases further divided into initiator (caspase-2, -8, -9, -10) and executioner (caspase-3, -6, -7) caspases [11]. Initiator caspases are responsible for cleaving the inactive proenzyme forms of effector caspases, thereby activating effector caspases, which in turn, proteolytically degrade a host of intracellular proteins to carry
out cell death [12]. During apoptosis, the activation of caspases occurs through two major pathways: the extrinsic and the intrinsic pathway [1, 12]. The extrinsic pathway is induced by death receptors belonging to the TNFR family which promote the formation of a death-inducing signaling complex [13] [12] responsible for activating the initiator caspases, caspase-8 and/or -10, and subsequent activation of the executioner caspases, caspase-3, -6 and -7 [14]. Similar to the extrinsic pathway, stimulation of the intrinsic pathway also ultimately results in activation of the executioner caspases -3, -6 and -7; however, intrinsic apoptosis is triggered by a variety of stress factors such as DNA damage or oxidative stress, and controlled through the mitochondria by the Bcl-2 family of proteins [15]. Activation of this pathway typically leads to permeabilization of the outer mitochondrial membrane (OMM), resulting in the release of cytochrome c (cyto c) and formation of an apoptosome complex responsible for activating the initiator caspase, caspase-9 [16]. A cell undergoing apoptosis exhibits distinct morphological characteristics which include cell shrinkage, membrane blebbing, chromatin condensation and the formation of apoptotic bodies [17]. These apoptotic bodies are quickly recognized and degraded by phagocytes allowing for the efficient removal of cellular components without eliciting an inflammatory response; thus, apoptosis is commonly referred to as a “silent” death [18].

Unlike apoptosis, necrosis is a caspase-independent form of cell death that results in the activation of the immune system. During necrotic cell death, the organelles swell and the cell membrane ruptures, releasing its cellular contents into the extracellular space, resulting in an inflammatory response [19]. This proinflammatory response is useful when a cell has been invaded by a foreign pathogen or has undergone physical injury by an external force such as bodily harm or toxins. Since necrosis is caspase-independent, it can often serve as a back-up or
substitute pathway when death is imperative and apoptosis is blocked by caspase inhibition. While necrosis is typically considered a chaotic form of cell death lacking distinct signaling events, accumulating evidence has begun to support the existence of structured necrotic pathways, such as necroptosis. Necroptosis occurs when extrinsic apoptosis is triggered by the death receptor ligand, TNFα, and caspase-8 activation is inhibited, resulting in necrosis [20]. Following TNFR stimulation, a DISC is formed to activate caspase-8 which includes FADD and receptor-interacting protein kinase 1 (RIP1) [21]. When caspase-8 activation is blocked or insufficient, FADD and RIP1 form an alternative complex with another receptor-interacting protein kinase, RIP3, and engage in signaling events that eventually lead to cell death [22].

Similar to necrosis, autophagic cell death is a caspase-independent pathway; however, like apoptosis, it does not elicit an immunological response [23]. The process of autophagy is typically considered a prosurvival mechanism; the degradation and recycling of cellular components through autophagic vacuoles can help to provide cellular energy when nutrients are depleted, remove excess or damaged organelles, eliminate long-lived proteins, and destroy invading organisms [23-25]. A dying cell showing an abundance of autophagic vacuoles, without displaying characteristics of apoptosis or necrosis, is labeled as undergoing ACD; however, it is unclear if autophagy is an accompaniment or the cause of cell death [26]. Macroautophagy is the main autophagic pathway employed in maintaining cellular homeostasis [27]. During this process, targeted cellular components are enveloped by a C-shaped membrane, known as a phagophore, to form an autophagosome [23]. These autophagosomes fuse with lysosomes, allowing the sequestered cellular contents to be degraded and recycled by lysosomal hydrolases. There are two forms of macroautophagy: non-selective and selective. Non-selective occurs when cells are deprived of nutrients, prompting the degradation of a wide range
of cytosolic contents which include organelles and proteins [28, 29]. In contrast, selective autophagy occurs under nutrient-rich conditions and results in the degradation of a specific, damaged cellular component, such as peroxisomes (pexophagy) or mitochondria (mitophagy) [30-35]. While both of these pathways work to promote the survival of a cell, excessive and/or dysregulated autophagy could also result in cell death.

In addition to the classical types of cell death (apoptosis, necrosis, and ACD) there are a variety of other lesser-understood mechanisms that promote cell death in an atypical manner. Tentatively refered to as atypical cell death pathways, this continuously growing category includes the processes of mitotic catastrophe, paraptosis, anoikis, pyroptosis, pyronecrosis, and entosis [36]. Atypical cell death pathways are usually unique to certain cell types or tissue(s), triggered in response to unusual or specific stimuli, and/or result in an assortment of classical death characteristics. For example, pyroptosis is a form of cell death discovered in macrophages that occurs specifically in response to flagellin from invading bacteria [36]. During the atypical response of pyroptosis, characteristics from all three major cell death pathways are represented. When the inflammasome detects bacterial flagellin, the cell’s first response is to trigger autophagy in an effort to destroy the invading organism [31]. If the level of infection becomes too great, then the typically apoptotic response of caspase activation occurs with the cleavage of caspase-1, a typically non-apoptotic caspase [37]. Once activated, caspase-1 goes on to trigger pyroptosis, resulting in the necrotic characteristics of cell membrane rupture and activation of the immune system. The emergence of atypical cell death pathways over the past decade not only demonstrates the ability of a cell to produce a tailored set of death characteristics based on specific needs, but exposes novel pro-death functions for proteins typically thought of as pro-survival molecules, as seen with caspase-1 in pyroptosis. If established pro-survival proteins are
turning out to have a lethal side, then it is possible customary pro-death proteins have the ability to serve a pro-survival purpose. Indeed, apoptosis-inducing factor (AIF), a protein whose discovery was linked to its pro-death function, has been shown to possess both vital and lethal activities.

The structure and processing of AIF

Apoptosis inducing factor (AIF) is a phylogenetically conserved flavoprotein that has been identified in mammals, bacteria, frogs, flies, worms, and yeast [38, 39]. In mice and humans, the AIF gene is located on the X chromosome and gives rise to a 67 kDa precursor protein (613 residues in humans, 612 in mice) containing a mitochondrial localization sequence (MLS), two nuclear localization sequences, 2 flavin adenine dinucleotide (FAD)-binding motifs, and an nicotinamide adenine dinucleotide (NAD)-binding domain (Figure 1) [40]. When imported into the mitochondria, the first 54 amino acids are cleaved to create the 62 kDa mature form of AIF (Δ54). This form of AIF is tethered to the inner mitochondrial membrane (IMM) with a majority of the protein extending into the inner membrane space (IMS) [38, 41-43]. Upon insertion into the IMM, AIF incorporates its FAD co-factor causing the oxidoreductase portion of AIF (the FAD- and NADH- binding domains) to adopt a glutathione reductase-like fold, similar to bacterial NAD-dependent oxidoreductases, that promotes stabilization of NAD and FAD and confers electron transfer activity to AIF [42]. Through a series of in vitro biochemical studies employing recombinant AIF protein, Miramar et al demonstrated the ability of AIF to oxidize both NADH and NADPH independently of its apoptotic activity [38, 41]. While AIF has been shown to possess enzymatic activity in vitro, the role for this function in vivo has yet to be established.
**Figure 1. Major forms and splice variants of AIF.** Schematic representation of the human AIF gene encoded products: full-length nuclear-encoded AIF precursor protein, mature AIF processed at amino acid 54 upon import into the mitochondria prior to insertion in the inner mitochondrial membrane (∆54), apoptotic AIF processed at amino acid 102 upon apoptotic insult (∆102), and the AIF splice variants AIF short (AIFsh), AIF short 2 (AIFsh2), and AIF short 3 (AIFsh3).
The C-terminal region of AIF is considered the pro-apoptotic domain, containing a DNA-binding motif required for inducing apoptosis [44, 45]. Following apoptotic insult, permeabilization of outer mitochondrial membrane (OMM) prompts the proteolytic cleavage of AIF at position 102 (Δ102), by calpain and/or cathepsin proteases, to yield a 57 kDa apoptotic form (Figure 1) [37, 38, 40, 45, 46]. Once released from the mitochondria, AIF translocates to the nucleus and promotes the apoptotic features of chromatin condensation and large-scale DNA cleavage without the involvement of caspases [41]. Although AIF has the ability to bind DNA, it does not possess intrinsic nuclease properties[47]; therefore, it must recruit partners such as EndoG [48], cyclophilin A [49], and/or H2AX [50] to initiate these death-inducing characteristics.

Since the initial description of AIF, there have been four splice variants identified: AIF2 (AIF-exB), AIFshort (AIFsh), AIFshort2 and AIFshort3 (Figure 1). AIF2 is a brain-specific variant containing an alternative exon 2b instead of the original exon 2a [51]. While the purpose of the alternative splicing is unknown, the usage of exon 2b in place of 2a does not affect mitochondrial import or function [52]. The AIFsh isoform results from an alternative transcriptional start site located at intron 9 of AIF, creating a cytosolic protein only possessing proapoptotic activity due to the lack of an MLS and oxidoreductase domain [53]. AIFsh2 and AIFsh3 are produced via alternative usage of exon 9b with both variants lacking the C terminal domain and NLS motifs found in AIF [54, 55]. Similar to AIFshort, AIFsh3 lacks the MLS sequence thus is confined to the cytoplasm; while contrary to AIFshort, AIFsh2 possesses oxidoreductase but no apoptotic activity. While these splice variants have not been shown to contribute significantly to the biology of AIF, the features of these isoforms validate the
apoptotic activity of the C-terminal domain and corroborate its independence from the oxidoreductase function of AIF.

**The pro-death activity of AIF.**

As its name implies, “apoptosis-inducing factor” was originally discovered for its ability to induce the apoptotic characteristic of DNA fragmentation in a caspase-independent manner [38, 47, 56, 57]. In the late-1990s, apoptosis had been linked to caspase activation as the primary means of biochemical signaling, and the cell-death field was just beginning to uncover factors that contributed to the regulation of this process. Being the first protein discovered to induce cell death without the activation of caspases, gained AIF notoriety as a novel cell death effector and prompted the study of its activity in a variety of model systems and cell types [55]. However, rather than emerging as a universal death molecule, the contribution of AIF to cell death has been shown to depend on the species, cell type and death stimulus. For example, human embryonic stem (ES) cells lacking AIF are relatively resistant to serum starvation-induced apoptosis, but remain sensitive to etoposide and azide treatment [58, 59]. On a similar note, knockout of the AIF homologue, AIF-1, in *Saccharomyces cerevisiae* (*S. cerevisiae*) renders cells resistant to oxidative stress, whereas the downregulation of AIF in mice sensitizes the myocardium to oxidative stress-induced cell death [56, 60-62].

Mostly, AIF has been shown to play a significant role in the death of neurons and cardiomyocytes, in response to excitotoxicity, DNA-damage, and hypoxia–ischemia [63]. High molecular weight DNA cleavage following the translocation of AIF and endonuclease G (EndoG) has been reported in human adult cardiomyocytes following ischemia-reperfusion [64, 65]. AIF has also been found to mediate poly (ADP-ribose) polymerase-1 (PARP-1)-dependent cardiac remodeling and impairment of ventricular contraction [65]; with PARP-1 knockout mice
showing reduced AIF translocation, cardiomyocyte death, and myocardial hypertrophy [58]. Contrary to these data, Harlequin (Hq) mice, a strain of mice exhibiting an 80% reduction in AIF expression due to a proviral insertion in the AIF gene, exhibit increased heart damage compared to control mice when subjected to myocardial ischemia reperfusion [56, 57, 60]. Along with PARP-1 mediated cardiomyocyte cell death, AIF has also been shown to mediate PARP-1 dependent excitotoxicity induced by stimulation of N-methyl D-aspartate (NMDA) receptors and p53-dependent neuronal cell death following treatment with camptothecin [60, 66, 67]. In correlation, cortical and hippocampal neurons from Hq mice show partial resistance to cell death induced by NMDA, PARP, and/or oxygen-glucose deprivation (OGD); further supporting the role of AIF in mediating neuronal cell death [60, 66, 68, 69]. Moreover, several studies have shown protection from glutamate-, hypoxia- and NMDA-induced neuronal cell death with either microinjection of AIF neutralizing antibodies or the genetic reduction of AIF expression [70, 71].

**AIF as promoter of mitochondrial homeostasis.**

While the pro-apoptotic functions of AIF have been well studied, the homeostatic mechanism(s) underlying the presence and enzymatic activity of AIF in the mitochondria has yet to be established. Findings from numerous gene ablation studies suggest AIF plays a role in oxidative phosphorylation by modulating the structure and function of the electron transport chain [72]. The genetic inactivation of aif in mice abolishes cavitation during the early stages of embryogenesis, making knockout of AIF embryonically lethal and underscoring the requirement of AIF in development [67]. Interestingly, Hq mice exhibiting a substantial reduction in AIF expression are viable yet display severe retinal and neuronal degeneration due to an increase in oxidative stress [71]. In a study examining the brain and retina of Hq mice, mitochondria were
shown to have a significant reduction in the expression and activity of complex I of the electron transport chain (ETC) as a consequence of AIF deficiency [71, 73-75]. This respiratory phenotype of altered complex I function with the ablation of AIF was further demonstrated in embryonic stem (ES) and HeLa cells, and has since been shown in a variety of cell types and tissues [76]. Tissue-specific ablation studies provide further evidence of the role of AIF in controlling mitochondrial function and structure. The muscle-specific deletion of AIF in mice has been shown to result in severe muscle atrophy, cardiomyopathy, and mitochondrial dysfunction due to impaired respiratory chain activity [70]; brain-specific deletion of AIF leads to the accumulation of fragmented mitochondria with aberrant cristae formation, indicating a novel role for AIF in controlling mitochondrial structure [77, 78].

Strikingly, the characteristics presented in $Hq$ mice and AIF-deficient cells and tissues, correspond with defects present in humans suffering from oxidative phosphorylation deficiencies. Oxidative phosphorylation disorders result in a variety of defects such as oxidative stress, ataxia and neurodegeneration and often affect tissues that require high energy production such as the retina, heart, brain and muscle [79-83]. A deficiency of complex I in the respiratory chain accounts for approximately 30-40% of all cases of mitochondrial disease, and can result in features such as cardiomyopathy, ataxia, blindness, deafness, progressive neurodegeneration, and seizures [84]. Recently, a deleterious mutation in the AIF gene, eliminating arginine at position 201, has been linked to patients with mitochondrial encephalomyopathy [70, 84]. Fibroblasts engineered with this mutation exhibit acute mitochondrial fragmentation indicative of faulty oxidative phosphorylation, and reflect the phenotype of abnormal cristae formation seen in mice with brain-specific deletion of AIF [75].
The protective role of AIF in cancer.

Along with AIF being implicated in mitochondrial disease, accumulating evidence suggests a role for AIF in the development and progression of cancer; which may be related to the ability to control mitochondrial function. In 2005, Porter et al. revealed that knockdown of AIF in human colorectal cancer cell lines led to the severe inhibition of tumor cell growth in vivo compared to wildtype cells [85]. This loss in tumorigenicity coincided with a significant decrease in the expression and activity of complex I, suggesting a mechanistic connection between cancer cell survival and the role of AIF in regulating oxidative phosphorylation. The protective function seen in colorectal cancer led our laboratory to examine the role of AIF in promoting prostate cancer cell growth and metastasis. Analysis of publically available gene expression data revealed AIF mRNA to be elevated in human prostate cancer, which correlated with an upregulation in protein levels found in tissue samples taken from patients with benign, localized, and metastatic prostate cancer [85]. The level of AIF expression directly correlated with the progression of the disease, suggesting elevated AIF levels to play a role in the growth and invasion of prostate cancer. In vitro analysis of the prostate cancer cell lines LNCaP, DU145 and PC3 showed that depletion of AIF from DU145 and PC3 cells led to a significant reduction in cell survival and growth under growth stress conditions, a characteristic that was not observed in LNCaP cells [85]. Similarly, DU145 and PC3 cell lines exhibited a decrease in complex I subunit expression and an increase in glucose consumption upon the depletion of AIF, while the LNCaP prostate cancer cells did not exhibit changes in either activity. This discrepancy in phenotype between the LNCaP cell line and DU145 and PC3 cell lines links the protective role of AIF to advanced prostate cancer and further supports a mechanistic connection between cancer cell survival and the ability of AIF to regulate mitochondrial metabolism. To help further
establish the protective role of AIF in the progression of PC, a xenograft study was employed using AIF deficient PC3 cells. While the loss of AIF had no effect on cell growth under normal conditions in vitro, a substantial delay and/or reduction in tumorigenic growth was detected in vivo [67]. Moreover, immunoblot analysis of tumors derived from PC3 xenografts showed both AIF and complex I protein levels to have remained repressed. The suppression of complex I subunit expression compromises mitochondrial energy production, forcing cells to switch to glycolysis to meet energy demands. In a nutrient-rich environment this metabolic change did not affect cell growth; however, under growth-stress conditions cancer cell growth was severely compromised. This may account for AIF playing a vital role in prostate cancer progression in advanced prostate cancer cells.

Overall, the data indicates a pro-survival role for AIF in maintaining healthy mitochondrial function through the regulation of mitochondrial energy metabolism. The ability of AIF to control energy production could be exploited by prostate cancer cells to the benefit of promoting cancer cell growth and metastasis. Determining the mechanisms behind the ability of AIF to support mitochondrial function and promote cancer cell survival could help further our understanding of the regulation of cell death in health and disease, and uncover potential avenues of treatment. In this study, we reveal novel AIF binding partners that have led us to investigate the role of AIF in various mitochondrial processes ranging from protein import to dephosphorylation. A selective and substantial decrease in mitochondrial mRNA levels seen with the loss of AIF in the prostate cancer cell line, PC3, correlates with a decrease in protein expression levels shown in previous studies, and implicates the regulation of mitochondrial transcription or mRNA stability as the means by which AIF is able to impact mitochondrial metabolism, potentially to the benefit of cancer cell growth and metastasis. To further examine
the significance of AIF in prostate cancer progression, we have developed and characterized AIF
deficient prostate stem cells for use in an innovative tissue regeneration mouse model that will be
utilized to define the temporal role of AIF in the development of prostate cancer.

In exploring the interaction between AIF and the newly discovered binding partner,
phosphoglycerate mutase family member 5 (PGAM5), we have uncovered a novel cell death
pathway in which AIF plays a protective role by attenuating caspase activation that occurs as a
consequence of PGAM5 upregulation. Contrary to the known functions of PGAM5 being
previously implicated in cell death, this newly uncovered pathway does not rely upon the ability
of PGAM5 to participate in dephosphorylation or interact with Keap1 and Bcl-XL. Transmission
electron microscopy reveals a detrimental loss in mitochondrial number and severe aberrant
mitochondrial morphology following the expression of PGAM5. These mitochondrial
characteristics implicate enhanced mitochondrial fission and subsequent mitochondrial clearance
in the ability of PGAM5 to promote cell death, and suggest the protective actions of AIF may be
connected to its potential role in stabilizing mitochondrial structure.

