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STUDIES ON FORTILIN – PROHIBITIN INTERACTION

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STUDIES ON FORTILIN – PROHIBITIN INTERACTION

by

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Dedication

To my family whose support and prayers have helped me in all my endeavors and to Bothland whose constant support has helped me to pursue that which is not always easy but is most definitely rewarding

STUDIES ON FORTILIN – PROHIBITIN INTERACTION

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Abstract: Apoptosis, programmed cell death, is a tightly regulated process that occurs in development, in tissue maintenance and turnover, and in regulating the immune system. Alterations in apoptosis regulation is known to be involved in various diseases including cancer, autoimmune diseases, and cardiovascular diseases including atherosclerosis initiation and maintenance. Fortilin is an antiapoptotic protein with a wide tissue distribution and a wide range of functions. fortilin has no sequence homology to other regulators of apoptosis, such as the Bcl-2 Proteins or the Inhibitors of Apoptosis proteins. In elucidating the mechanism of Fortilin-mediated cellular protection, fortilin protein interactions have been previously shown to modulate the cellular response to apoptotic stimuli. The goal of this dissertation is to investigate novel fortilin protein interactions in order to further shed light on the mechanism of fortilin mediated protection. In a proteomic screen fortilin was shown to interact with the anti-proliferative protein, prohibitin. I demonstrate that fortilin specifically interacts with this protein through *in vitro* studies. fortilin co-localizes with prohibitin in a perinuclear distribution and subcellular studies showed that fortilin and prohibitin are found in the nucleus, cytosol, and to a lesser degree in the endoplasmic reticulum. Fortilin was shown to mutually stabilize prohibitin. Finally, cells overexpressing fortilin and prohibitin attenuate the apoptosis response of cells compared to cells overexpressing either protein alone. In summary, these findings demonstrate a novel protein interaction between fortilin and prohibitin and shows a functional significance in modulating apoptosis after inducing cell stress.

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My goal of becoming a physician-scientist was initially one of intellectual curiosty delving into the secrets of nature. Since that time, my view has evolved to one where the wonders of science can offer hope to humanity. I hope that through work such as this I can make but a small imprint on the great landscape of science and in so doing, progress knowledge ever closer towards this hope.

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Cardiovascular disease (CVD) is the leading cause of death in developed countries including the United States and is projected to be the most prevalent disease in the world. An estimated 81 million Americans are currently suffereing from at least 1 or more CVDs with an estimated 2300 deaths per day, of which 1 in 6 deaths are caused by coronary artery disease. According to recent reports, the burden to society through direct and indirect costs in morbidity of CVD is approximately $503.2 billion. This estimate includes health expenditures, medications, lost productivity, and total direct costs for this year alone is estimated at $155 billion (Lloyd-Jones et al., 2010). A combination of a lipid-rich diet and sedentary lifestyle contribute to CVD with atherosclerosis being the most recognized process. Atherosclerosis is the most identifiable disease causing CVD- related mortality including heart attacks and strokes. Despite current advances in lipid control therapies, CVD is estimated to affect some 785,000 Americans this yearwith atherosclerosis being a silent contributor directly affecting the progression of CVD (Lloyd-Jones et al., 2010).

1.1.1 Microscopic Morphology of the Atheroma

Atherosclerosis is a systemic disease involving fatty deposits, inflammation, and scar tissue affectingmedium to large arteries. Atherogenesis, the process of atherosclerotic plaque formation on the inner lining of an artery, is thought to occur through a distinct series of pathologic-clinical phases, 1-5. Atheroma formation begins from the initial fatty streak typical of Phase 1 lesions, with no clinical sequaelae. The fatty streak occurs throughout the arterial vasculature and is found in all age groups. At this stage, these fatty

streaks can even regress. The progression to Phase 2, or the late phase, atheroma is not well understood. However, what is observed in Phase 2 lesions is the increase in cellularity and disorder along with the deposition of lipid and extracellular matrix. Histologic analysis has shown that Phase 1 and 2 atheromas contain a progressive increase in immune cells, VSMCs, and matrix deposition; *in vitro* studies show that this environment drives the recruitment of additional inflammatory cells and promotes the proliferation of VSMCs and subsequently the expansion of extracellular matrix proteins within the atheroma. Phase 2 lesions may or may not be stenotic (occluding vessels) but are friable and are prone to rupture precisely because of the extracellular matrix deposition within the medial lining of the arterial wall which forms a friable fibrous cap encompassing the lesion

Phase 3 through 5 lesions describe more aggressive and acute disease-causing plaques which include the unstable plaque and the thrombotic plaque—that is, a plaque that has already ruptured activating the coagulation process forming the atherothrombotic lesion. Included in these later plaques is the persistence of lipid-laden foam cells of Mϕ lineage, a ruptured thin fibrous cap, and an exposed necrotic core consisting of deposited cholesterol crystals and necrotic foam cell remnants. These histological findings and the clinical correlates (myocardial infarction, stroke, etc) have corresponding changes in the composition of the cellular components within the atheromatous tissue.