Overall, the data presented in this study demonstrate the biological significance of the
survival functions of AIF in the control of oxidative phosphorylation and through the regulation
of mitochondrial transcription. Depending on the context and cell type, these functions may be
manipulated to the benefit of cancer cell survival and progression. Along with a vital role in
mitochondrial metabolism, we have also revealed the ability of AIF to attenuate caspase activity
in a novel PGAM5-mediated cell death pathway, which could be connected to a previously
implicated role for AIF in regulating mitochondrial dynamics/structure.
CHAPTER 2

THE IMPACT OF AIF UPON REGULATION OF THE MITOCHONDRIAL PROTEOME
INTRODUCTION

Mitochondrial dysfunction is a key element to a variety of neurodegenerative disorders including Alzheimer’s disease, retinal degeneration, Parkinson’s disease and amyotrophic lateral sclerosis (ALS) [86, 87]. Alteration to the activity or expression of mitochondrial proteins can result in abnormal energy production, generation of reactive oxygen species (ROS), aberrant dynamics, and a variety of other complications that ultimately result in cell death. AIF is a mitochondrial protein whose loss in expression and/or activity has been shown to promote neurodegeneration due to disruption of the electron transport chain. In 2002, study of the Harlequin mouse revealed severe neuronal and retinal degeneration due to an increase in oxidative stress as a consequence of AIF deficiency [71]. The mitochondria of the brain and retina of the Harlequin mouse was later shown to have compromised oxidative phosphorylation due to a decrease in expression of the 17 kDa, 39 kDa and 20 kDa subunits of complex I, a phenotype that was also seen in AIF deficient HeLa and embryonic stem (ES) cells [84]. More recently, a deleterious mutation of AIF has been linked to patients with mitochondrial encephalomyopathy due to oxidative phosphorylation failure that results from a substantial loss in complex III and complex IV function [70, 71, 73, 85]. Overall, these data indicate the significance of AIF in promoting cell survival through its ability to regulate the assembly and function of the electron transport chain, and highlights the need for further studies exploring the role of AIF outside the framework of cell death in the hopes of providing further understanding of mitochondrial disease and potentially new therapeutic treatment(s).

The impaired oxidative phosphorylation seen with the cellular loss of AIF stems from the disruption of complex I activity due to a decrease in expression of complex I subunits. The most basic mechanisms by which AIF may affect mitochondrial protein expression are through
controlling the processes of transcription and/or translation. Although a majority of respiratory chain subunits are imported from the nucleus, a select few are encoded by the mitochondrial genome and are vital to the assembly and function of the electron transport chain. While the impact of AIF ablation on nuclear-encoded subunit expression has been investigated in several studies, the comprehensive assessment of mitochondrial gene expression following the loss of AIF has yet to be examined [88]. The mitochondrial genome consists of circular, double-stranded DNA containing 37 genes encoding 22 tRNAs, 2 rRNAs and 13 subunits of the electron transport chain [89-91]. With seven of the thirteen mitochondrial-encoded subunits belonging to complex I specifically; AIF may be controlling complex I expression and activity through the regulation of mitochondrial transcription and/or mitochondrial translation. The mitochondrial genome is transcribed by a mitochondrial RNA polymerase (POLRMT), core transcription factors (TFAM, TFB1M, TFB2M), and additional regulatory factors, including the mitochondrial transcription termination factor, mTERF [92]. Interestingly, heart and skeletal muscle specific ablation of mTERF3 in mice results in shortened lifespan related to cardiomyopathy, aberrant mitochondria, and severe respiratory chain deficiencies [76]. These phenotypes are nearly identical to those observed in similar experiments evaluating the targeted deletion of AIF, suggesting that these two genes reside in a common biological pathway [93].

Since a majority of the mitochondrial proteome is transcribed in the nucleus, including the transcription and translation machinery necessary for mitochondrial protein synthesis, AIF could impact the mitochondrial proteome along with mitochondrial transcription/translation through the regulation of mitochondrial protein import. Once proteins are imported into the mitochondria, their expression levels and activity can also be controlled through post-translational modification (PTM). There are various forms of PTM that a peptide may undergo
which include phosphorylation, methylation, and ubiquitination; however, ETC proteins have been shown to be affected in particular by acetylation [94, 95]. Acetylation of a protein occurs when a hydroxyl group, typically located on a lysine residue, is replaced by an acetyl group. The addition of an acetyl group can modify the activity of the protein, as well as create crosstalk with other forms of PTM such as ubiquitination and methylation [96]. In order to reverse this activity, a deacetylase enzyme must remove the acetyl group from the peptide. Sirtuins are class III deacetylases homologous to the yeast transcriptional repressor, silent information regulator 2 (Sir2). Of the seven sirtuins found in mammals; three reside in the mitochondria (SIRT3, -4 and -5), and two of those require NAD to function (SIRT3, SIRT5) [97]. Similar to AIF, cellular loss of SIRT3 results in a decrease in complex I activity, attributed to an increase in acetylation; suggesting acetylation plays a role in regulating oxidative phosphorylation [71, 75, 97, 98]. SIRT3 has also been shown to target several subunits of complex I in the electron transport chain including the 39 kDa subunit, a subunit whose expression levels have been shown to decrease with the loss of AIF [42]. AIF possesses NADH oxidase activity \textit{in vitro}; however, the purpose of this activity \textit{in vivo} remains to be determined [99]. The phenotypic parallels between AIF and SIRT3 open the possibility that AIF regulates complex I expression and activity, potentially through an NAD-dependent mechanism targeting sirtuin-mediated deacetylation.

In this study, we investigate the role of AIF in mediating the processes of mitochondrial transcription, translation, protein import, and PTM of mitochondrial proteins. The control of any one these avenues would allow AIF to regulate mitochondrial activity, and subsequent cell survival, through the establishment of the mitochondrial proteome. Although the loss of AIF was found to be inconsequential to mitochondrial protein import and the acetylation of mitochondrial proteins, a decrease in mitochondrial transcript levels was seen upon the loss of
AIF in the prostate cancer cell line, PC3. While these data suggest a role for AIF in regulating mitochondrial transcription, a similar decrease of mRNA levels was not observed in AIF deficient LNCaP cells, indicating AIF-mediated control of transcription to be selective and its role in regulating metabolism to be more complex than previously envisioned.

EXPERIMENTAL PROCEDURES

Materials. Reagents were obtained as follows: protein G-coupled agarose, GlutaMAX, Dulbecco modified Eagle medium (DMEM), methionine-free DMEM, Roswell Park Memorial Institute (RPMI) 1640 and phosphate-buffered saline (PBS) from Invitrogen; TnT® Quick Coupled Transcription/Translation System and FluoroTect™ GreenLys in vitro Translation Labeling System from Promega; fetal bovine serum (FBS) from HyClone. All other chemicals were from Sigma. Antibodies were obtained as follows: anti-SMAC/diablo (#567365) from Calbiochem; anti-acetylated lysine (#9814 and #9681) from Cell Signaling, anti-cytochrome c (#556433) from Pharmagen; and anti-Sirt 5 (#AV32390) from Sigma-Aldrich.

Cell culture, transfection and plasmids. HEK 293T, HeLa, MEF cell lines were grown in DMEM containing 10% FBS supplemented with 2 mM Glutamax, PC3 and MDA-MB-231 cell lines were grown in RPMI 1640 containing 10% FBS supplemented with 2 mM GlutaMAX. All cells were maintained at 37°C in an atmosphere of 95% air and 5% CO₂ except during lentivirus infection as described below. Transfections were performed by the method of calcium phosphate precipitation as described previously [100]. pEBB-AIF-TAP (full-length) and pEBB-AIF-N102-TAP were generated by subcloning WT and N102 AIF into the pEBB tandem-affinity purification (TAP) plasmid. pEBB SMAC/Diablo was constructed by subcloning SMAC/Diablo from pUb-Smac/Diablo [101] into the pEBB vector. pcDNA3 AIF, pcDNA3 EF-Tu, pcDNA3
TFAM, pcDNA3 NDUFA9, pcDNA3 beta actin, and pcDNA3 citrate synthase were generated by PCR using an expressed sequence tag clone containing human full-length AIF (Image clone 5740894), EF-Tu (Image clone 3536952), TFAM (Image clone 5538151), NDUFA9 (Image clone 5761015), beta actin (Image clone 2961617), or citrate synthase (Image clone 6484456) from Thermo Scientific.

**Cell lysis, immunoblot analysis and immunoprecipitations.** Cell lysates were prepared in either Nonidet P-40 (NP-40) lysis buffer containing histone deacetylase (HDAC) inhibitors (50 mM Tris-HCl, 0.5% NP-40, 50 mM NaCl, 500 mM EDTA, 10 mM nicotinamide, 1 mM Trichostatin A, 1 mM PMSF, and 1 protease inhibitor mixture tablet per 10 mL) or radioimmune precipitation assay (RIPA) lysis buffer (PBS containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM DTT, 1 mM PMSF, and 1 protease inhibitor mixture tablet per 10 mL). Extracts were normalized for protein content, and then separated by SDS-polyacrylamide gel electrophoresis (PAGE) [102] using 4 to 12% gradient SDS-polyacrylamide gels (Invitrogen). For immunoblot analysis, SDS-PAGE was followed by transfer to nitrocellulose membranes (Invitrogen), which were then blocked with 5% milk in Tris-buffered saline containing 0.02 to 0.2% Tween, followed by incubation with the indicated antibodies for 1 h at room temperature. Following washing, membranes were incubated with HRP-conjugated anti-mouse IgG or anti-rabbit IgG secondary antibodies for 45 min at room temperature and visualized by enhanced chemiluminescence. For immunoprecipitation experiments, cell lysates (NP-40 HDAC lysis buffer) were normalized for protein content and incubated with indicated antibodies for 2 h at 4°C. Protein G-coupled agarose beads were then added and incubated for 1 h. Agarose beads were recovered by centrifugation and washed in NP-40 HDAC lysis buffer, precipitated proteins were eluted by adding lithium dodecyl sulfate (LDS) sample buffer and
heating the mixture to 95°C for 5 min. Recovered proteins were then separated by electrophoresis, and immunoblot analysis was carried out as described above.

**Tandem Affinity Purification.** Cells were seeded into two groups of five 15 cm plates each and then transfected with 12 µg of pEBB-AIF-TAP (full-length) or pEBB-AIF-N102-TAP per plate. The AIF-N102 construct produces a protein containing 102 amino acid residues corresponding to the amino-terminus of AIF in fusion with the full TAP tag, and was used as a background control for proteomic analysis. Two days after transfection cells were fractionated by dounce homogenization, protein was extracted from the crude mitochondrial fraction, and extracts were subjected to tandem affinity purification as described [103]. Final purified samples were submitted to the Proteomics Centre at University of Victoria for further processing including trypsin digestion, HPLC separation and MS/MS to determine peptide sequences.

**Lentiviral production and infection.** Lentiviral knockdown AIF in HEK 293T, HeLa, PC3, MEF and MDA cells was performed as previously described [100]. Briefly, FG12 derived plasmids were combined with equal amounts of lentiviral packaging plasmids and transfected into the various cell lines by calcium phosphate precipitation as described [85]. 48 h after transfection, supernatants were harvested, filtered using 0.45-µm-pore size Millex HV PVDF filter units (Millipore), and concentrated by centrifugation at 60,000 × g for 90 min. The supernatants were aspirated, and virus-containing pellets were resuspended in PBS overnight at 4 °C. One day before infection, target cells were seeded at 60,000 cells/well in 12-well plates. At the time of infection, polybrene was added to a final concentration of 25 mM, resuspended virus was added, and cells were incubated for 4 h at 37 °C in an atmosphere of 93% air, 7% CO₂. Virus containing supernatants were then removed, fresh medium was added to cells, and cells were incubated for an additional 2–3 days in an atmosphere of 95% air, 5% CO₂. Stable
incorporation of FG12-based lentiviral DNA was determined by immunoblot analysis and/or puromycin selection and flow cytometry assessing GFP fluorescence as shown/described previously [104].

\[ ^{35}S \text{ mitochondrial translation experiment.} \] Mitochondrial translation products were labeled with \(^{35}\)S-methionine as described previously [105]. Briefly, cells were seeded at 3 million cells/per plate in 10 cm plates for each cell line and allowed to attach overnight. The cells were then rinsed and incubated with methionine-free media at 37°C for 5 min. Following incubation, media was replaced with methionine-free media containing emetine (100 μg/mL) and incubated for 6 min at 37°C. \(^{35}\)S-methionine (100 μCi final) was then added to each plate and incubated for 2 hr at 37°C. Cells were washed (2X) with isotonic buffer (25 mM Tris-HCl, 137 mM NaCl, 10 mM KCl, and 0.7 mM Na₂HPO₄), harvested by trypsinization, and lysed in modified Laemmli lysis buffer (25 mM Tris-HCl, 1% SDS, 8.5% glycerol, 1% beta-mercaptoethanol, 2 mM EDTA, and 1 mM PMSF). Extracts were normalized for protein content and then separated by SDS-polyacrylamide gel electrophoresis (PAGE) using 4 to 12% gradient SDS-polyacrylamide gels (Invitrogen). Gels were fixed for 1 hr in 30% methanol/10% acetic acid prior to placement in gel drier; autoradiography was employed to visualize translation products.

\[ \text{Mitochondrial isolation.} \] Cells were seeded at 12 million cells/per plate in 150 mm plates and allowed to attach overnight. Cells were then harvested by trypsinization, centrifuged at 500xg for 5 min at 4°C, resuspended in 500 μL of isolation buffer (5 mM Tris, 250 mM sucrose, and 0.1 mM PMSF), and placed on ice for 2 min. Mitochondria were then isolated by dounce homogenization and differential centrifugation [106] and resuspended in mannitol buffer (225 mM mannitol, 25 mM sucrose, 10 mM Tris, and 0.1 mM EDTA). Concentration of isolated
mitochondria was determined through lysis and Bradford measurement using a portion of the resuspended mitochondria.

**Fluorescent labeling of nuclear-encoded mitochondrial proteins.** Fluorescent labeling of nuclear-encoded mitochondrial proteins was performed using Fluorotect™ Green<sub>lys</sub> and TNT® Quick Coupled Transcription/Translation System (Promega) as per the manufacturer’s instructions. Briefly, a 50 μL reticulocyte reaction containing 40 μL TNT® master mix, 6 μL nuclease-free water, 1 μL Fluorotect™ Green<sub>lys</sub>, 1 μL <sup>35</sup>S-methionine (1 mM), and 1 μg of designated DNA was incubated for 1 hr at 30°C. Labeling was confirmed by SDS-PAGE and fluorescence imaging.

**Mitochondrial import assay.** Mitochondria were isolated from cells and fluorescently labeled nuclear-encoded mitochondrial proteins were created using in vitro transcription/translation. 50 μg isolated mitochondria were incubated with 1 mM ATP, 2 mM sodium succinate and 10 μL reticulocyte reaction containing designated nuclear-encoded mitochondrial protein for 30 min at 37°C. Samples were then centrifuged at 13000xg for 1 min, mitochondrial pellet was washed with mannitol buffer (see mitochondrial isolation method) and resuspended in 2X LDS loading buffer. Mitochondrial proteins were then separated by SDS-PAGE using 4 to 12% gradient SDS-polyacrylamide gels and visualized using fluorescence imager.

**Real-time polymerase chain reaction.** RT-PCR was carried out on the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). The standard curve method was used for quantification. Total RNA was isolated using TRIzol reagent (Invitrogen), according to the manufacturer's instructions. 30 μg of RNA was treated with 35 units of DNase I (Promega) for 30 min at 37 °C. After DNase I digestion, RNA was purified using an Absolutely
RNA RT-PCR Miniprep kit (Stratagene) following the manufacturer's protocol. Oligo(dT) primer was then used in cDNA synthesis. Briefly, 200 ng of RNA was reverse transcribed in a total volume of 50 μl with a reverse transcription reagents kit (Applied Biosystems). To make a standard curve, serial dilutions of RNA from one sample (160, 40, 10, 2.5 ng) were added to the RT reaction. Aliquots (3.5 μl) of cDNA were added to a 31.5-μl reaction mixture containing 17.5 μl of 2× SYBR® Green PCR Master Mix (Applied Biosystems) and 200–400 nm primers. Absence of DNA contamination was verified by performing amplification from cDNA without reverse transcriptase. The primers for PCR were designed with IDT PrimerQuest software (Integrated DNA Technologies, Inc.).

RESULTS

Novel interactions of AIF in healthy mitochondria. While a number of AIF binding partners exist that could provide insight into the mechanisms behind the pro-death role of AIF, there is a lack of validated AIF interactions in a healthy cell that may serve in developing the general paradigm of AIF biology under homeostatic conditions. In an effort to resolve this impediment, we employed a biochemical screen to identify potential AIF associated proteins from healthy mitochondria using the tandem affinity purification (TAP) method [107, 108]. This screen employed as bait full length and truncated AIF (N102) in fusion with a carboxy-terminal TAP tag (Figure 2). Following transfection in HEK 293 cells, tagged proteins were captured using IgG beads, eluted by TEV protease digestion, and captured a second time using calmodulin beads. Purified material was precipitated from calmodulin binding eluates by trichloroacetic acid and digested with trypsin, and potential AIF-interacting proteins were identified by liquid chromatography-tandem mass spectrometry analysis of generated peptides. Western blot
Figure 2. TAP Screen for AIF binding proteins. HEK 293 cells were transiently transfected with plasmids encoding either control (AIF-N102, panel A) or AIF TAP (panel B) fusion proteins. 48h after transfection cells were fractionated, and mitochondrial extracts were prepared. Tagged proteins were then captured using IgG beads, eluted by TEV protease digestion, and captured a second time using calmodulin beads. Final eluates from calmodulin binding were then precipitated with trichloroacetic acid prior to proteomic analysis. Shown is immunoblot analysis of samples following each step of the TAP procedure. Immunoblotting was performed using anti-AIF followed by HRP secondary (panel A, panel B top), or HRP secondary alone (panel B bottom). Note the HRP secondary will detect the protein A domain of the TAP tag and is the source of signal observed in Panel A, not AIF immunoreactivity.
analysis was performed on samples following each step of the TAP procedure to monitor the purification process (Figure 2). This TAP approach identified a number of novel AIF-associated proteins (Table I) involved a variety of processes ranging from mitochondrial transport to dephosphorylation. The spectrum of factors identified revealed a variety of avenues through which AIF can impact multiple aspects of mitochondrial gene expression including transcription, translation, and protein import.

**AIF in regulating mitochondrial protein import.** An overwhelming majority of mitochondrial proteins are transcribed in the nucleus, translated in the cytosol, and then imported into the mitochondria. Among these imported proteins is the transcription and translation machinery needed to carry out expression of the mitochondrial genome, as well as nuclear-encoded subunits of the various enzyme complexes necessary for oxidative phosphorylation. Two major translocases are responsible for the import of mitochondrial proteins: the translocase of the outer membrane (TOM) and the translocase of the inner membrane (TIM) [109, 110]. The TAP screen revealed an association between AIF and a component of the TIM complex, known as heat shock protein 70 (Hsp70) (Table I). Hsp70 is responsible for using ATP-hydrolysis to facilitate the import of proteins across the inner membrane into the matrix, in order to be processed and sent to the correct compartment for assembly [111]. The ability of AIF to regulate this process may explain how loss of AIF results in decreased levels of nuclear-encoded ETC subunits.

To test the impact of AIF ablation on the import of nuclear-encoded mitochondrial proteins, we designed an *in organello* experiment using fluorescently labeled nuclear-encoded mitochondrial proteins and isolated mitochondria from AIF proficient and deficient cells. Although radioactive isotopes are commonly used to tag and trace proteins, the use of
Table I. Novel AIF-binding partners identified by Tandem Affinity Purification (TAP) screen.

<table>
<thead>
<tr>
<th>Name</th>
<th>Peptides</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Programmed cell death protein 8, (AIF)</td>
<td>15</td>
<td>Bait</td>
</tr>
<tr>
<td>heat shock protein 70</td>
<td>8</td>
<td>Stress/Heat Shock, known AIF binding protein</td>
</tr>
<tr>
<td>phosphoglycerate mutase family member 5</td>
<td>2</td>
<td>Oxidative stress response, Phosphatase Activity</td>
</tr>
<tr>
<td>ADP/ATP carrier protein (ANT2)</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Sideroflexin 1</td>
<td>1</td>
<td>Mitochondrial Solute Transport</td>
</tr>
<tr>
<td>2-oxoglutarate carrier</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Phosphate carrier</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Chain G, RNA polymerase II</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Elongation factor Tu</td>
<td>1</td>
<td>Mitochondrial Transcription/Translation</td>
</tr>
<tr>
<td>60S ribosomal protein L18</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Beta-tubulin</td>
<td>3</td>
<td>Misc</td>
</tr>
<tr>
<td>Alpha-tubulin</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Sodium/potassium-transporting ATPase</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>T-cell receptor fragment</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>
radioactivity in experiments is costly, requires specialized training, and generates substantial hazardous waste. The use of a fluorescent tag allows us to circumvent these issues and still visualize the proteins post-import. Select nuclear-encoded proteins were chosen to assess mitochondrial import which include the mitochondrial transcription factor A (TFAM), elongation factor Tu (EF-Tu), the 39 kDa subunit of complex I (NDUFA9), beta actin (β-actin) and citrate synthase. TFAM is a key activator of mitochondrial transcription and is responsible for bending mitochondrial promoter DNA to aid transcription of the mitochondrial genome [112]; a defect in import of TFAM may implicate impaired transcription in the decrease of mitochondrial-encoded proteins seen with knockdown of AIF. EF-Tu is a component of the mitochondrial translation machinery needed to carry out elongation of the peptide during translation of the mitochondrial genome [71, 73, 74]; EF-Tu will help provide the same insight as TFAM with impaired translation being revealed instead of transcription. As previously mentioned, NDUFA9 is a component of complex I whose expression has been shown to decrease due to loss of AIF [113]; this protein serves as a way to determine if loss of subunit expression is arising from a lack of import. β-actin is a cytoskeletal protein not found in the mitochondria, that serves as a negative control [75, 114]; and citrate synthase is an enzyme that resides in the mitochondrial matrix and expression has been shown to remain unaltered during loss of AIF, serving as a positive control [101]. To create fluorescently labeled versions of these selected proteins, plasmids containing the corresponding gene sequence were transcribed and translated using the TNT Reticulocyte Lysate System (Promega) in the presence of FluoroTect™ GreenLys (Promega). FluoroTect™ GreenLys is a modified charged lysine transfer RNA (tRNA) marked with a fluorophore, that is incorporated into nascent proteins during translation, eliminating the need for [35S]-methionine or other radioactive amino acids. FluoroTect™ GreenLys labeled
Figure 3. Lentiviral knockdown of AIF in MEF, MDA-MB-231 and HeLa cell lines. Protein expression of AIF in A) MEF, B) MDA and C) HeLa cell lines following lentiviral infection with AIF human or mouse shRNA. AIF levels were detected by western blot. Beta-actin serves as a loading control.
nuclear-encoded proteins were easily detected by a fluorescence imager following 1 hr of *in vitro* translation and SDS-page gel electrophoresis (Figure 4A). Once fluorescent labeling was confirmed, translation products were incubated with mitochondria isolated from wildtype and AIF deficient cells by for approximately 30 minutes at 37°C in the presence of ATP and sodium succinate. The mitochondria were then washed and lysed; samples were separated by SDS-PAGE and import of labeled proteins visualized using fluorescence imager [115]. Lamentably, the import assay was unable to provide any definitive answers due to a loss in detection seen upon incubation of the reticulocyte translation products with isolated mitochondria. In Figure 4B, Fluorotect™ GreenLys labeled AIF and TFAM show a clear detectable band following *in vitro* transcription/translation (input), prior to incubation with mitochondria isolated from AIF proficient HeLa cells. Following interaction with the mitochondria, however, the signal disappears from both the supernatant and the mitochondrial pellet; suggesting that the presence of mitochondria leads to a quenching of the fluorescent signal. To test this possiblity, fluorescently labeled TFAM was incubated with adenosine triphosphate (ATP) and sodium succinate for 30 minutes at 37°C in the presence and absence of mitochondria. TFAM was easily detected following *in vitro* transcription/translation both prior to and after incubation in the absence of mitochondria (Figure 4C). However, detection of fluorescence was lost upon incubation in the presence of isolated mitochondria (Figure 4C), indicating the mitochondria to be the factor leading to the loss in signal. The specific cause of this fluorescence quenching remains an open question, and could be due to a variety of factors including mitochondrial activity, ROS, and/or the electrochemical gradient. While this question remains open, an alternative approach was employed to assess the ability of AIF deficient cells to import nuclear-encoded mitochondrial proteins.
**Figure 4. Establishing the role of AIF in mitochondrial protein import.** A) Flurotect™ GreenLys labeled proteins following 1 hr of in vitro transcription/translation. Proteins were run on 4-12% SDS-PAGE gel following translation and detected using a fluorescence imager. B) Flurotect™ GreenLys labeled AIF or TFAM prior to (input) and following incubation with mitochondria (mito. sup, mito. pellet). Flurotect™ GreenLys labeled AIF or TFAM were incubated for 30 min with mitochondria isolated from HeLa shLacZ cells in the presence of ATP and sodium succinate. Following incubation, mitochondria were pelleted, supernatant was collected prior to lysis of the pellet, and samples run on an SDS-PAGE gel. C) Fluorescence image of Flurotect™ GreenLys labeled TFAM incubated for 30 min with or without mitochondria isolated from HeLa shLacZ cells in the presence of ATP and sodium succinate. In vitro transcription/translation of TFAM in the absence of DNA [no template] and Flurotect™ GreenLys [(-) Fl. Green] served as controls. D) Protein expression of SMAC/diablo in HeLa shLacZ, and shAIF cell lines detected by immunoblotting with SMAC/diablo antibody.
All nuclear-encoded mitochondrial proteins have a 10-70 amino acid long peptide sequence found at the N-terminus known as a mitochondrial localization signal (MLS) [116]. The MLS directs a newly synthesized protein to the mitochondria and can contain additional signals that target the protein to different regions such as the inner membrane or the mitochondrial matrix [117]. Like most signal peptides, the MLS is cleaved when the protein is imported and localized to its specific residence within the mitochondria [118]. Second mitochondria-derived activator of caspases (SMAC), also known as diablo, is a mitochondrial protein that promotes cytochrome-c dependent activation of apoptosis by preventing inhibitors of apoptosis [119] proteins [120] from inhibiting caspases [103]. SMAC/diablo is translated as a 29 kDa mitochondrial precursor protein which is proteolytically processed into a 23 kDa mature protein upon import into the mitochondria [85]. To assess if the loss of AIF has an effect on mitochondrial protein import in general, processing of SMAC/diablo was assessed in AIF proficient and deficient HEK 293T cells. Lentivirus was used to stably infect HEK 293T cells with shRNA sequences targeting either AIF or LacZ as a negative control [103]. Cells stably incorporating hairpin DNA were detected by GFP fluorescence and AIF ablation was confirmed by immunoblot analysis (Figure 12A). Once knockdown of AIF was confirmed, the H2T shLacZ and H2T shAIF cell lines were transiently transfected with SMAC/diablo and processing was examined by western blot analysis. The mature 23 kDa form of SMAC/diablo was detected in the presence and absence of AIF (Figure 4D), suggesting mitochondrial import to be unaffected by the loss of AIF.

**AIF in regulating mitochondrial transcription and translation.** Although a majority of the mitochondrial proteome is imported from the nucleus, a small number of proteins are encoded by
the mitochondrial genome. The biochemical screen employed by our laboratory revealed an association between AIF and various components necessary for mitochondrial transcription and translation. The ability of AIF to regulate these processes could explain the impact AIF ablation has on the assembly and function of the electron transport chain. While changes in expression of nuclear-encoded genes have been observed, no study has examined the extent to which AIF affects expression of the mitochondrial genome. To establish the role of AIF in regulating mitochondrial genes at the transcriptional level, we performed quantitative RT-PCR to assess changes in transcript levels of mitochondrial-encoded genes upon the loss of AIF in LNCaP and PC3 cells. A decrease in mRNA levels of the 39 kDa nuclear-encoded complex I subunit was detected with the ablation of AIF in PC3 cells (Figure 5A), correlating to the decrease in expression shown in a previous study [38, 47]. Interestingly, several mitochondrial-encoded subunits exhibited a significant reduction in transcript levels upon the loss of AIF, including cytochrome b (CYB) and four complex I subunits (Figure 5A), revealing the ability of AIF to regulate transcription of the mitochondrial genome. Conversely, the only protein to produce a change in transcription with the loss of AIF in LNCaP cells was ND1, a mitochondrial-encoded complex I subunit (Figure 5B), indicating the potential role of AIF in regulating mitochondrial transcription to be circumstantial.

To determine if this impact on transcription has a subsequent effect on mitochondrial translation, in vivo radiolabeling of newly synthesized mitochondrial encoded products was used to assess mitochondrial protein synthesis in AIF proficient and deficient HeLa cells. HeLa cells possess an abundance of mitochondria which is useful for obtaining detectable levels of translation products; and similar to PC3, the loss of AIF in HeLa cells results in decreased expression of nuclear-encoded complex I subunits. As in PC3 cells, lentivirus was used to stably
Figure 5. AIF ablation selectively reduces mitochondrial gene expression. RNA levels of nuclear- and mitochondrial-encoded genes were determined in control (black) or AIF ablated (gray) PC3 (A) and LNCaP (B) cells by real time PCR analysis. Mitochondrial-encoded subunits included complex I (ND1-6, ND4L), complex III (CYB), complex IV (CO1-3) and complex V (ATP6, ATP8) with nuclear-encoded complex I (20 kDa, 17kDa, 39 kDa) and AIF RNA levels assessed for comparison. GAPDH and β-actin primers were used as normalization controls and varied by less than 10% between samples.
infect HeLa cells with shRNA sequences targeting either AIF or LacZ as a negative control [103]. Cells stably incorporating hairpin DNA were detected by GFP fluorescence and AIF ablation was confirmed by immunoblot analysis (Figure 3C). Once knockdown of AIF was confirmed, both cell lines were incubated for 2 hr with methionine-free media containing emetine, a cytosolic protein synthesis inhibitor, and $^{35}$S-methionine. Following incubation, cells were lysed, normalized for protein content and mitochondrial proteins were separated by SDS-PAGE. Autoradiography was then employed to visualize newly synthesized mitochondrial products with incorporated $^{35}$S-methionine. Although a disparity in mitochondrial transcript levels was seen in PC3 cells upon the depletion of AIF, no significant change in translation products was detected in HeLa cells under the same conditions (Figure 6), suggesting AIF does not play an essential role in mitochondrial translation, or similar to its cell death activity, involvement in regulating mitochondrial transcription and translation may be cell type/context specific.

**AIF in regulating the acetylation of mitochondrial proteins.** Along with the processes of mitochondrial import, mitochondrial transcription and mitochondrial translation, AIF may control ETC protein levels and activity through the post-translational modification of acetylation. To establish if the depletion of AIF induces a change in the acetylation status of the entire cell and/or mitochondrial specific proteome, whole cell and mitochondrial lysates from AIF proficient and deficient cell lines were analyzed by western blot analysis using an antibody against acetylated lysine. No significant change in overall acetylation was detected in PC3 or MDA cells upon the loss of AIF (Figure 7A), nor was a change seen looking specifically at mitochondrial proteins isolated from AIF deficient MEF or HeLa cells (Figure 7B). While these data suggest AIF is not involved in regulating general protein acetylation, AIF may still be
Figure 6. Mitochondrial protein synthesis in AIF proficient and deficient HeLa cells. $^{35}$S-labeled human mitochondrial translation products separated by electrophoresis on a 4-12% gradient SDS-PAGE gel. AIF proficient (shLacZ) and deficient (shAIF) HeLa cells were incubated with $^{35}$S-methionine for 2 hr in the presence of emetine. Whole cell lysates were normalized for protein content and proteins separated by SDS-PAGE. Gel was then fixed in a methanol/acetic acid solution, dried, and exposed for fluorography for 4 days at -80°C.
Figure 7. **AIF does not play a role in mitochondrial protein acetylation.** A) Western blot of AIF proficient (shLacZ) and deficient (shAIF) PC3 and MDA whole cell lysates detecting acetylated proteins using an anti-acetylated lysine antibody. B) Western blot of AIF proficient (shLacZ) and deficient (shAIF) MEF and HeLa mitochondrial lysates detecting acetylated proteins using an anti-acetylated lysine antibody. C) Protein expression of SIRT5 in the HeLa shLacZ and shAIF cell lines detected by immunoblotting with SIRT5 antibody. D) Acetylated proteins were immunoprecipitated from HeLa shLacZ and shAIF mitochondria using an anti-acetylated lysine antibody. Immunoprecipitates were then subjected to immunoblot analysis using an anti-cytochrome c antibody.

In the context of controlling the acetylation of specific SIRT3 or SIRT5 substrates, a known substrate of SIRT5 activity is cytochrome c [70]; SIRT5 expression remains unaffected by a loss
in AIF (Figure 7C), therefore, any change in cytochrome c acetylation detected upon depletion of AIF would implicate the involvement of AIF in SIRT5 function. To examine the acetylation status of cytochrome c, mitochondria were isolated from AIF proficient and deficient HeLa cells and acetylated proteins were immunoprecipitated using acetylated lysine antibody. Following precipitation, cytochrome c acetylation was analyzed by western blot. Similar to global and mitochondrial protein acetylation, the acetylation of cytochrome c remained impervious to a decrease in AIF expression (Figure 7D), consistent with the hypothesis that AIF does not control acetylation to any significant degree.

DISCUSSION

AIF was originally discovered through its ability to trigger caspase-independent cell death in a variety of model systems [71]. However, multiple studies examining the loss of AIF in vivo suggest a more critical role for this protein in supporting normal mitochondrial function. A loss in AIF protein can lead to a variety of mitochondria-specific alterations which include defects in mitochondrial structure and abnormal mitochondrial fission and fusion dynamics [85]. However, the most notable and frequent abnormality seen with the depletion of AIF is a decrease to the protein complement of the electron transport chain resulting in severely impaired oxidative phosphorylation [71, 85]. We performed a biochemical screen to uncover factors that participate in AIF biology under homeostatic conditions that may help to discern the mechanisms governing the ability of AIF to impact mitochondrial function. Several candidate AIF-interacting partners were obtained which display a diverse range of cellular functions including regulation of mitochondrial transcription/translation and molecular transport into and within the mitochondria.
Association with these particular proteins provides distinct avenues through which AIF would be able to direct the mitochondrial proteome, and thus influence mitochondrial respiration.

The interaction between AIF and Hsp70 offers a potential opportunity for AIF to affect mitochondrial protein import through the regulation of TIM. While our method for assessing mitochondrial protein import requires further development in order to provide definitive data, similar processing of the mitochondrial resident protein SMAC/diablo under AIF proficient and deficient conditions suggest AIF to be uninvolved in mitochondrial protein transport. A more in-depth look into the relationship between AIF and Hsp70 would help to establish the physiological nature of this association and subsequently determine if AIF depletion could have a genuine impact on protein import.

In a study recently published by our laboratory examining the role of AIF in supporting prostate cancer cell growth and metastasis, AIF deficient PC3 cells exhibited a decrease in expression of the 39 kDa subunit of complex I and an increase in glucose consumption indicative of a metabolic switch from oxidative phosphorylation to glycolysis [85]. In LNCaP cells, however, neither a decrease in the 39 kDa subunit nor an increase in glucose consumption was seen following the ablation of AIF [85]. These observations mimic what was observed when we assessed the impact of AIF ablation on mitochondrial transcription. AIF deficient PC3 cells displayed a significant reduction in mRNA levels of mitochondrial-encoded complex I (ND1, ND2, ND3, ND6), complex III (cytochrome b) and complex IV (CO1 and CO3) subunits; along with the nuclear-encoded 39 kDa complex I subunit. In contrast to these data, the depletion of AIF in LNCaP cells did not produce a substantial change in transcript levels of either nuclear- or mitochondrial-encoded subunits. The difference in transcriptional phenotype between PC3 and LNCaP correlates with the difference in expression of complex I subunits found in our previous
study, and suggests the ability of AIF to regulate mitochondrial transcription is cell type and context specific [85]. Although PC3 and LNCaP are both considered prostate cancer cell lines, LNCaP cells share common features with adenocarcinoma such as androgen dependence and expression of prostate-specific antigen (PSA), while PC3 cells are more characteristic of small cell neuroendocrine carcinoma displaying androgen independence and a lack of PSA expression [18, 36]. Thus, while both cell lines fall under the category of “prostate cancer” they represent different stages of disease progression.