As mentioned above, the histopathological appearance and associated cellular changes within the atherosclerotic plaque drives the progression of the disease. The trigger that tips the relatively benign fatty streak towards atheroma formation is thought to occur through coordinated events involving a sedentary diet in a pro-inflammatory background.

1.1.2 PATHOPHYSIOLOGY OF ATHEROMA FORMATION

Low-density lipoprotein, LDL, is a naturally occurring lipid transport protein elevations of which is clinically associated with obesity, type 2 diabetes, hypertension, and various forms of heart and cardiovascular diseases (Kannel and Vasan, 2009). LDL functions to transport cholesterol to the liver and is synthesized in the liver. In the context of heart disease, several mechanistic studies have evaluated how LDL can participate in the progression of atherosclerosis. High serum levels of LDL from a sedentary diet promotes LDL deposition within tissues where a relative abundance leads to the nonenzymatic formation of oxidized LDL, OxLDL (Palinski et al., 1989). In this model, as the inflammatory process progresses, monocytes are recruited to the plaque-prone site. Macrophage-derived foam cells begin to form and accumulate within the fatty streak (Napoli et al., 1997). These cells accumulate the oxLDL cholesteryl esters without sufficient processing of oxLDL (Itabe et al., 1994). As a result the lipid-laden foam cell persists within the plaque further driving inflammation (Kikuchi et al., 2000; Martinet and Kockx, 2001).

Late atherosclerotic plaques (Phase 2) are composed of cholesterol crystals in a large necrotic core containing large numbers of macrophages and foam cells. This plaque progression typically causes clinical manifestation through narrowing of the

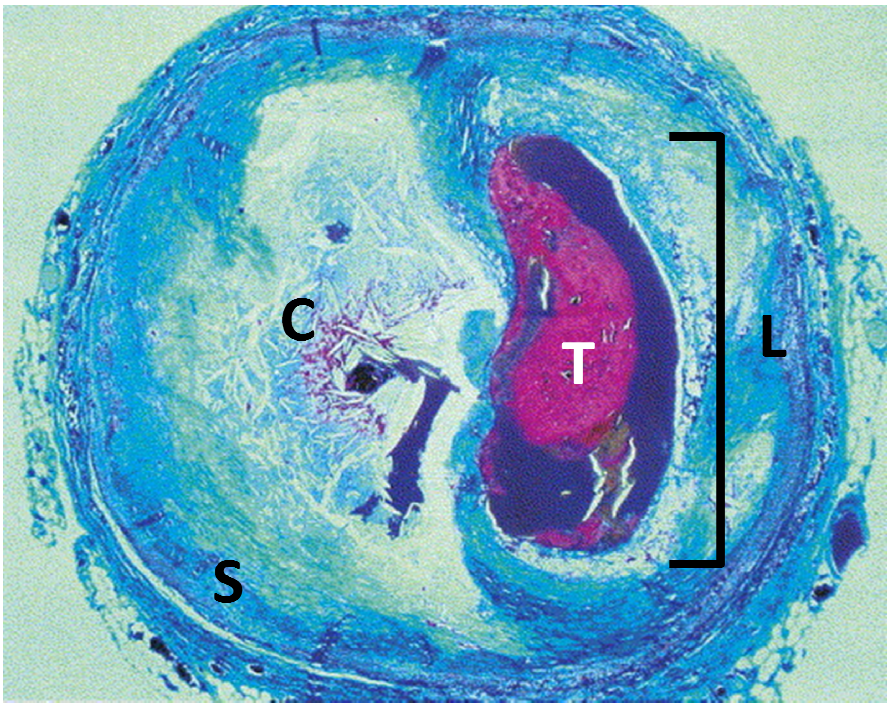


Figure 1. Atherothrombotic plaque. Masson Trichrome stain of a cross sectioned coronary artery containing a ruptured and thrombotic lesion illustrating all the components of a typical atherosclerotic plaque including the large necrotic core (C) containing cholesterol crystals, extracellular matrix typical of Phase 1 and 2 lesions. A thrombus (T) occludes the vessel which is typical of late phase lesions (Phase 3-5) (Fuster et al., 2005b). L, lumen of the vessel. S, smooth muscle cells.