In addition to providing further insight into the selective support of AIF within prostate cancer; the transcriptional data pose a challenge to the current mechanism and regulation of mitochondrial transcription. As mentioned previously, the mitochondrial genome consists of circular, double-stranded DNA encoding 22 tRNAs, 2 rRNAs and 13 subunits of the electron transport chain [90]. The individual genomic strands are designated as the light strand (L) which encodes 8 tRNAs and the complex I subunit, ND6, and the heavy strand (H) encoding the 2 rRNAs, 14 tRNAs and the remaining 12 ETC subunits. Transcription of the L-strand is initiated from one single promoter [122], whereas the H-strand is initiated from two promoters (HSP1, HSP2) [123]. The HSP1 transcription-initiation site is located upstream of HSP2 and is responsible for producing a transcript that covers the 2 rRNAs and 2 tRNAs; while HSP2 transcribes almost the entire H-strand covering the 2 rRNAs, 14 tRNAs, and 12 respiratory subunits [124]. The loss of AIF was shown to have an impact on the selective subunits ND1, ND2, ND3, ND6 (complex I) along with CO1, CO3 (complex IV) and cytochrome b (complex III). The ability of AIF to control transcription of the L-strand could explain the loss in ND6 mRNA levels with the ablation of AIF. However, the impact of AIF deficiency on the other selective subunits encoded by the H-strand is perplexing. The mitochondrial genome lacks
introns, thus transcription produces a polycistronic precursor RNA that is processed to produce the individual tRNA and mRNA molecules [125-127]. If the selective subunits affected by AIF ablation were encoded continuously at the end of the strand, the data would suggest dysregulation of transcription termination, potentially through interaction with mTERF. However, the genes encoding these selective subunits are spread throughout the strand from beginning to end, providing no rationale or correlation between the current mechanism of mitochondrial transcription and the ability of AIF to regulate transcription of these specific proteins.

Further supporting the complexity of AIF in regulating mitochondrial transcription is the lack of effect AIF ablation has on mitochondrial translation in HeLa cells. Similar to PC3, the loss of AIF in HeLa cells results in decreased expression and activity of complex I of the respiratory chain [71], suggesting AIF plays a similar physiological role in both PC3 and HeLa cells. However, no significant change in mitochondrial translation products was seen upon the depletion of AIF in HeLa cells to correlate with the substantial loss in mitochondrial transcript levels seen in AIF deficient PC3 cells. Therefore, the role of AIF in controlling mitochondrial metabolism through the regulation of mitochondrial transcription appears to be highly contextual, at least within the scope of cancer cells.

As an alternative to the mechanisms of mitochondrial transcription/translation and mitochondrial protein transport, AIF may control ETC protein levels and activity through post-translational modification. The ablation of AIF results in phenotypes similar to what is seen with cellular loss of SIRT3, an NAD-dependent mitochondrial deacetylase whose activity has been shown to regulate oxidative phosphorylation. The ability of AIF to generate NAD+ makes it feasible for AIF to regulate acetylation/deacetylation through sirtuins, and thus control mitochondrial metabolism. In examining whole cell and mitochondrial protein specific
acetylation, however, the overall acetylation status of proteins was found to be unaffected following the knockdown of AIF. And a similar result was seen in assessing the acetylation level of the specific sirtuin substrate, cytochrome c, further suggesting AIF to be uninvolved in regulating the post-translational modification of acetylation/deacetylation.

Overall, the data presented in this study suggests that the mechanisms employed by AIF to direct the mitochondrial proteome do not rely on the regulation of mitochondrial protein import or the acetylation/deacetylation of mitochondrial proteins. The control of mitochondrial transcription and/or mitochondrial translation remains open avenues through which AIF is able to direct energy production to the benefit of prostate cancer cells. The extent to which AIF is able to control these mitochondrial mechanisms appears to be dependent on the context of the cancer environment; further studies are therefore needed to more fully define the scope of AIF influence upon mitochondrial gene expression.
CHAPTER 3

AIF IN THE REGULATION OF A NOVEL, PGAM5-MEDIATED CELL DEATH PATHWAY DISPLAYING CHARACTERISTICS OF MITOPHAGY
INTRODUCTION

The emergence of atypical cell death pathways over the past decade has begun to show that while the different forms of cell death can be distinguished based on certain criteria, there is a significant level of crossover between these pathways that would suggest them to be interrelated. During the atypical response of pyroptosis, for example, characteristics from all three major cell death pathways are represented. When stimulated by bacterial flagellin, the cells first response is to trigger autophagy in an effort to destroy the invading organism [36]. If the level of infection becomes too high, however, the typical apoptotic response of caspase activation occurs, leading to the necrotic characteristics of cell membrane rupture and activation of the immune system [12, 18, 36]. These atypical reactions challenge the current view of seeing cell death as a collection of individual pathways, and argue instead, for viewing it as one extensive cell signaling network. There are a wide variety of stimuli and conditions that can trigger cell death, but not all cells will necessarily die in response to the same stimulus. Therefore, viewing cell death as one complete system allows for the outcome of triggering such a network to be completely dependent on the cell type, stimulus and desired immunological response. This alternative view allows for proteins that commonly serve a pro-survival role, such as caspase-1 in pyroptosis, to occasionally facilitate cell death under specific conditions, and explains how proteins thought to operate strictly within one cell death pathway are emerging as players in more than one pathway with the ability to induce a tailored set of death characteristics depending on the above criteria.

As previously mentioned, AIF is a mitochondrial flavoprotein discovered for its ability to promote apoptosis through chromatin condensation and DNA cleavage [36, 37, 39, 44, 45]. Over the past decade, this pro-death function has been shown to serve a pivotal role only in
certain cell types such as neurons and cardiomyocytes, and is limited to specific stimuli, including DNA-damaging agents, engagement of death receptors, oxidative stress, excitotoxins, and hypoxia–ischemia [57, 58, 60, 61]. Along with an ever expanding list of death signaling pathways that converge on AIF, there have been various characteristics that suggest AIF is customarily a pro-survival molecule that only serves a pro-death function under definitive circumstances (see Chapter 1). In an effort to distinguish the pro-life functions of AIF from its pro-death activities, we performed a biochemical screen to isolate proteins that interact with AIF in healthy mitochondria under normal conditions. Among the proteins identified was phosphoglycerate mutase family member 5 (PGAM5) (Table I), a newly emerging protein gaining notice for its potential involvement in regulating multiple cell death pathways [128, 129]. Similar to AIF, PGAM5 has been reported to reside in the inner membrane of the mitochondria and exhibits dual characteristics as a pro-survival and pro-death molecule.

PGAM5 exists in two isoforms due to alternative splicing of a 3’ exon: PGAM5 short (PGAM5s) and PGAM5 long (PGAM5l). The first 239 amino acids are identical containing an N-terminal mitochondrial localization sequence (MLS), a highly conserved PGAM domain, an NXESGE motif required for binding the Nrf-2 inhibitor, Keap1, and a BH3-like domain necessary for interacting with the Bcl-2 family member, Bcl-XL (Figure 8A) [130]. Although the PGAM domain is similar to that seen in other phosphoglycerate mutases, neither PGAM5 isoform possesses the typical mutase activity common to members of the PGAM family [129]. Instead, PGAM5l has been shown to utilize the catalytic core found within this domain to function as a Ser/Thr phosphatase, with a known substrate for this activity being the mitogen-activated protein kinase kinase kinase (MAPKKK), apoptosis signal regulating kinase 1 (ASK1) [129]. ASK1 is responsible for activating the JNK and p38 MAPK pathways which, depending
on the cell type and stimulus, leads to a variety of cellular responses that include apoptosis [131]. The phosphatase function of the PGAM5\textsubscript{S} isoform remains elusive due to a significant lack in research compared to PGAM5\textsubscript{L}. However, a recent study by Wang et al. proposed a potential role for both PGAM5\textsubscript{S} and PGAM5\textsubscript{L} phosphatase activity in the atypical cell death pathway of necroptosis [19]. Both isoforms have been shown to associate with the RIP1/RIP3 complex during necroptosis, leading to the dephosphorylation and subsequent activation of the mitochondrial fission protein, Drp1 [128]. Drp1 was shown to coprecipitate with PGAM5\textsubscript{S} but not PGAM5\textsubscript{L}, suggesting the PGAM5\textsubscript{S} isoform to be ultimately responsible for the activation of Drp1. However, the ability of PGAM5\textsubscript{S} to dephosphorylate Drp1 was never demonstrated due to recombinant PGAM5\textsubscript{S} being insoluble under assay conditions. Therefore, it has yet to be established if PGAM5\textsubscript{S} possesses the same ser/thr phosphatase function as PGAM5\textsubscript{L}.

Distinct from possessing phosphatase activity, PGAM5\textsubscript{S} and PGAM5\textsubscript{L} have been shown to associate with the antioxidant response inhibitor, Keap1, and the anti-apoptotic Bcl-2 family member, Bcl-X\textsubscript{L} [130]. Keap1 is a BTB-Kelch substrate adaptor protein for a Cul3-dependent E3 ubiquitin ligase that targets the anti-oxidant transcription factor, Nrf2 [132]. Nrf2 is a major regulator of cytoprotective genes which encode enzymes that neutralize reactive molecules, eliminate damaged macromolecules and restore cellular redox homeostasis [133]. Under normal conditions, Keap1 maintains a low level of expression and activity of Nrf2 by marking it for proteosomal degradation, and subsequently repressing Nrf-2 dependent gene expression. During oxidative stress, the rise in cellular levels of reactive oxygen species (ROS) induces a modification of Keap1 which results in the decreased ubiquitination of Nrf2 and an increase in steady-state levels [134]. Although both PGAM5 isoforms have been shown to bind to Keap1 only PGAM5\textsubscript{L} has been reported to form a ternary complex with Keap1 and Nrf2, sequestering
these proteins at the mitochondrial membrane and contributing to the repression of Nrf2-dependent gene expression [135]. While the physiological purpose of this activity has yet to be determined this data implicates a potential role for PGAM5 in regulating anti-oxidant response.

The ability of PGAM5 to associate with Bcl-X\textsubscript{L} was first identified when PGAM5 was captured from a selective screen for potential Bcl-X\textsubscript{L}-binding partners [136]. Bcl-X\textsubscript{L} is a pro-survival protein responsible for preventing the permeabilization of the outer mitochondrial membrane and subsequent release of mitochondrial death proteins [137]. While the efficacy of the association between PGAM5 and Bcl-X\textsubscript{L} remains unknown, interaction between these two proteins has been shown to lead to the indirect ubiquitination and degradation of Bcl-X\textsubscript{L} due to PGAM5 bringing Bcl-X\textsubscript{L} into the proximity of Keap1 [138]. This Keap1-dependent degradation of Bcl-X\textsubscript{L} results in increased sensitivity to drug-induced apoptosis, and provides a potential avenue through which PGAM5 may be inducing cell death [138].

The \textit{Drosophila melanogaster} ortholog of mammalian PGAM5 (dPGAM5) has been shown to exacerbate mitochondrial degeneration and neuronal cell death in the \textit{Drosophila} model of Parkinson’s disease induced by mutation of PTEN-induced kinase 1 (PINK1), a serine/threonine kinase associated with early-onset autosomal recessive Parkinson’s disease [139, 140]. Because PINK1 has been shown to play a critical role in the induction of mitophagy, the genetic interaction of PINK1 and PGAM5 suggests that PGAM5 might be involved in mitochondrial quality control [141]. Furthermore, the inactivation of dPGAM5 function results in elongated mitochondria while transgenic expression promotes mitochondrial fragmentation, suggesting dPGAM5 to play a role in the dynamic process of mitochondrial fission [139].
In this study, we identify PGAM5\textsubscript{S} and PGAM5\textsubscript{L} to be novel binding partners of AIF. While exploring the interaction between these proteins, both PGAM5 isoforms were shown to induce cell death unrelated to the caspase-independent apoptotic function of AIF. Rather than helping to facilitate this newfound death characteristic of PGAM5, AIF plays a protective role by attenuating caspase activation that occurs as a consequence of this activity. Contrary to the known functions of PGAM5 previously implicated in cell death, this newly uncovered pathway does not rely upon the ability of PGAM5 to participate in dephosphorylation or interact with Keap1 and Bcl\textsubscript{-}XL. Transmission electron microscopy reveals a detrimental loss in mitochondrial number and severe aberrant mitochondrial morphology following the expression of either PGAM5 isoform. Overall, the data reveal a novel cell death role for PGAM5\textsubscript{S} and PGAM5\textsubscript{L} that may be connected to the potential activities of PGAM5 in mitochondrial dynamics and/or clearance.

**EXPERIMENTAL PROCEDURES**

**Materials.** Reagents were obtained as follows: protein G-coupled agarose, Glutamax, tetramethylrhodamine methyl ester (TMRM) and phosphate-buffered saline from Invitrogen; fetal bovine serum from HyClone; Dulbecco modified Eagle medium (DMEM) from Mediatech; Ser/Thr phosphatase assay system from Promega; DEVD-7-amino-4-trifluoromethyl coumarin from BioMol; and site-directed mutagenesis kit from Stratagene. All other chemicals were from Sigma. Antibodies were obtained as follows: anti-AIF (#SC13116) from Santa Cruz Biotechnology; anti-ASK1 (#sc-7931) from Santa Cruz; anti-Keap1 [142], anti-Bcl-X\textsubscript{L} [89], anti-phosphorylated JNK [143] and anti-phosphorylated p38 (#9216) from Cell Signaling; anti-
Bcl-XL (#610746) from BD Transduction Laboratories; horseradish peroxidase (HRP)-conjugated anti-FLAG (#A8592), HRP-conjugated anti-hemagglutinin (anti-HA) (#H6533) and anti-Flag (#F3165) from Sigma; anti-HA from Covance (#MMS-101P); and HRP-conjugated anti-mouse (#NA931V) and anti-rabbit (#NA934V) from Amersham.

**Cell culture, transfection and plasmids.** HEK 293 cells were grown in DMEM containing 10% fetal bovine serum supplemented with 2 mM Glutamax at 37°C in an atmosphere of 95% air and 5% CO₂. Transfections were performed by the method of calcium phosphate precipitation as described previously [100]. pEBB-AIF-TAP (full-length) and pEBB-AIF-N102-TAP were generated by subcloning WT and N102 AIF into the pEBB tandem-affinity purification (TAP) plasmid. pCDNA3-ASK1-HA [131] was obtained with permission from Jonathan D. Ashwell at the National Institute of Health. pEBB PGAM5₅, pEBB PGAM5₇, pEBB AIF were generated by PCR using an expressed sequence tag clone containing human PGAM5₅ (Image clone #3048106) from Open Biosystems and PGAM5₇ (Image clone #4525757) from ATCC, and full length AIF (Image clone #5740894). pEBB PGAM5₅ and pEBB PGAM5₇ were used to further subclone pEBB HA-PGAM5₅, pEBB PGAM5₅-HA, pEBB Flag-PGAM5₅, pEBB HA-PGAM5₇, pEBB PGAM5₇-HA and pEBB Flag-PGAM5₇. pEBB Δ54AIF and pEBB Δ102AIF were generated by PCR using pEBB AIF as template. pEBB PGAM5 H105A, pEBB PGAM5 E79A/S80A, and pEBB PGAM5 L135E/F139E mutants were generated by site-directed mutagenesis (Stratagene) using pEBB HA-PGAM5₅ and HA-PGAM5₇ as template.

**Cell lysis, immunoblot analysis and immunoprecipitations.** Cell lysates were prepared in either Laemmlı (625 nM Tris-HCl pH 6.8, 2% SDS, 10% Glycerol, 5% β-mercaptoethanol) or 0.5% SDS lysis buffer (50 mM Tris-HCl, 0.5% SDS, 150 mM NaCl, 1% Na-Deoxycholate, 1% Triton X-100, 10 mM EDTA, 1 mM PMSF), normalized for protein content, and then separated by
SDS-polyacrylamide gel electrophoresis (PAGE) [128] using 4 to 12% gradient SDS-polyacrylamide gels (Invitrogen). For immunoblot analysis, SDS-PAGE was followed by transfer to nitrocellulose membranes (Invitrogen), which were then blocked with 5% milk in Tris-buffered saline containing 0.02 to 0.2% Tween, followed by incubation with the indicated antibodies for 1 h at room temperature. Following washing, membranes were incubated with HRP-conjugated anti-mouse IgG or anti-rabbit IgG secondary antibodies for 45 min at room temperature and visualized by enhanced chemiluminescence. For immunoprecipitation experiments, cell lysates (0.5% SDS lysis buffer) were normalized for protein content and incubated with indicated antibodies for 2 h at 4°C. Protein G-coupled agarose beads were then added and incubated for 1 h. Agarose beads were recovered by centrifugation and washed in 0.5% SDS buffer, precipitated proteins were eluted by adding lithium dodecyl sulfate sample buffer and heating the mixture to 95°C for 5 min. Recovered proteins were then separated by electrophoresis, and immunoblot analysis was carried out as described above.

**Tandem Affinity Purification.** As described in Chapter 2.

**Lentiviral knockdown.** Lentiviral knockdown AIF in HEKT 293 cells was performed as previously described [144]. Briefly, FG12 derived plasmids were combined with equal amounts of lentiviral packaging plasmids and transfected into HEK293T cells by calcium phosphate precipitation as described [103]. 48 h after transfection, supernatants were harvested, filtered using 0.45-μm-pore size Millex HV PVDF filter units (Millipore), and concentrated by centrifugation at 60,000 × g for 90 min. The supernatants were aspirated, and virus-containing pellets were resuspended in PBS overnight at 4 °C. One day before infection, target cells were seeded at 60,000 cells/well in 12-well plates. At the time of infection, polybrene was added to a final concentration of 25 mM, resuspended virus was added, and cells were incubated for 4 h at
37 °C in an atmosphere of 93% air, 7% CO₂. Virus containing supernatants were then removed, fresh medium was added to cells, and cells were incubated for an additional 2–3 days in an atmosphere of 95% air, 5% CO₂. Stable incorporation of FG12-based lentiviral DNA was determined by immunoblot analysis.

**Viability experiments.** Cells were seeded at 300,000 cells per well in 6-well plates and allowed to attach overnight. Cells were then transfected with pEBB empty vector, pEBB HA-PGAM5S, pEBB HA-PGAM5L, pCDNA3-Bax, and other plasmids; then incubated at 37°C for 48 hours. Cells were harvested, washed, and resuspended in PBS containing 2μg/mL propidium iodide. Cell viability was then determined by flow cytometry using and Accuri C6 flow cytometer.

**Mitochondrial membrane potential experiment.** Cells were seeded in six-well plates; transfected 24 hours later with pEBB empty, pEBB HA-PGAM5S, pEBB HA-PGAM5L, and pCDNA3-Bax; then incubated at 37°C for 24 hours. Mitochondrial membrane potential was assessed by resuspending cells in 200 nM tetramethylrhodamine methyl ester (TMRM) and measuring fluorescence with an Accuri C6 flow cytometer.

**Caspase activity assay and inhibition.** Cells were seeded at 300,000 cells per well in 6-well plates and allowed to attached overnight. Cells were transfected with pEBB empty vector, pEBB HA-PGAM5S, pEBB HA-PGAM5L, pCDNA3-Bax, and other plasmids; then incubated at 37°C for 48 hours. Floating and attached cells were harvested, and caspase 3 assays were performed as described previously [145].

**Phosphatase assay.** Cells were seeded in 10 cm plates; transfected 24 hours later with HA or Flag tagged PGAM5S and PGAM5L wildtype and mutant plasmids. Cell lysates prepared 24 hours later (0.5% SDS lysis buffer) were normalized for protein content and incubated with
indicated antibodies for 2 h at 4°C. Protein G-coupled agarose beads were then added and incubated for 1 h. Agarose beads were recovered by centrifugation and phosphatase activity measured according to manufacturers protocol (Promega) using PPase-2C buffer (250 mM Imidazole pH 7.2, 1 mM EGTA, 25 mM MgCl₂, 0.1% β-mercaptoethanol, 0.5 mg/mL BSA).

Transmission electron microscopy. Cells were seeded in 10 cm plates; transfected 24 hours later with pEBB empty, pEBB HA-PGAM5S, or pEBB HA-PGAM5L. Cells were harvested between 24 and 36 hr post-transfection, fixed in 2.5% glutaraldehyde, and embedded in Spurr’s resin. Sections were cut with a Reichert Ultracut E ultramicrotome and counterstained with lead citrate and uranyl acetate. Digital images were taken using a FEI Tecnai Spirit 12 Bio Twin TEM at 80 kV adapted with a AMT 2 Vu digital camera and AMT proprietary software.

RESULTS

PGAM5S and PGAM5L are novel interacting partners of AIF. While a majority of AIF research has centered on its cell death activity, significant questions remain regarding the biological activity of AIF within a healthy cell. Currently there are few validated factors with which AIF interacts in a healthy cell that could help to establish a general paradigm for AIF biology under homeostatic conditions. In an effort to resolve this discrepancy, we employed a biochemical screen to identify potential AIF associated proteins from healthy mitochondria (see Chapter 2). A number of novel AIF-associated proteins were identified which included the mitochondrial phosphoglycerate mutase, PGAM5 (Table I). To further validate the interaction between AIF and PGAM5, hemagglutinin (HA)-tagged PGAM5S and PGAM5L were co-expressed with FLAG-tagged proteins of either an empty vector (control) or the mature form of AIF (Δ54
AIF) in HEK 293T cells. AIF was then immunoprecipitated using an anti-FLAG antibody and the presence of PGAM5\textsubscript{S} and PGAM5\textsubscript{L} detected by immunoblot. Both PGAM5\textsubscript{S} and PGAM5\textsubscript{L} co-precipitated with AIF, confirming the association between these two proteins indicated by the TAP screen (Figure 8B).

**PGAM5\textsubscript{S} and PGAM5\textsubscript{L} trigger novel cell death pathway.** While investigating potential interactions between AIF and PGAM5, we discovered overexpression of PGAM5\textsubscript{S} or PGAM5\textsubscript{L} triggers morphological changes indicative of apoptosis. To measure cell death following PGAM5\textsubscript{S} and PGAM5\textsubscript{L} expression, a propidium iodide fluorescence exclusion assay was performed. HEK 293T cells were transfected with an empty vector (control), PGAM5\textsubscript{S}, PGAM5\textsubscript{L}, or the Bcl-2 associated protein, Bax. Bax is a pro-apoptotic member of the Bcl-2 family responsible for forming pores in the mitochondrial membrane which allow for the release of cytochrome c and subsequent induction of cell death; it is used to serve as a positive control [14]. Following transfection, all cells were harvested and resuspended in propodium iodide, a cell impermeable dye whose fluorescence is enhanced upon intercalation with DNA [146]. Fluorescence was then assessed using flow cytometry and cell viability was calculated as the percent of cells with no change in fluorescence. Expression of both PGAM5\textsubscript{S} and PGAM5\textsubscript{L} resulted in more than a 50 percent loss of cell viability compared to control (Figure 9A), similar to what is seen with Bax, confirming the morphological changes following PGAM5 overexpression are coincident with cell death.

Previous studies have implicated PGAM5\textsubscript{S} and PGAM5\textsubscript{L} in the cell death pathways of apoptosis and necrosis; therefore we tested the activity of PGAM5 within the apoptotic pathway [128, 129]. During apoptosis, the activation of caspases occurs through two major pathways:
Figure 8. PGAM5\textsubscript{S} and PGAM5\textsubscript{L} associate with AIF. A) Schematic representation of the two isoforms of PGAM5 containing relevant regions of the protein. B) Plasmids encoding an empty vector (control), HA-tagged PGAM5\textsubscript{S} or HA-tagged PGAM5\textsubscript{L} were co-transfected with either an empty vector (control), Flag-tagged full length AIF (FL AIF), or Flag-tagged mature AIF (Δ54 AIF) in HEK 293T cells. AIF was immunoprecipitated using an anti-Flag antibody 24 hr post-transfection. Input lysates and immunoprecipitates were run on SDS-PAGE gel and analyzed by western blotting for the presence of PGAM5 using an anti-HA antibody.
extrinsic and the intrinsic pathway (see Chapter 1). The relationship between PGAM5 and the Bcl-2 family member, Bcl-X<sub>L</sub>, along with a lack of TNFR receptor stimulation, suggests PGAM5<sub>S</sub>- and PGAM5<sub>L</sub>-mediated cell death to be occurring in an intrinsic manner. A way to assess activation of the intrinsic pathway is to establish permeabilization of the outer mitochondrial membrane (OMM). To determine if permeabilization of the OMM occurs following PGAM5 expression, HEK 293T cells were transfected with an empty vector (control), PGAM5<sub>S</sub>, PGAM5<sub>L</sub>, or Bax. Following transfection, cells were incubated with tetramethylrhodamine methyl ester (TMRM), a fluorescent dye used to label and measure the membrane potential of mitochondria, and analyzed by flow cytometry [147]. A loss in mitochondrial membrane potential (MMP) indicates permeabilization of the mitochondrial membrane. The expression of both PGAM5 isoforms leads to almost a 20% decrease in MMP compared to control (Figure 9B). While it does not produce as significant of a loss in MMP as Bax, the permeabilization of the mitochondrial membrane is significant enough to lead to the activation of caspase-3. The activity of caspase-3 was assessed 48 hr post-transfection using an AFC-derived caspase-3 substrate [145]. Both PGAM5 isoforms were able to induce a significant amount of caspase-3 activity compared to control (Figure 9C). These data are consistent with a role for PGAM5<sub>S</sub> and PGAM5<sub>L</sub> in promoting cell death through the intrinsic pathway.

**Labeling and extraction of PGAM5<sub>S</sub> and PGAM5<sub>L</sub>.** For isolation and detection of PGAM5<sub>S</sub> and PGAM5<sub>L</sub>, we engineered HA-epitope tagged versions of each isoform. To ensure placement of the tag did not interfere with the expression or activity of either isoform, we performed a caspase assay and immunoblot analysis on both N-terminal and C-terminal tagged variations of these proteins. Placing an HA-tag at the N-terminus of PGAM5<sub>S</sub> and PGAM5<sub>L</sub> resulted in a
Figure 9. PGAM5_S and PGAM5_L promote intrinsic cell death. A) HEK 293T cells were transfected with an empty vector (control), PGAM5_S, PGAM5_L or the pro-apoptotic Bcl-2 family member, Bax. Forty-eight hours following transfection, cell viability was assessed using propidium iodide staining and flow cytometry. Data were derived from three independent experiments and are presented as the means ± SEM. B) Mitochondrial membrane potential (MMP) was assessed using tetramethylrhodamine (TMRM) and flow cytometry following overnight transfection. Data were derived from triplicate samples and represented as percentage of cells maintaining high MMP (ΔΨ_m). C) HEK293T cells were transfected with empty vector (control), PGAM5_S, or PGAM5_L. Cell lysates were subjected to a caspase 3 DEVD-AFC assay 48 hours post transfection, data presented as the means ± SEM obtained from three individual experiments. *p < 0.05 compared to control.
level of expression significantly lower than what is seen when the tag is placed at the C-terminus (Figure 10A). Moreover, cells expressing N-terminal tagged PGAM5<sub>S</sub> and PGAM5<sub>L</sub> showed diminished caspase activity compared to PGAM5 C-terminal tagged proteins, with N-terminal tagged PGAM5<sub>L</sub> in particular showing a level of activity only slightly above control (Figure 10C). Like all nuclear-encoded mitochondrial proteins, PGAM5<sub>S</sub> and PGAM5<sub>L</sub> have a mitochondrial localization signal (MLS) located at the N-terminus [116, 117]. The loss in expression and activity of N-terminal tagged protein compared to the C-terminal tagged version may be due to the epitope interfering with the import and localization of the protein. In light of these observations, C-terminal tagged versions of PGAM5<sub>S</sub> and PGAM5<sub>L</sub> were chosen for use in these studies.

While both isoforms are confirmed mitochondrial residents, there is controversy over their specific localization to the outer or inner mitochondrial membrane [135, 148]. In working with both isoforms, we have discovered the PGAM5<sub>S</sub> isoform requires a more stringent buffer for extraction than PGAM5<sub>L</sub>, an observation supported in a recent publication by Wang et al [128]. Following transfection with either PGAM5<sub>S</sub> or PGAM5<sub>L</sub>, HEK 293T cells were harvested and lysed with buffers containing various detergent concentrations; immunoblot analysis was then used to determine the level of PGAM5 extraction. While PGAM5<sub>L</sub> was easily detected under both low and high detergent concentrations, PGAM5<sub>S</sub> was only extracted by the buffer containing 2% sodium dodecyl sulfate (SDS) (Figure 10B); indicating a potential difference in localization, hydrophobicity and/or protein-protein interactions between PGAM5<sub>S</sub> and PGAM5<sub>L</sub>.

**PGAM5<sub>S</sub> and PGAM5<sub>L</sub> induce caspase-independent cell death unrelated to AIF.** Although intrinsic apoptosis is typically a caspase-dependent pathway there are alternative models, notably
Figure 10. Labeling and extraction of PGAM5<sub>S</sub> and PGAM5<sub>L</sub>. A) HEK293T cells were transfected with C-terminal or N-terminal HA-tagged PGAM5<sub>S</sub> and PGAM5<sub>L</sub>. Whole cell lysates were prepared 24 hours post-transfection and analyzed by immunoblotting with anti-HA antibody. B) HEK 293T cells were transfected with C-terminal HA-tagged PGAM5<sub>S</sub> or PGAM5<sub>L</sub>. Whole cell lysates were prepared using a range of buffers containing various detergent concentrations and extraction efficiency was analyzed by immunoblotting with anti-HA antibody. C) HEK293T cells were transfected with empty vector (control), C-terminal HA-tagged PGAM5<sub>S</sub> and PGAM5<sub>L</sub>, or N-terminal HA-tagged PGAM5<sub>S</sub> and PGAM5<sub>L</sub>. Caspase 3 activation was assessed in cell lysates 48 hours post transfection using DEVD-AFC derived substrate and time resolved fluorescence. *, p < 0.01 compared to N-terminal tagged PGAM5<sub>S</sub>; **, p < 0.05 compared to N-terminal tagged PGAM5<sub>L</sub>. 
AIF-induced cell death, which can occur in the absence of caspase activation [149]. To determine if PGAM5 S and PGAM5 L are promoting cell death in a caspase-dependent manner, cell viability was assessed following expression in the absence and presence of ZVAD, a pan-caspase inhibitor. While treatment with ZVAD completely blocked caspase-3 activation following PGAM5 S and PGAM5 L expression (Figure 11A), it failed to protect the cells from PGAM5-induced cell death (Figure 11B). Therefore, while caspase activation occurs following PGAM5 expression it is not required in order for PGAM5 to trigger cell death.

Since both isoforms associate with AIF, a well-known caspase-independent pro-apoptotic protein, PGAM5 S and PGAM5 L could be promoting cell death through interaction with AIF. To determine if AIF plays a role in PGAM5-induced cell death, cell viability following PGAM5 expression was assessed in AIF deficient HEK 293T cells. Lentivirus was used to stably infect HEK 293T cells with shRNA sequences targeting either AIF or LacZ as a negative control [103]. Cells stably incorporating hairpin DNA were detected by GFP fluorescence and AIF ablation was confirmed by immunoblot analysis (Figure 12A). Once knockdown of AIF was confirmed, the parental (H2T parental), shLacZ (H2T shLacZ) and shAIF (H2T shAIF) cell lines were transfected with PGAM5 S or PGAM5 L and cell viability was assessed at 48 hours post-transfection. Both PGAM5 isoforms were able to promote a significant loss in cell viability in the presence and absence of AIF (Figure 12B), suggesting PGAM5 does not induce caspase-independent cell death through its association with AIF.

Surprisingly, however, AIF does have the ability to partially inhibit caspase-3 activation resulting from expression of either PGAM5 isoform. The unprocessed form (F.L), mature form (Δ54) or apoptotic form (Δ102) of AIF were co-expressed with PGAM5 S and caspase activity was assessed 48 hours post-transfection. All three forms of AIF were able to produce a
Figure 11. PGAM5<sub>S</sub> and PGAM5<sub>L</sub> promote caspase-independent cell death. A) Empty vector (control), PGAM5<sub>S</sub>, or PGAM5<sub>L</sub> was transfected into HEK 293T cells. Cells were treated with 50 μM ZVAD at 10 hr post-transfection. Cell lysates were subjected to a caspase 3 DEVD-AFC assay 48 hours post transfection, data presented as the means ± SEM obtained from three individual experiments. B) Empty vector (control), PGAM5<sub>S</sub>, or PGAM5<sub>L</sub> was transfected into HEK 293T cells. Cells were treated with 50 μM ZVAD at 10 hr post-transfection. 48h post-transfection cells were harvested and viability was measured using propidium iodide and flow cytometry. *, p < 0.001 compared to control without ZVAD; **, p < 0.001 compared to control with ZVAD.
**Figure 12. AIF is not required for PGAM5<sub>S</sub> and PGAM5<sub>L</sub> to promote cell death.**  A) Protein expression of AIF in the H2T parental, shLacZ, and shAIF cell lines was detected by immunoblotting with AIF antibody. Beta-actin was detected as a loading control. B) H2T parental, shLacZ and shAIF cells were transfected with empty vector (control), PGAM5<sub>S</sub>, or PGAM5<sub>L</sub>. 48 hours post transfection cells were harvested and viability measured using propidium iodide and flow cytometry, data presented as the means ± SEM obtained from three individual experiments. *, p < 0.05 compared to H2T Parental control; ** p < 0.05 compared to H2T shLacZ control; ‡, p < 0.05 compared to H2T shAIF control.
significant reduction in PGAM5S-induced caspase activity compared to wildtype (Figure 13A); however, this partial inhibition of caspase-3 is not significant enough to prevent a loss in cell viability (Figure 13B). The same results were seen for the long isoform when co-expressed with the different forms of AIF (Figure 13C and 13D). Overall, this data reveals an unexpected, protective role for AIF in attenuating caspase-activation. Strikingly, this activity does not prevent PGAM5-mediated cell death, and moreover, the presence of AIF is not essential to the ability of PGAM5 to kill.

**PGAM5S and PGAM5L phosphatase function does not play a role in cell death activity.** The phosphatase activity of PGAM5L has been shown to trigger the ser/thr kinase, ASK1, which plays a significant role in regulating the p38 and JNK pathways (Figure 14A) [129, 131]. Activation of these pathways can trigger a variety of cellular responses, depending on stimulus and cell type, which include apoptosis [150]. To determine if the cell death being triggered by PGAM5S and PGAM5L is a result of increased activation of these pathways we performed a western blot using antibodies against phosphorylated p38 and JNK following the expression of each isoform. The overexpression of ASK1 resulted in increased phosphorylation of both p38 and JNK, while PGAM5S and PGAM5L expression did not have an effect on either pathway (Figure 14B). These data conflict with what has been previously reported for PGAM5L, and suggests the activation of ASK1 is not involved in PGAM5-mediated cell death. The reasons for this discrepancy are unclear, but may center on the ability of exogenous PGAM5L expression to promote the activation of p38 and JNK, an activity that may exhibit a dependence upon cell type and level of expression. Nonetheless, the level of exogenous PGAM5 used to promote cell death presented in this study is not sufficient enough to promote the activation of ASK1; discounting
Figure 13. AIF inhibits caspase activation following PGAM5 upregulation. A and B) Empty vector (control), full-length AIF (FL-AIF), mature AIF (∆54 AIF), or apoptotic AIF (∆102 AIF) were transfected with or without PGAM5 in HEK 293T cells. Cell lysates were subjected to a caspase 3 DEVD-AFC (A) and cell viability (B) assay 48 hours post transfection. C and D) Empty vector (control), full-length AIF (FL-AIF), mature AIF (∆54 AIF), or apoptotic AIF (∆102 AIF) were transfected with or without PGAM5 in HEK 293T cells. Cell lysates were subjected to a caspase 3 DEVD-AFC (C) and cell viability (D) assay 48 hours post transfection. *, p < 0.05 compared to control; **, p < 0.05 compared to wildtype; †, p > 0.05 compared to wildtype.
Figure 14. The upregulation PGAM5<sub>S</sub> and PGAM5<sub>L</sub> does not activate ASK1-mediated MAPK pathway. A) Cartoon schematic illustrating the role of PGAM5 in the ASK1-mediated MAPK pathway, adapted from the literature. B) HEK 293 cells were transiently transfected with an empty vector (control), ASK1, PGAM5<sub>S</sub> or PGAM5<sub>L</sub>. Whole cell lysates were prepared at 48 hours post-transfection and western blot analysis was performed using phosphorylated p38 and JNK antibodies.
ASK1-mediated apoptosis in the ability of PGAM5 to induce cell death.

Along with ASK1, the phosphatase activity of PGAM5S and PGAM5L has been implicated in the activation of Drp1 during necroptosis; providing another avenue through which PGAM5 could promote cell death [128]. On the same note, it may be an unknown substrate of PGAM5 phosphatase activity allowing for PGAM5-mediated apoptosis. To establish the overall relevance of PGAM5S or PGAM5L phosphatase function in its ability to promote cell death we created a point mutant lacking phosphatase activity. Mutating a histidine within the PGAM domain at position 105 to alanine has been shown to inhibit phosphatase activity in PGAM5L [129]. We were able to recreate this loss in activity seen previously with the long isoform (Figure 15A) while maintaining similar levels of expression (Figure 15B). To assess phosphatase activity, PGAM5L wildtype and the PGAM5L H105A mutant were immunoprecipitated at 24 hours post-transfection, incubated with phosphorylated Ser/Thr substrate, and the amount of free phosphate available was measured after a period of 15 minutes. PGAM5L wildtype produced approximately 450 pmol of free phosphate, while PGAM5L H105A showed activity similar to control (Figure 15A); confirming a loss in phosphatase function of the H105A mutant. Once inhibition of phosphatase activity in the PGAM5L H105A mutant was confirmed, caspase activation and cell viability were reassessed. PGAM5L H105A was able to activate caspase 3 and induce cell death similar to wildtype (Figure 15C and 15D), indicating the phosphatase function of PGAM5L to be unrelated its ability to induce cell death.