arterial walls in high-shear stress areas. These stenoses can occur within the small lumen of the coronary vasculature; as such, vessel narrowing can lead to tissue ischemia. As the disease progresses, the occlusion will cause severe decreases in oxygen delivery to cardiac tissue precipitating myocardial ischemia—clinically angina attacks. Additionally, due to the necrotic core, the fibrous cap overlying the plaque thins to a point where local shear stress promotes rupture, causing flow-limiting thrombi or emboli characteristic of Phase 3-5 lesions. These lesions lodge within the vasculature either locally or distally disrupting blood flow (Fuster et al., 2005a; Libby et al., 2011). Clinically, this embolization within the coronary vasculature is the principle cause of acute coronary syndromes including unstable angina, myocardial infarction, and if not treated sufficiently, sudden cardiac death. Additionally, atherosclerosis is associated with the etiology of many other vaso-occlusive disorders such as peripheral artery disease, renal artery stenosis, and cerebrovascular diseases including transient ischemic attacks (TIA), syncope, and stroke (Aronow and Ahn, 1994; Dormandy and Rutherford, 2000; Fowkes et al., 1991; Jackson and Clagett, 2001; Ogren et al., 1993; Sander et al., 2011).

Historically, atherosclerosis was viewed as a passive disorder of insidious lipid deposition at key plaque-prone sites. In the last twenty years, this view has largely been replaced with the view of the atherosclerotic plaque as being a dynamic inflammatory lesion (Libby, 2006). More importantly, the clinically relevant consequence of atherosclerosis is plaque rupture. As outlined above, Phase 3-5 plaques precisely describe the morphologies of plaques which have acutely undergone rupture promoting a cascade of thrombosisformation with fibrin deposition and embolization. It is evident from the present discussion that cellularity and the abnormal cellular composition within the plaque plays an etiologic role in the establishment and progression of the atheroma. In the next

section I outline several studies which demonstrate a clear role of the abnormal regulation of cell death and how this deregulated process tips a lesion towards atherosclerosis.

1.2 The Role of Apoptosis in Atherosclerosis

1.2.1 APOPTOSIS DEREGULATION IN CELLS REGULATING PLAQUE PROGRESSION

Apoptosis, programmed cell death, is a highly conserved process for normal cellular turnover of tissues. Deregulation of this tightly regulated process has been implicated in autoimmune diseases, neurological disease states, cancer, and cardiovascular disease (Bredesen, 2008; Hanahan and Weinberg, 2011; Maniati et al., 2008; Whelan et al., 2010). As outlined above, it is quite evident that alterations in cellularity and thus cell number play a unique role on the progression of an atheroma (Geng and Libby, 2002; Kockx and Herman, 2000). The progression of a fatty streak to the atheroma involves the proliferation of VSMCs, the recruitment of inflammatory cells, and the aberrant persistence of these cells. For decades, the focus of atherosclerosis was on the involvement of VSMC proliferation and on finding mitogenic mediators such as the platelet derived growth factor (PDGF) mediating this effect. However, PDGF was shown to function as a chemoattractant with PDGF-mediated bursts of proliferation during acute injury (Badimon et al., 1993; Fuster et al., 1992a; Graf et al., 1997). In the ensuing years, a better understanding of atherosclerosis has lead researchers to revisit the idea that atherosclerosis involves cell death, as first postulated by Virchow's *fibro-fatty degeneration* even going so far as to use the term *endarteririts deformans* impliying these lesions as being involved in inflammation (Fuster et al., 1992b, c; Virchow, 1856). In order to fully appreciate the involvement of cellular proliferation as a mechanism of atheroma formation, a discussion on the regulation of cellularity should take into account mechanisms regulating cell death. In fact, early

studies into apoptosis in atherosclerosis demonstrated apoptotic cell death in MΦ and VSMCs as determined by the finding that Caspase-1 and Fas/TNF-Caspase pathways were involved both in human atherosclerotic specimens and experimental models of atherosclerosis (Geng et al., 1997; Geng et al., 1996; Kockx et al., 1996)

Roughly, the progression of atherosclerotic plaques can be divided into two phases, an early phase and a late phase, each involving deregulated apoptosis. Indeed there are rough correlations between the histopathologic plaque lesions and alterations in the cells of those lesions—both cellular and intracellular. The early phase (fatty streak and early atheroma formation) involves deregulated cell proliferation with minimal apoptotic clearance of VSMCs and endothelial cells (EC) with concomitant EC dysfunction—essentially an antiapoptotic mechanism (Geng and Libby, 2002). In this early stage, injury to the vascular endothelium is an important initating event that promotes an increase in EC turnover which involves cycles of apoptosis and proliferation (Dimmeler et al., 1998b). In fact, the atherosclerotic milieu which consists of high glucose concentration, OxLDL, oxidative stress, and angiotensin II can all stimulate EC apoptosis (Dimmeler et al., 1997a; Dimmeler et al., 1997b; Dimmeler et al., 1998a; Du et al., 1998; Lizard et al., 1997a; Lizard et al., 1997b).