In contrast to PGAM5L, we were unable to detect any phosphatase activity for the PGAM5S isoform. The expression and immunoprecipitation of PGAM5S was confirmed by western blot preceding incubation with phosphorylated substrate (Figure 16B inset); however, no activity was seen by PGAM5S compared to the PGAM5L isoform (Figure 16A). Therefore, we
Figure 15. PGAM5L phosphatase activity is not relevant to cell death function. A) Empty plasmid (control), PGAM5L wild-type, and PGAM5L H105A were transiently expressed in HEK 293T cells. PGAM5L proteins were precipitated at 24h post-transfection and incubated with a phosphorylated ser/thr peptide substrate. Phosphatase activity was measured as the amount of free phosphate available following 15 minutes of incubation. B) Immunoblot analysis of PGAM5L wildtype and H105A expression in phosphatase assay. C) HEK293T cells were transfected with empty vector (control), PGAM5L wildtype, or PGAM5L H105A. Cell lysates were subjected to a caspase 3 DEVD-AFC assay 48 hours post transfection, data presented as the means ± SEM obtained from three individual experiments. D) PGAM5L variants or plasmid control were transiently expressed in HEK293 cells. 48h after transfection cells were harvested and viability measured using propidium iodide and flow cytometry. *, p < 0.001 compared to control; ** p < 0.01 compared to wildtype; † p > 0.05 compared to wildtype.
must assume either PGAM5\textsubscript{S} does not possess Ser/Thr phosphatase activity, or similar to another family member, Sts-1/TULA2, it prefers tyrosine as its substrate [151]. Regardless, to help overall negate the potential phosphatase activity of PGAM5\textsubscript{S} playing a role in PGAM5-induced cell death, we created and assessed a PGAM5\textsubscript{S} H105A mutant. As seen with PGAM5\textsubscript{L} H105A, PGAM5\textsubscript{S} H105A was able to activate caspase-3 and promote a loss in cell viability similar to wildtype (Figure 19A and 19B); therefore, any potential phosphatase function related to PGAM5\textsubscript{S} is unrelated to its ability to induce cell death.

**AIF interacts with PGAM5 substrate, ASK1.** Given that both ASK1 and AIF have the ability to promote apoptosis in response to oxidative stress and have been shown to interact with PGAM5\textsubscript{S} and PGAM5\textsubscript{L}, we wanted to investigate a potential association between ASK1 and AIF. To determine if these two proteins interact within the cell, we immunoprecipitated ASK1 from HEK 293T cells and blotted for the presence of AIF. While endogenous ASK1 basal levels are virtually undetectable (input), immunoprecipitation allowed for the concentration of ASK1 and the detection of AIF (Figure 17A); confirming the ability of these two proteins to bind one another. The molecular size of AIF detected is between 64 and 51 kDa, indicating ASK1 to associate with either the mature form of AIF (62 kDa) found in the mitochondria, or the apoptotic form (57 kDa) found in the cytoplasm. ASK1 is localized to both the cytoplasm and the mitochondria, however, the lack of death stimulus prior to immunoprecipitation would favor that ASK1 is interacting with the mature form of AIF rather than the apoptotic form [152, 153]. ASK1 has been shown to promote apoptosis through the release of cytochrome c and the subsequent activation of caspase-9 and caspase-3 [153]. To establish if AIF is able to augment or attenuate ASK1-mediated cell death, we co-expressed ASK1 with the unprocessed form (F.L),
Figure 16. **PGAM5\textsubscript{S} phosphatase activity remains undetected.** A) Empty plasmid (control), Flag-tagged PGAM5\textsubscript{S} wild-type, and Flag-tagged PGAM5\textsubscript{L} wild-type were transiently expressed in HEK 293 cells. PGAM5 proteins were precipitated at 24h post-transfection, and incubated with a phosphorylated peptide substrate. Phosphatase activity was measured as the amount of free phosphate available following 15 minutes of incubation. B) Immunoblot confirming expression and precipitation of PGAM5\textsubscript{S} and PGAM5\textsubscript{L} prior to incubation with phosphorylated substrate.
Figure 17. A novel interaction between ASK1 and AIF. A) Immunoprecipitation of endogenous ASK1 together with endogenous AIF in HEK 293T cells. Input lysates, control IP with IgG, and ASK1 IP were run on SDS-PAGE gel and analyzed by western blotting. B) Empty vector (control), full-length AIF (FL-AIF), mature AIF (∆54 AIF), or apoptotic AIF (∆102 AIF) were transfected with or without ASK1 in HEK 293T cells. Cell lysates were subjected to a caspase 3 DEVD-AFC assay 48 hours post transfection, data presented as the means ± SEM obtained from three individual experiments. C) Empty vector (control), PGAM5_S and PGAM5_L were transfected with or without ASK1 in HEK 293T cells. Cell lysates were subjected to a caspase 3 DEVD-AFC assay 48 hours post transfection, data presented as the means ± SEM obtained from three individual experiments.
mature form (Δ54) or apoptotic form (Δ102) of AIF and measured caspase-3 activity at 48 hours post-transfection. Concurrently, we co-expressed ASK1 with either PGAM5 isoform to assess the effect PGAM5 has on ASK1-induced caspase activity.

The expression of ASK1 alone resulted in a significant amount of caspase-3 activation compared to control, with the addition of either PGAM5 isoform producing a level of caspase activity suggestive of an additive effect rather than synergistic (Figure 17C). These data indicate ASK1 and PGAM5 are promoting caspase activation through separate mechanisms, further supporting PGAM5-mediated cell death being independent of ASK1. Co-expression with the various forms of AIF had no affect on ASK1-induced caspase activity (Figure 17B), suggesting AIF to be uninvolved in this aspect of ASK1-mediated cell death. Therefore, while ASK1 and AIF associate with one another, the purpose of this interaction appears to be unrelated to ASK1 apoptotic activity.

Interaction with Keap1 and Bcl-XL does not play a role in PGAM5-induced cell death.
PGAM5\(_S\) and PGAM5\(_L\) could be mediating cell death through the ability to promote a loss in Bcl-X\(_L\) levels and/or repress Nrf2-dependent gene expression. To determine if the association of PGAM5 with Keap1 and/or Bcl-X\(_L\) plays a role in PGAM5-mediated cell death, caspase activation and cell viability was assessed using inactive binding mutants. As previously shown, mutating amino acids 79 and 80 within the Keap1 binding motif to alanine inhibits PGAM5\(_S\) and PGAM5\(_L\) from binding Keap1 (Figure 18A and 18B), while mutating two residues within a BH3-like binding motif found in the PGAM domain of both isoforms is sufficient to block interaction with Bcl-X\(_L\) (Figure 18C) [130]. Cell death activity of the PGAM5\(_S\) Keap1 (E79A/S80A) and
Figure 18. Point mutations disrupt PGAM5\textsubscript{S} and PGAM5\textsubscript{L} interaction with Keap1 and Bcl-\textsubscript{XL}. A) Empty vector (control), PGAM5\textsubscript{S} wildtype, or PGAM5\textsubscript{S} E79A/S80A (Keap1 binding mutant) were transiently expressed in HEK 293T cells. Whole cell lysates were analyzed by immunoblotting with anti-HA(PGAM5) and anti-Keap1 antibodies (bottom two panels). Anti-HA immunoprecipitates (IP) were subjected to immunoblot analysis using an anti-Keap1 antibody (top panel). B) Empty vector (control), PGAM5\textsubscript{L} wildtype, or PGAM5\textsubscript{L} E79A/S80A were transiently expressed in HEK 293T cells. Whole cell lysates were analyzed by immunoblotting with anti-HA(PGAM5) and anti-Keap1 antibodies (bottom two panels). Anti-HA immunoprecipitates (IP) were subjected to immunoblot analysis using an anti-Keap1 antibody (top panel). C) Empty vector (control), PGAM5\textsubscript{S} wildtype, PGAM5\textsubscript{L} wildtype, PGAM5\textsubscript{S} L135E/G139E (Bcl-\textsubscript{XL} binding mutant), or PGAM5\textsubscript{L} L135E/G139E (Bcl-\textsubscript{XL} binding mutant) were transiently expressed in HEK 293T cells. Whole cell lysates were analyzed by immunoblotting with anti-HA (PGAM5) and anti-Bcl-\textsubscript{XL} antibodies (bottom two panels). Anti-Bcl-\textsubscript{XL} immunoprecipitates (IP) were subjected to immunoblot analysis using an anti-HA (PGAM5) antibody (top panel).
Figure 19. PGAM5S and PGAM5L interaction with Keap1 and Bcl-XL is unrelated to cell death activity. A) Empty vector (control), PGAM5S wildtype, PGAM5S H105A, PGAM5S E79A/S80A or PGAM5S L135E/G139E was transiently expressed in HEK 293T cells. Cell lysates were subjected to a caspase 3 DEVD-AFC assay at 48 hours post transfection, data presented as the means ± SEM obtained from three individual experiments. B) PGAM5S variants or plasmid control were transiently expressed in HEK 293T cells. Cells were harvested at 48h post-transfection and cell viability was measured using propidium iodide and flow cytometry. C) Empty vector (control), PGAM5L wildtype, PGAM5L H105A, PGAM5L E79A/S80A or PGAM5L L135E/G139E was transiently expressed in HEK 293T cells. Cell lysates were subjected to a caspase 3 DEVD-AFC assay at 48 hours post transfection, data presented as the means ± SEM obtained from three individual experiments. D) PGAM5L variants or plasmid control were transiently expressed in HEK 293T cells. Cells were harvested at 48h post-transfection and cell viability was measured using propidium iodide and flow cytometry. *, p < 0.01 compared to control; **, p > 0.05 compared to wildtype; ‡, p < 0.05 compared to wildtype.
Bcl-X\textsubscript{L} (L135E/G139E) binding mutants was assessed alongside of wildtype and PGAM5\textsubscript{S} H105A. Both PGAM5\textsubscript{S} E79A/S80A and PGAM5\textsubscript{S} L135E/G139E were able to achieve the same level of caspase activation (Figure 19A) and loss in cell viability (Figure 19B) seen with wildtype. PGAM5\textsubscript{L} E79A/S80A and PGAM5\textsubscript{L} L135E/G139E produced results similar to PGAM5\textsubscript{S} mutants with caspase activation (Figure 19C) and decreased viability (Figure 19D) equal to control. PGAM5\textsubscript{L} H105A achieved significantly more caspase activation compared to wildtype (Figure 19C); however, this may be due to an increase in expression (data not shown). Overall, the data indicates PGAM5\textsubscript{S} and PGAM5\textsubscript{L} interaction with Keap1 and Bcl-X\textsubscript{L} is dispensable in the ability of PGAM5 to promote cell death.

**PGAM5\textsubscript{S} and PGAM5\textsubscript{L} promote mitochondrial abnormalities and mitophagy.** As mentioned previously, transgenic expression of PGAM5 in the flight muscles of *Drosophila* promotes mitochondrial fragmentation, indicating PGAM5 plays a role in the process of mitochondrial fission [139]. The ectopic expression of human PGAM5\textsubscript{S} and PGAM5\textsubscript{L} in COS-1 and HeLa cells also results in altered mitochondrial morphology, further supporting a role for PGAM5 in regulating mitochondrial dynamics [135]. In an effort to establish if PGAM5\textsubscript{S} and PGAM5\textsubscript{L} overexpression is disrupting mitochondrial dynamics to the detriment of the cell, transmission electron microscopy (TEM) was performed on HEK 293T cells between 24 and 36 hours post-transfection with an empty vector (control), PGAM5\textsubscript{S}, or PGAM5\textsubscript{L}. HEK 293T cells transfected with an empty vector displayed an abundance of mitochondria distributed throughout each cell (Figure 20A) that exhibited healthy dynamics and normal cristae formation (Figure 20B). Upon exogenous expression of PGAM5\textsubscript{S}, cells displayed a range of phenotypes; all containing highly abnormal mitochondria. One population of cells possessed an abundance of small, spherical
mitochondria (Figure 21A left panel) with abnormal or absent cristae formation (Figure 21A right panel); compared to another cell population which contained a limited number of large, spherical mitochondria (Figure 21B left panel) also exhibiting highly aberrant or absent cristae (Figure 21B right panel). A small population of cells expressing PGAM5S demonstrated a perinuclear aggregation of irregular mitochondria (Figure 22A and 22B) with the outer mitochondrial membrane either indiscernible (Figure 22A right panel) or visibly fragmented (Figure 22B). This aggregation was seen following the expression of PGAM5L (Figure 23B) along with another population of cells containing an abundance of large, spherical mitochondria distributed throughout the intracellular space containing abnormal or deficient cristae (Figure 23A). Taken together these images show severely abnormal mitochondrial number, distribution, morphology and structure following the upregulation of PGAM5S and PGAM5L, and implicate the dysregulation of mitochondrial dynamics and clearance in PGAM5-mediated cell death.
Figure 20. Transmission electron microscopy (TEM) images of healthy mitochondria. A) Representative transmission electron microscopy (TEM) image of HEK 293T cell transfected with empty vector (control) at 36 hours post-transfection. Asterisks designate mitochondria. B) Magnification of image in Figure A contained within the red box. Arrows highlight normal cristae formation.
Figure 21. **PGAM5**<sub>s</sub> promotes fragmented mitochondria and abnormal cristae formation.  
A) *Left*: Whole cell TEM image of HEK293T cell transfected with PGAM5<sub>s</sub> at 36 hours post-transfection. *Right*: Magnification of mitochondria on the left contained within the red box. Asterisks designate mitochondria, triangles highlight lack of cristae.  
B) *Left*: Whole cell TEM image of HEK293T cell transfected with PGAM5<sub>s</sub> at 36 hours post-transfection. *Right*: Magnification of mitochondria on the left contained within the red box. Asterisks designate mitochondria, triangles highlight lack of cristae, and arrows illustrate abnormal cristae patterns.
Figure 22. PGAM5$_S$ promotes aggregation of mitochondria, abnormal cristae formation and disruption of OMM. A) Left: Whole cell TEM image of HEK293T cell transfected with PGAM5$_S$ at 36 hours post-transfection. Right: Magnification of mitochondria on the left contained within the red box. Circle outlines aggregated mitochondria, arrows highlight abnormal cristae formation. B) Left and Right: TEM images of mitochondria found in HEK293T cells transfected with PGAM5$_S$ at 36 hours post-transfection. Mitochondria designated with lower case (m), triangles highlight disruption of mitochondrial membrane.
Figure 23. PGAM5L promotes aggregation of mitochondria and abnormal cristae formation. A) Left: Whole cell TEM image of HEK293T cell transfected with PGAM5L at 36 hours post-transfection. Right: Magnification of mitochondria on the left contained within the red box. Asterisks designate mitochondria, arrows highlight abnormal cristae patterns. B) Left: Whole cell TEM image of HEK293T cell transfected with PGAM5L at 36 hours post-transfection. Right: Magnification of mitochondria on the left contained within the red box. Oval outlines aggregated mitochondria, lower case (m) designate mitochondria, and arrows illustrate aberrant cristae formation.
DISCUSSION

AIF was originally discovered through its ability to trigger caspase-independent cell death in a variety of model systems [70]. Although recognized for this apoptotic function, accumulating evidence suggests AIF plays a more significant role in cell survival. While a number of AIF binding partners are available to help elucidate the mechanisms behind the pro-death role of AIF, there is a lack of validated factors that interact with AIF under homeostatic conditions. To resolve this issue, we employed a biochemical screen to uncover potential AIF associated proteins from healthy mitochondria, and identified PGAM5 as a potential binding partner. PGAM5 is a mitochondrial protein whose phosphatase activity and interaction with Keap1 has been shown to play a role in various apoptotic and necrotic cell death pathways [128, 129, 138]. In this study, we uncover an additional role for PGAM5<sub>S</sub> and PGAM5<sub>L</sub> in promoting cell death through a non-apoptotic mechanism unrelated to any previously established functions of PGAM5. Following PGAM5<sub>S</sub> and PGAM5<sub>L</sub> expression, the outer mitochondrial membrane becomes permeabilized and caspase-3 is activated, initially suggesting PGAM5 triggers cell death through the intrinsic apoptotic pathway. While the activation of caspases is the typical response during intrinsic apoptosis, there are instances where the pathway can be initiated without caspases playing a fundamental role [149]. Indeed, PGAM5<sub>S</sub> and PGAM5<sub>L</sub> promote cell death following chemical caspase inhibition, suggesting they fall within the category of caspase-independent death effectors. Although PGAM5<sub>S</sub> and PGAM5<sub>L</sub> associate with AIF, a well-known intrinsic caspase-independent cell death molecule, AIF does not play a role in the ability of PGAM5 to induce caspase-independent cell death. Markedly, AIF serves a protective role in attenuating caspase activation that occurs as a consequence of PGAM5 expression. Interestingly, however, this inhibition is not sufficient to prevent PGAM5-mediated cell death.
Although the phosphatase activity of PGAM5\textsubscript{S} and PGAM5\textsubscript{L} has been previously implicated in the cell death pathways of apoptosis and necroptosis, we found this function to be unrelated to the PGAM5-mediated cell death activity presented in this report. The lack in phosphatase activity exhibited by PGAM5\textsubscript{S}, along with the inability of the PGAM5\textsubscript{L} H105A phosphatase mutant to promote cell death, demonstrates dephosphorylation to be irrelevant to the cell death activity revealed in this study, whereas, it is crucial to the previously established apoptotic and necrotic pathways involving PGAM5 [128, 129]. Furthermore, a lack of TNF-alpha stimulation and inhibition of caspase-8 following transfection makes it unlikely that the cell death activity seen by PGAM5\textsubscript{S} or PGAM5\textsubscript{L} is due to necroptosis.

In addition to PGAM5 phosphatase function, the previously established association of PGAM5\textsubscript{S} and PGAM5\textsubscript{L} with Bcl-X\textsubscript{L} and Keap1 also proved to be non-essential in the ability of PGAM5 to kill. Through association with PGAM5, Keap1 is able to indirectly ubiquitinate and degrade Bcl-X\textsubscript{L} resulting in a significant loss in Bcl-X\textsubscript{L} expression [138]. A loss in Bcl-X\textsubscript{L} expression can result in permeabilization of the OMM and subsequent activation of the intrinsic pathway. Inactivating the ability of PGAM5 to bind to Keap1 and/or Bcl-X\textsubscript{L} did not have an affect on the ability of PGAM5 to activate caspase-3 and promote cell death. Therefore, the interaction of PGAM5\textsubscript{S} and PGAM5\textsubscript{L} with Keap1 and Bcl-X\textsubscript{L} is not responsible for the ability of PGAM5 to trigger intrinsic cell death. Although loss of the Keap1-PGAM5 interaction did not protect cells from PGAM5-mediated cell death, it is surprising at least an increase in cell death was not detected since binding to Keap1 has been shown to promote the degradation of PGAM5 [130]. On a similar note, the direct interaction of PGAM5 with Bcl-X\textsubscript{L} would suggest PGAM5 has the ability to exert a regulatory influence on Bcl-X\textsubscript{L}, whether negative or positive. Therefore, it is interesting that neither inhibition nor promotion of cell death was seen upon loss of this
interaction, indicating the various functions of PGAM5 to serve fairly separate physiological purposes, and further exposing PGAM5 as a multi-faceted protein.

Since both isoforms have been implicated in the processes of mitochondrial fission and mitophagy through their ability to promote mitochondrial fragmentation and associate with the mitophagic protein, PINK1, we decided to assess the impact of increased PGAM5 expression on the physical state of the mitochondria using transmission electron microscopy [139]. TEM images following PGAM5\(_S\) and PGAM5\(_L\) expression not only revealed a loss in mitochondrial number, but the mitochondria present are spherical in shape and have aberrant cristae formation, indicative of faulty mitochondrial dynamics. An upregulation in mitochondrial fission can result in a loss of mitochondrial number, mitochondrial fragmentation, and an increase in mitophagy [154, 155]. Without a sufficient number of healthy functioning mitochondria a cell cannot produce the energy necessary to sustain itself and thus will eventually die. A side consequence of dysfunctional dynamics is increased ROS production due to defective electron transport, which can result in permeabilization of the outer mitochondrial membrane and the release of mitochondrial death proteins [154, 155]. This may explain why caspase-3 activation occurs following the upregulation of PGAM5, but is not ultimately responsible for PGAM5-induced cell death. An increase in mitochondrial fission may also explain why AIF is able to significantly inhibit caspase activation following expression of PGAM5\(_S\) and PGAM5\(_L\), but is unable to protect cells from PGAM5-induced cell death. A loss of AIF in neurons has been shown to result in fragmented mitochondria with aberrant cristae, indicating a potential role for AIF in controlling mitochondrial structure [70]. By expressing AIF along with PGAM5, AIF is able to promote a level of structural stabilization that prevents mitochondrial membrane
permeabilization, and thus caspase activation, but does not provide protection against a loss in energy resulting from increased mitochondrial fission and mitophagy.

Overall, data presented in this study highlight a novel cell death role for PGAM5S and PGAM5L. We have shown this activity to be unrelated to a newfound association with AIF or any previously established functions of PGAM5. While the exact mechanism of this unique cell death activity of PGAM5 remains unclear, mitochondrial abnormalities resulting from the overexpression of both isoforms suggests aberrant mitochondrial dynamics and/or mitophagy to be involved. In addition, the ability of AIF to oppose PGAM5-mediated caspase activation illuminates a protective role for AIF and indicates the potential ability of AIF to regulate permeabilization of the OMM. Further elucidation of the molecular function of PGAM5 in regulating mitochondrial dynamics and/or quality control may help provide a better understanding of mitochondrial disease pathogenesis and lead to more effective therapeutic treatment strategies.
CHAPTER 4

DEVELOPMENT OF AN IN VIVO TISSUE RECOMBINATION APPROACH TO STUDY THE TEMPORAL IMPACT OF AIF UPON THE PROGRESSION OF PROSTATE CANCER
INTRODUCTION

Cancer is a disease characterized by a lack of cell death. Cancer cells are able to survive and replicate by evading the cell-suicide process, leading to the formation of tumors [156]. As a cell death molecule, AIF has the potential to serve an inhibitory role in cancer cell development and progression. However, studies show elevated AIF levels to serve a protective role in promoting cancer cell growth and survival, which appears to be related to the ability of AIF to regulate energy production. In a study by Porter et al. investigating the mechanistic connection between the NADH oxidase activity of AIF and cell survival, human colorectal cancer cell lines were assessed in which the aif gene had been knocked out [75]. AIF-deficient carcinoma cells showed a significant decrease in the expression and activity of complex I, as was previously reported for AIF-deficient ES and HeLa cells; and demonstrated a severe reduction of tumor cell growth in vivo compared to wildtype cell lines [71, 75]. These data not only demonstrate the ability of AIF to play a pro-survival role in cancer, they also suggest a mechanistic connection between cancer cell survival and the role of AIF in regulating oxidative phosphorylation. This protective function seen in colorectal cancer was reiterated in a study recently published by our laboratory investigating the role of AIF in prostate cancer. Publically available gene expression data revealed AIF mRNA to be elevated in human prostate cancer, which correlated with elevated protein levels found in tissue samples taken from patients with benign, localized, and metastatic prostate cancer [85]. The level of AIF expression directly coincided with the progression of the disease, suggesting the upregulation of AIF to play a role in the growth and invasion of prostate cancer. The ablation of AIF from the prostate cancer cell lines, PC3 and DU145, led to a significant reduction in cell survival and growth, not observed in AIF-deficient LNCaP cells, and a xenograft model revealed AIF deficient PC3 cells to exhibit a substantial
reduction of tumorigenic growth in vivo [85]. The reduced survival demonstrated by the AIF-deficient DU145 and PC3 cell lines correlated with decreased expression of mitochondrial complex I subunits and changes in glucose metabolism, providing further evidence of a connection between cancer cell survival and the ability of AIF to control mitochondrial metabolism. Overall, the data highlight the significance of AIF in promoting advanced prostate cancer cell survival, making AIF a potential target for a new avenue of treatment.

Prostate cancer is the second leading cause of cancer-related death in adult men worldwide, and the most common type of cancer found in American men. If diagnosed early there are a variety of treatments available for localized prostate cancer that can result in long-term patient survival [157, 158]. However, if the cancer has advanced beyond the prostate gland, and/or has become metastatic, localized treatment options become unsuitable. Therefore, finding new, alternative avenues of treatment for advanced prostate cancer is crucial to increasing patient health and reducing mortality. Elucidating the role of AIF in prostate cancer progression could help uncover an alternative pathway to target in the treatment of metastatic disease. While the xenograft model performed by our laboratory helped establish the relevance of AIF in the development and progression of prostate cancer, there are limitations to this model which advocate the need for employing an alternative model system. In the xenograft model, tumor growth was propagated by implanting PC3 cells subcutaneously into the hind flank of immunodeficient mice [85]. While the use of human prostate cancer cells has the benefit of recapitulating the genetic complexity of human tumors, these tumors do not form in the prostate and PC3 cells are metastatic prior to implantation, thus there is limited ability to define the temporal development of tumorigenesis.
In contrast, a genetically engineered mouse (GEM) model would allow for the development of tumors in situ that can be analyzed from early to late stage tumorogenesis. In a GEM model, genes thought to be involved in transformation or malignancy are mutated, deleted, or overexpressed; the effect of altering these genes is studied over time and therapeutic responses to tumors are followed in vivo [159]. Since we are interested in determining the temporal effects of AIF deficiency on prostate cancer driven by a loss-in-function of PTEN, we would need to generate a multigene transgenic mouse with prostate specific loss of both AIF and PTEN. While this model would be useful in assessing the role of AIF at various stages in prostate cancer, this method would require a substantial amount of time and expense to develop.

To overcome the disadvantages of both a xenograft and GEM mouse model, we propose an innovative tissue regeneration model that allows for the growth of a prostate-like structure in a short period of time, in which the development of cancer can be induced, providing a more robust environment for establishing temporal changes in prostate tumorogenesis. Although the prostate of a mouse is anatomically distinct from humans, it is a ductal structure that contains luminal and basal epithelial cells, neuroendocrine cells, and stroma similar to a human prostate which makes it a valuable tool in studying prostatic disease. In the proposed alternative model, adult mouse prostatic stem cells (MPSCs), in combination with rat urogenital mesenchyme (rUGM), are able to form prostatic ductal structures containing basal and luminal epithelial cell layers when implanted under the renal capsule of immunocompromised mice [160, 161]. The development of prostate cancer is encouraged in these prostatic structures through knockdown of a tumor suppressor, such as phosphatase and tensin homolog (PTEN), which has previously been shown to be successful in inducing cancer within this model (data unpublished). The mouse prostatic basal and luminal cells express markers associated with human basal cells and human
luminal cells. Thus, the developmental stages of murine prostate cancer to be histologically assessed are similar to human prostate cancer. To establish the temporal role of AIF in prostate cancer progression within this model system, cancerous prostatic structures will be grown in the absence and presence of AIF and analyzed at various timepoints post-implantation. In this study, we establish and characterize AIF-deficient mouse prostate stem cells for use in the proposed tissue regeneration mouse model.

**EXPERIMENTAL PROCEDURES**

**Materials.** Reagents were obtained as follows: phosphate-buffered saline from Invitrogen; fetal bovine serum from HyClone; Dulbecco modified Eagle medium (DMEM) from Mediatech; and the QuantichromTM glucose assay kit from BioAssay Systems. All other chemicals were from Sigma. Antibodies were obtained as follows: anti-AIF (#SC13116) from Santa Cruz Biotechnology; 17 kDa (#A21359), 20 kDa (#459210) and 39 kDa (#459100) antibodies from Invitrogen; and HRP-conjugated anti-mouse (#NA931V) and anti-rabbit (#NA934V) from Amersham.

**Cell culture.** Wildtype and PTEN deficient mouse prostate stem cell lines were obtained from Dr. Scott Cramer and grown in DMEM/F12 supplemented with Fraction V BSA, cholera toxin, bovine pituitary extract, gentamycin, insulin, vitamin E, transferrin, trace elements and EGF. All cells were maintained at 37°C in an atmosphere of 95% air and 5% CO₂ except during lentivirus infections as described below.

**Cell lysis and immunoblot analysis.** Cell lysates were prepared in RIPA buffer (phosphate buffered saline containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate), normalized for protein content, and then separated by SDS-polyacrylamide gel electrophoresis.
(PAGE) using 4 to 12% gradient SDS-polyacrylamide gels (Invitrogen). For immunoblot analysis, SDS-PAGE was followed by transfer to nitrocellulose membranes (Invitrogen), which were then blocked with 5% milk in Tris-buffered saline containing 0.05% to 0.2% Tween, followed by incubation with the indicated antibodies for 1 h at room temperature. Following washing, membranes were incubated with HRP-conjugated anti-mouse IgG or anti-rabbit IgG secondary antibodies for 45 min at room temperature and visualized by enhanced chemiluminescence.

**Lentiviral knockdown.** Lentiviral knockdown of AIF in mouse prostate stem cell lines was performed as previously described [103]. Briefly, FG12 derived plasmids were combined with equal amounts of lentiviral packaging plasmids and transfected into mouse prostate stem cells by calcium phosphate precipitation as described [100]. 48 h after transfection, supernatants were harvested, filtered using 0.45-μm-pore size Millex HV PVDF filter units (Millipore), and concentrated by centrifugation at 60,000 × g for 90 min. The supernatants were aspirated, and virus-containing pellets were resuspended in PBS overnight at 4 °C. One day before infection, target cells were seeded at 60,000 cells/well in 12-well plates. At the time of infection, polybrene was added to a final concentration of 25 mM, resuspended virus was added, and cells were incubated for 4 h at 37 °C in an atmosphere of 93% air, 7% CO₂. Virus containing supernatants were then removed, fresh medium was added to cells, and cells were incubated for an additional 2–3 days in an atmosphere of 95% air, 5% CO₂. Stable incorporation of FG12-based lentiviral DNA was determined by immunoblot analysis.

**Cell growth rate measurements.** Wildtype control (WT shLacZ), wildtype AIF deficient (WT shAIF), PTEN deficient control (PTEN −/− shLacZ), and PTEN deficient AIF deficient (PTEN −/− shAIF) mouse prostatic stem cell lines were seeded in 6-well plates, 50K cells per well. Three
wells from each cell type were harvested by trypsinization at 24 hr intervals for a total of 96 h, and the total number of cells in each replicate was determined by Coulter counting.

**Glucose consumption.** Wildtype control (WT shLacZ), wildtype AIF deficient (WT shAIF), PTEN deficient control (PTEN \(^{-/-}\) shLacZ), and PTEN deficient AIF deficient (PTEN \(^{-/-}\) shAIF) mouse prostatic stem cell lines were seeded in replicate 6-well plates, 100K cells per well. The cells were then incubated for 48 hr at 37°C, medium was collected from each well, and total glucose was determined using the QuantiChrom™ glucose assay kit per manufacturer’s instructions. The glucose present in medium incubated without cells was determined and used to calculate glucose consumption of each sample. The cells from each well were harvested, and the total cell number was determined by Coulter counting. Total glucose consumption per sample was then divided by the number of cells to generate final glucose consumption per cell values for each cell line.

**RESULTS**

**Generation of AIF-deficient mouse prostate stem cell lines.** The wildtype mouse prostatic epithelial cells used for this study were previously isolated, characterized, and infected with lentivirus to achieve knockdown of PTEN [160]. Briefly, isolated adult mouse prostatic cells expressing markers associated with prostate stem cells (Sca 1 and CD49f) *in vitro* were shown to undergo tissue-specific multilineage differentiation to form basal and luminal epithelial cells when grafted under the renal capsules of nude mice in the presence of fetal rat urogenital mesenchyme [160, 161]. *In vivo*, these fully differentiated basal and luminal epithelial cells express markers normally associated with human prostatic basal (p63, cytokeratin 14) and
luminal cells (androgen receptor, cytokeratin 8, and cytokeratin 18) [160, 162]. To establish AIF deficiency within these cell lines, lentivirus was used to infect cells with shRNA sequences targeting either mouse AIF or LacZ as a negative control [85, 103]. Cells stably incorporating hairpin DNA were detected by GFP fluorescence and western blot analysis were performed to demonstrate the presence of AIF in all control cell lines and the absence of AIF in all knockdown cell lines (Figure 24A). The four cell lines generated are as follows: wildtype control (WT shLacZ), wildtype AIF deficient (WT shAIF), PTEN deficient control (PTEN −/− shLacZ), and PTEN deficient AIF deficient (PTEN −/− shAIF).

**Characterization of AIF-deficient mouse prostate stem cell lines.** Once knockdown of AIF was confirmed, all MPSC cell lines were assessed prior to implantation to establish any consequences of AIF depletion that may affect the outcome of the model. To assess growth rate, each cell line was seeded at a certain density in a 6-well format, cells were harvested and counted at 24 hr intervals for a total of 96 hr, and growth rate measured as cells per hour. Initial characterization of these cells in vitro has revealed a minimal affect on growth rate with the loss of PTEN and/or AIF compared to wildtype (Figure 24B). Glucose consumption, however, showed a significant increase with the knockdown of PTEN (Figure 24C); however, this increase does not appear to be hindered or enhanced by the ablation of AIF (Figure 24C). Glucose consumption was measured per manufacturer’s instructions over a period of 48 hr using a QuantiChrom™ glucose assay kit; consumption is shown as glucose consumption per cell. Of notable interest, immunoblot analysis revealed no change in complex I subunit expression (Figure 24A) with the depletion of AIF, contrary to the decrease observed in a variety of other cell lines.
Figure 24. Characterization of AIF deficient mouse prostatic epithelial stem cells. A) Immunoblot analysis was performed on cell lysates generated from wildtype, AIF deficient wildtype, PTEN deficient, and PTEN deficient + AIF deficient mouse prostatic stem cells using an α-AIF antibody and α-17kDa, α-20kDa, α-39 kDa complex I subunit antibodies. B) Wildtype, AIF deficient wildtype, PTEN deficient and PTEN deficient + AIF deficient cell lines were plated at a density of 15K cells with cell number measured once a day for four days to assess cell growth. C) Wildtype, AIF deficient wildtype, PTEN deficient and PTEN deficient + AIF deficient cell lines were plated at a density of 50K cells and glucose consumption was measured over a 48 hour period.
DISCUSSION

Although tissue culture models are extremely useful in understanding the biology of prostate cancer, they do not recapitulate the complex cellular interactions within the tumor microenvironment that play a significant role in cancer development and progression. Therefore, the use of animal models is critical for defining the molecular basis of prostate cancer pathogenesis. While the mouse is one of the best animals in which to model cancer, there are challenges to the use of mice for the study of human prostate cancer. The prostate of a mouse is anatomically different from humans, consisting of four distinct lobes (anterior, ventral, dorsal, and lateral) while the human prostate has one lobe divided into three zones (central, transitional, and peripheral) [163]. Also, the metastasis of prostate cancer in mice has a tendency to originate from mesenchymal cells, as opposed to epithelial cells in humans, and it has been proven difficult to induce bone metastasis in mice, which is the most common type of metastasis found in human prostate cancer patients [157-159]. In spite of these obstacles, mouse models have made a significant contribution in elucidating the molecular mechanisms of prostate cancer development and disease. The two most commonly used mouse prostate cancer models are the xenograft and genetically engineered mouse (GEM) models. In a xenograft model, tumor growth is propagated by implanting human cancer cells or tumor fragments either under the skin or into an organ of immunodeficient mice such as athymic or severe combined immune deficient (SCID) mice [164]. While xenografts allow for the quick acquisition of data and the use of human cancer cells has the benefit of recapitulating the genetic complexity of human tumors, tumor growth occurs in a host with an impaired immune system and there is limited ability to define the temporal development of tumorigenesis. In contrast, GEM models can be used to develop tumors in situ under normal immune conditions and allow for the analysis of tumors
from early to late stage cancer. However, GEM models can take as long as a year to develop and may be unfeasible depending on the gene(s) of interest.

Despite the contribution xenograft and GEM mouse models have made in validating gene functions, identifying novel cancer genes and gaining insight into the molecular and cellular mechanisms underlying tumorigenesis, they are still limited in recapitulation of de novo human tumor development. An ideal mouse model would emulate the molecular, cellular, and genomic events of human cancers, reproducing all the stages of human prostate cancer, with progression from prostate intraepithelial neoplasia (PIN) through androgen-dependent (AD) to androgen-independent (AI) disease with metastases. Along with the stages of cancer, the histopathologic features and molecular pathways that are changed in humans should also be observed in the mouse. The innovative mouse model proposed in this study allows for the formation of a prostatic structure that exhibits similar cellular, structural and molecular characteristics to the human prostate, along with a histopathology that reflects the natural progression of prostate cancer leading from PIN to invasive carcinoma.

The prostatic epithelium consists of two major epithelial cells: basal epithelial cells and luminal epithelial cells. Basal epithelial cells are androgen-independent, undifferentiated cells with high proliferative capacity and low apoptotic index [165]. They are characterized by the expression of cytokeratins 5, 14, and 15, as well as p63 and the antiapoptotic protein, Bcl-2 [162]. Luminal epithelial cells are more differentiated, androgen-dependent cells with low proliferative capacity and high apoptotic index [166]. These cells are typically characterized by the expression of androgen receptor (AR) and cytokeratins 8 and 18 [162]. The mouse prostate stem cells used in the proposed cancer model have been shown to undergo tissue-specific multilineage differentiation to form fully differentiated basal and luminal cells with secretion of
prostatic secretory proteins into the lumen. These cells also express markers associated with human basal cells including CK5 and p63, and luminal cells such as AR, CK8 and CK18, allowing for the developmental stages of mouse prostate cancer to be histologically assessed similar to human prostate cancer.