Throughout atheroma progression, the late-stage advanced plaque contains numerous inflammatory cells with increased oxidizing enzymes affecting the local microenvironment. This localized environment further promotes oxidation of LDL. Numerous studies indicate that oxidized LDL is a potent inducer of atherosclerosis and is associated with apoptosis signaling in the early and advanced lesion (Martinet and Kockx, 2001). OxLDL can stimulate endothelial cells to produce proinflammatory molecules such as adhesion factors on the endothelium including ICAM and VCAM-1 and the

chemoattractants TNF, IL-1B, and M-CSF recruiting inflammatory cells to these sites. Concurrently, altered ECs and VSMCs recruit inflammatory cells through the expression of adhesion molecules. Apoptosis deregulation of vascular smooth muscle cells has vascular sequelae seen in coronary artery disease and atherosclerogenesis including upregulation of pathways promoting cell survival and inflammation (Bombeli et al., 1997; Shah, 2000). VSMCs show an activation of NF-kB signaling and matrix metalloprotease proteins (MMP) 1 and 9 which are necessary for the expression of ICAM and VCAM-1 (Couffinhal, 1994; Miyoshi, 2006; Barlic, 2007}. Furthermore, alterations in the EC involve deregulation of TGF-beta and p38 pathways through an OxLDL-mediated pathway playing an additional a role in promoting the inflammatory environment seen in atherogenesis (Dimmeler et al., 1997a; Dimmeler et al., 1997b). These events are thought to further drive atheroma formation through the additional production of chemoattractant cytokines from recruited immune cells.

Finally, the instability of the atherosclerotic plaque itself is a product of altered cell clearance and deregulated apoptosis. Proliferation of macrophages and vascular smooth muscle cells without subsequent clearance in a pro-inflammatory background promotes atheroma formation (Clarke et al., 2006). Intriguingly, the subsequent necrotic cores arising from the increased cellularity within late atherosclerotic plaques promotes apoptosis within the cells, causing plaque rupture and atherothrombosis through the exposure of pro-thrombotic cholesterol crystals, tissue factor, and necrotic cellular debris (Libby et al., 2011; Virmani et al., 2002). Thus apoptosis deregulation is implicated at all stages of atheroma formation from the initial streak to the ruptured necrotic core and modulates VSMCs, ECs, and inflammatory cells.

1.2.2 APOPTOSIS DEREGULATION IN MACROPHAGE-DERIVED FOAM CELLS

From a cellular perspective, the macrophage plays an important central role in both the progression and in the rupture of a plaque (Clarke et al., 2006). While deregulated apoptosis appears important in VSMCs and ECs in atherosclerosis progression, it is the MP and the MP derived foam cell which has been extensively studied in atherogenesis. Particuarly, apoptosis deregulation has been evaluated as having a central role inpromoting the abnormal persistence of the macrophage within the atheroma (Geng and Libby, 1995). Mechanisms include alterations in cell signaling of endoplasmic reticulum stress mediated apoptosis. Additionally, the aberrant activation of the tumor suppressor p53 in macrophages is well understood in the initiation and progression of atherosclerosis, presumably through alterations in p53-mediated apoptosis signaling (Boesten et al., 2009; Yuan et al., 2011). Importantly, the formation of macrophage-derived foam cells from internalizing circulating oxidized cholesterol and oxy-sterols is implicated as a key event in the formation and persistence of the foam cell. In the establishment and progression aspects, dysfunctional ECs and the retention of LDL and its oxidized product promotes the migration of macrophages to plaque prone sites.

Several studies have shown that as a corollary, elevated serum cholesterol and intercellular inflammatory cell signaling in plaque-prone sites, further promotes the formation of oxidized-LDL, which is then internalized by the macrophage. The inability of the macrophage to process the oxidized-LDL leads to a persistent localized inflammation and the differentiation of macrophages to foam cells. It is the aberrant apoptosis of these macrophages coupled with a destabilized fibrous cap characteristic of late lesions which destabilize the plaque (Phase 4-6).

In turn, these foam cells are not quiescent but are known to secrete pro-inflammatory mediators such as interleukin (IL)-1, macrophage-colony stimulating factor (M-CSF), and tumor necrosis factor (TNF)-alpha. This secretion of inflammatory mediators recruits circulating monocytes into plaque-prone sites thereby exacerbating the lesion and further promoting atheroma formation. Ultimately, the combination of an abnormal presistence of the macrophage lineage foam cells and the altered persistence and migration of VSMCs in the plaque leads to progression into the late atherosclerotic plaque.

The molecular mechanisms regulating the macrophage-to-foam-cell formation involves the combination of an