Although the proposed model provides many advantages for the study of prostate cancer pathogenesis, a challenge may arise from the lack in complex I phenotype demonstrated by the AIF deficient MPSC cell line. AIF has frequently been shown to contribute to the activity of mitochondrial complex I, and subsequently energy production, via its ability to impact subunit expression. This role in regulating mitochondrial metabolism is believed to be connected to the ability of AIF to promote the development and progression of cancer. In characterizing the AIF deficient mouse prostate stem cells, we revealed a lack of change in complex I subunit expression, contrary to what has been demonstrated in a variety of cell types which include MEF and ES cells [71, 75, 85]. In a study recently published by our laboratory examining the role of AIF in supporting prostate cancer cell growth and metastasis, AIF deficient PC3 cells exhibited a decrease in expression of the 39 kDa subunit of complex I, which correlates to a decrease in transcript levels shown in Chapter 2 (Figure 5A) [85]. Unlike PC3 cells, however, LNCaP prostate cancer cells did not show in a change in complex I subunit expression or transcript levels upon the depletion of AIF. Moreover, the significant reduction in cell survival and growth demonstrated by AIF-deficient PC3 cells was not similarly observed with AIF-deficient LNCaP cells, suggesting a correlation between reduced cancer cell survival and decreased expression of mitochondrial complex I subunits. If a connection exists between the ability of AIF regulate the mitochondrial proteome and its ability to promote prostate cancer, then the lack of decreased complex I subunit expression exhibited by the AIF deficient MPSCs prior to implantation may
prove to be a counter-indication for using this alternative model to assess the temporal role of AIF in prostate cancer progression.
CHAPTER 5

SUMMARY

Apoptosis-inducing factor (AIF) is a mitochondrial NADH oxidase originally discovered for its ability to trigger apoptosis through chromatin condensation and DNA cleavage [38, 47, 57]. Similar to other pro-apoptotic proteins, AIF was primarily considered to be a key factor solely in cell death signaling. However, accumulating evidence suggests the enzymatic activity of AIF to play an even more dominant prosurvival role in promoting healthy cellular metabolism and mitochondrial function. The notable impact on the electron transport chain upon the loss of AIF has been shown both \textit{in vitro} and \textit{in vivo} in a variety of cell types and systems, with loss-of-function only being restored by enzymatically active forms of AIF [71, 73, 75, 85]. Moreover, a deleterious mutation of AIF lacking enzymatic function has recently been linked to patients with mitochondrial encephalomyopathy resulting from severe oxidative phosphorylation deficiency [84]. These data provide a rationale for the localization and enzymatic activity of AIF in supporting the ability to control mitochondrial energy metabolism.

Along with a supportive role in promoting healthy mitochondrial function, the ability of AIF to regulate energy production has been reported to serve a survival role in cancer cell growth. The knockdown of AIF in human colorectal and prostate cancer cell lines results in the substantial inhibition of tumor cell growth \textit{in vivo} compared to wildtype cells; with a loss in tumorigenicity coinciding with a significant decrease in the expression and activity of complex I of the electron transport chain [75, 85]. While this supports a mechanistic connection between cancer cell survival and the role of AIF in regulating oxidative phosphorylation, the activity of AIF in supporting tumorogenesis appears to be dependent on the stage of the disease; at least in the context of prostate cancer.
While it is clear the enzymatic activity of AIF plays a role in maintaining cellular levels of electron transport chain proteins, how AIF controls the expression of these selective proteins has yet to be established. Furthermore, while changes in expression of mitochondrial respiratory chain subunits encoded by nuclear genes have been examined in multiple studies, there is no study addressing the extent to which AIF affects expression of mitochondrial-encoded subunits. Thus, one of the major goals of this work was to investigate various avenues through which AIF could control the mitochondrial proteome in an attempt to elucidate the mechanism(s) behind the ability of AIF to control oxidative phosphorylation.

Through a biochemical screen we identified a variety of potential proteins which associate with AIF in healthy mitochondria under homeostatic conditions. Among the potential factors identified were components of the mitochondrial transcription and translation machinery along with the TIM23 translocase protein, mtHsp70. Interaction with these various proteins led us to test the ability of AIF to control mitochondrial protein expression through the regulation of mitochondrial transcription, mitochondrial translation, and/or mitochondrial protein import. As an alternative to these mechanisms we also examined the involvement of AIF in directing mitochondrial protein acetylation as a way of controlling complex I activity. The knockdown of AIF did not have a significant impact on mitochondrial protein import or the acetylation/deacetylation of mitochondrial proteins, suggesting the mechanism employed by AIF to direct the mitochondrial proteome does not rely on the regulation of these processes. However, the depletion of AIF in PC3 cells resulted in a significant reduction in mRNA transcript levels of a variety of proteins which included the nuclear-encoded 39 kDa subunit of complex I, NDUFA9. NDUFA9 has been previously demonstrated to suffer a decrease in expression following the loss of AIF in a variety of cell lines, including PC3 cells, indicating the
loss in transcript levels to correlate with a loss in translation [71, 75, 85]. In contrast to this transcriptional phenotype observed in PC3 cells, the knockdown of AIF in the less aggressive prostate cancer cell line, LNCaP, did not produce a substantial change in transcript levels of either nuclear- or mitochondrial-encoded proteins. This observation mimics complex I phenotypic differences between PC3 and LNCaP cells seen in a study recently published by our laboratory, suggesting that similar to the cell death function of AIF, its ability to control mitochondrial transcription is not universal [85]. Although our data provides further insight into the selective support of AIF in the progression of highly metastatic, androgen-sensitive prostate cancer cells (PC3) compared to androgen-insensitive prostate cancer cells with low metastatic potential (LNCaP), the conditional circumstances surrounding AIF-mediated control of respiratory chain expression and function remain unclear. The biochemical screen identifying mitochondrial transcription proteins as potential binding partners of AIF utilized mitochondria from HEK 293 cells, a cell line shown to exhibit a decrease in expression of complex I subunits upon the loss of AIF. These interactions may be the source of AIF-mediated control of mitochondrial transcription and may not be present in cells lines that do not exhibit changes in electron transport subunit levels following the loss of AIF. Exploration of the association between AIF and its potential mitochondrial transcription partners could give insight into the mechanism behind the ability of AIF to regulate transcription of the mitochondrial genome, and help provide rationale for the selective support of AIF in prostate cancer. At the same time, analysis of prostatic growths derived from the innovative mouse model proposed in this study could expose temporal molecular changes in prostate cancer that may explain the circumstantial ability of AIF to control mitochondrial metabolism.
Intriguingly, four out of the seven mitochondrial-encoded complex I subunits (ND1, ND2, ND3, ND6) showed a substantial loss in mRNA levels with the ablation of AIF, along with the complex III subunit, cytochrome b, and complex IV subunits, CO1 and CO3. These data demonstrate for the first time the ability of AIF to control the transcription of both nuclear- and mitochondrial-encoded electron transport chain proteins, and create a challenge to the current paradigm of mitochondrial transcription. The mitochondrial genome lacks introns, thus transcription produces a polycistronic precursor RNA that is processed to produce the individual tRNA and mRNA molecules. Therefore, the ability of AIF to impact selective subunits encoded by the heavy strand of the mitochondrial genome remains puzzling. If these subunits were encoded continuously at the end of the strand, premature termination of transcription could be implicated in this selective phenotype. However, these subunits are spread throughout the genome from beginning to end, providing no rationale or correlation between the current mechanism of mitochondrial transcription and the ability of AIF to impact the transcription of specific proteins. A potential explanation for this perplexing outcome may be that the decrease in mRNA levels seen upon the ablation of AIF is due to a loss in mRNA stability and not a loss in transcription. Following processing from the RNA precursor, mitochondrial mRNAs undergo polyadenylation, creating a 3'-end poly(A) tail that confers stabilization and promotes the initiation of translation [167]. Shortening or truncation of this poly(A) tail has been shown to result in the degradation and decreased steady state levels of some mt mRNAs as well as their translational products [168]. Therefore, AIF may be promoting a loss in selective subunits through regulation of polyadenylation and subsequent mRNA stability.
Along with a loss in transcription of multiple mitochondrial-encoded complex I subunits, mRNA levels of complex III (cytochrome b) and complex IV (CO1, CO3) subunits were also significantly reduced as a consequence of AIF depletion. A considerable reduction in complex III and IV activity was found in muscle and fibroblasts taken from patients with mitochondrial encephalomyopathy due to a deleterious mutation in AIF, providing a potential correlation between mitochondrial disease and this newfound ability of AIF to control mitochondrial transcription [84]. In order to establish the extent to which AIF is able to regulate transcription, to both the benefit and detriment of cells, analysis needs to be performed on a variety of AIF proficient and deficient cell lines.

A loss in transcript levels often correlates with a decrease in translation, and vice versa. While we have yet to establish protein expression levels for the mitochondrial-encoded subunits exhibiting diminished transcription with the loss of AIF, the decrease in mRNA levels of the 39 kDa subunit of complex I coincide with a loss in expression shown previously in multiple studies [71, 73, 85]. Similar to PC3, HeLa cells are highly aggressive cancer cells that exhibit decreased expression of nuclear-encoded complex I subunits with the loss of AIF [71]. Therefore, we would expect AIF to produce characteristics and/or behave in a similar manner in both of these cell lines. However, no significant change in mitochondrial translation was seen upon the depletion of AIF in HeLa cells to correlate with the substantial loss in mitochondrial transcript levels seen in AIF deficient PC3 cells. Since we have yet to establish mitochondrial translation in the AIF deficient PC3 cells, these data may be interpreted in more than one way. Firstly, while the ablation of AIF in PC3 cells results in decreased transcription, it does not completely abolish mRNA transcript levels. Therefore, there is still mRNA present to allow for a certain level of protein expression. If mitochondria maintain a limited basal level of expression it could
be difficult to detect a change in translation between AIF proficient and deficient cells reflective of the substantial change in transcript levels seen in PC3 cells. On the other hand, AIF could simply be uninvolved in controlling mitochondrial transcription in HeLa cells; therefore, we do not see a change in translation products with the loss of AIF. If so, the mechanism employed by AIF to regulate the assembly and function of complex I in HeLa cells remains an open question. Again, a difference in regulatory ability may stem from AIF protein interactions between these cell lines or merely from a difference in cell type. Analysis of mitochondrial translation in PC3 cells and mitochondrial transcription in HeLa cells is necessary to establish rationale. Overall, the data uncovered through exploration of AIF in controlling mitochondrial transcription and translation lead us to conclude the vital ability of AIF in regulating mitochondrial respiration to be as complex as it lethal function.

In accordance with its capacity to promote healthy mitochondrial function, AIF has been implicated in maintaining mitochondrial structure. The deletion of AIF in neuronal cells has been shown to lead to the accumulation of fragmented mitochondria with aberrant cristae formation, while elevated AIF levels produce elongated mitochondria with tighter cristae [70]. These data suggest AIF plays a role in stabilizing mitochondrial structure through the regulation of mitochondrial fusion. Mitochondria constantly undergo the dynamic processes of fission and fusion in response to physiological and pathological changes [155, 169]. Fusion is necessary for the exchanging of contents between healthy and damaged mitochondria, providing a level of complementation to alleviate stress [170]; while fission is responsible for segregating severely damaged parts of mitochondria, targeting them for elimination through the selective autophagic pathway of mitophagy (see chapter 1) [154]. In exploring the interaction between AIF and the newly discovered binding partner, PGAM5, we uncovered a novel cell death pathway in which
AIF plays an inhibitory role that may be connected to its ability to stabilize mitochondrial structure.

The upregulation of PGAM5S and PGAM5L results in permeabilization of the OMM and subsequent activation of caspase-3, suggesting PGAM5 triggers cell death through the intrinsic apoptotic pathway. While the activation of caspases is the typical response during intrinsic apoptosis, there are instances where the pathway can be initiated without caspases playing a fundamental role [149]. Indeed, PGAM5S and PGAM5L promote cell death following chemical caspase inhibition, suggesting they fall within the category of caspase-independent death effectors. While the association of PGAM5S and PGAM5L with AIF does not play a role in the ability of PGAM5 to induce caspase-independent cell death, AIF does possess the ability to inhibit caspase activation that occurs as a consequence of PGAM5 expression. Although this attenuation of caspase activity is not sufficient to prevent PGAM5-mediated cell death, it does reveal a novel survival activity of AIF in a cell death pathway, contrary to its known function as a promoter of apoptosis.

PGAM5 has been previously connected to the processes of mitochondrial fission and mitophagy through association with the mitochondrial fission protein, dynamin related protein 1 (Drp1), and the mitophagic protein, PINK1 [139]. PGAM5S and PGAM5L have the ability to bind and activate Drp1 during TNFα-induced necroptosis; and the inactivation of dPGAM5 has been shown to alter mitochondrial morphology and rescues defects seen in PINK1 -/- Drosophila in a way that suggests potential involvement in promoting mitochondrial fission [139]. An upregulation in mitochondrial fission can result in a loss of mitochondrial number, mitochondrial fragmentation, and an increase in mitophagy [154, 155]. Without a sufficient number of healthy functioning mitochondria a cell cannot produce the energy necessary to sustain itself and thus
will eventually die. TEM images following PGAM5$_S$ and PGAM5$_L$ expression not only revealed a loss in mitochondrial number, but the mitochondria present are spherical in shape and have aberrant cristae formation, indicating faulty mitochondrial dynamics and increased mitochondrial clearance. Therefore, the cell death seen following the overexpression of PGAM5$_S$ and PGAM5$_L$ presented in this study may be due to the ability of these isoforms to promote the process of mitochondrial fission.

An increase in mitochondrial fission may also explain why AIF is able to significantly inhibit caspase activation following expression of PGAM5$_S$ and PGAM5$_L$, but is unable to protect cells from PGAM5-induced cell death. Mitochondria are dynamic organelles that constantly undergo fission and fusion in response to physiological and pathological changes. While fission segregates the severely damaged parts of mitochondria and targets them for elimination by mitophagy, fusion is necessary for the exchanging of contents between healthy and damaged mitochondria to provide a level of complementation to alleviate stress [169]. In mammalian cells, mitochondrial fusion is regulated by the GTPase proteins Mfn1, Mfn2, and OPA1 [171]. OPA1 is an IMM resident protein with the ability to control the remodeling of mitochondrial cristae during apoptosis, preventing the release of cytochrome c and subsequent cell death [174]. The downregulation of OPA1 can result in a variety of phenotypes which include fragmented mitochondria, abnormal cristae formation, and loss in activity of complex I and IV of the electron transport chain resulting in defective oxidative phosphorylation [170, 172, 173]. These characteristics parallel what is observed under AIF deficient conditions, suggesting OPA1 and AIF to potentially reside within the same dynamic pathway. If AIF and OPA1 reside in the same pathway, AIF may be attenuating PGAM5-mediated caspase activation by preventing and/or delaying the release of cytochrome c. While the ability of AIF to stabilize
mitochondrial structure would prevent permeabilization of the OMM, delaying the release of mitochondrial death factors and subsequent activation of caspases, it would not protect against a loss in energy resulting from increased mitochondrial fission and mitophagy. Therefore, we still see cell death following PGAM5 upregulation even though AIF is able to attenuate caspase activity. The potential roles of PGAM5 in mediating mitochondrial fission and AIF in mediating mitochondrial fusion/cristae formation are two highly interesting avenues for future research.

In this study, we have identified several novel interactions of AIF that have helped to provide insight into the protective functions of AIF in maintaining mitochondrial function and structure. It is clear from the range of data obtained in this report that the vital ability of AIF in regulating mitochondrial energy production is as complex as it lethal actions, with AIF-mediated control of the mitochondrial genome being dependent on cell type and circumstance. Of most interest, is the inhibitory role AIF plays in a newly uncovered cell death pathway mediated by PGAM5. AIF is able to alleviate caspase activation following the upregulation of PGAM5; however, this inhibition does not prevent the induction of cell death. Previous studies implicating a role for AIF in the process of mitochondrial fusion has led us to speculate that AIF is able to prevent the activation of caspases by preventing the release of mitochondrial death factors through stabilization of mitochondrial structure. Overall, we have contributed to the prosurvival aspect of AIF and have opened up new avenues for future research in exploring the role of AIF in regulating mitochondrial transcription and dynamics.
REFERENCES


Audrey M. Lenhausen
2045 Craig St
Winston-Salem, NC  27103
alenhausen@gmail.com

EDUCATION:
Wake Forest School of Medicine, Winston-Salem, NC
Doctor of Philosophy (PhD), Biochemistry & Molecular Biology, GPA:  3.7  Sept 2013

Meredith College, Raleigh, NC
Bachelor of Science (BS), Biology, GPA:  3.6  May 2009

RESEARCH EXPERIENCE:

Life Science Researcher 2009-present
Wake Forest School of Medicine, Winston-Salem, NC
Advisor: John Wilkinson, PhD

• Investigated the role of apoptosis-inducing factor (AIF) in maintaining mitochondrial energy
  metabolism through regulation of mitochondrial import, mitochondrial transcription, mitochondrial
  translation, and acetylation of mitochondrial proteins
• Examined the ability of the AIF to promote prostate cancer progression using an innovative mouse
  model
• Discovered a novel caspase-independent cell death pathway mediated by phosphoglycerate mutase
  member 5 (PGAM5)

Peer-Reviewed Publication
mechanism characteristic of mitophagy. (manuscript in preparation)

Biomedical Science Mentorship 2008-2009
Duke University, Durham, NC
Mentor: Jacoba Slagter-Jager, PhD & Bruce Sullenger, PhD

• Worked with post-doctoral research fellow in developing aptamer-antagomir constructs used to inhibit
  specific microRNAs upregulated in cancer cells

Independent Study 2007-2008
Meredith College, Raleigh, NC
Advisor: Karthik Aghoram, PhD

• Established the use of green-fluorescent protein (GFP) as a tool to teach biomanufacturing techniques in
  an undergraduate biochemistry laboratory

Field Study Summer 2007
School for Field Studies (SFS), Kenya, Africa

• Participated in a pilot environmental and public health field study in the Loitoktok district of Kenya
• Collected and analyzed data on the health status and needs of the Maasai community for the Ministry of
  Health, with a focus on women and children under 5 yrs
• Assisted in the organization and implementation of mobile immunization clinics
LEADERSHIP/TEACHING EXPERIENCE:

Representative
Wake Forest School of Medicine, Winston-Salem, NC
- Served as a Graduate Student Association (GSA) representative for the Dept. of Biochemistry and Molecular Biology
- Helped plan, promote, and execute all social and philanthropic events sponsored by the GSA

Teaching Assistant
Meredith College, Raleigh NC
- Developed protocols for protein expression and purification
- Composed laboratory manual chapters for protein expression and purification labs
- Organized and set up weekly laboratory modules
- Instructed students in fundamental techniques in biochemistry laboratory

HIGHLIGHTED SKILLS:
- scientific writing
- oral & written communication
- cell culture
- western blot
- flow cytometry
- RNA interference
- Sigma Plot
- mutagenesis
- cell death assays
- transfection
- subcellular fractionation
- PCR
- protein expression/purification
- mitochondrion isolation

SELECTED ABSTRACTS/PRESENTATIONS: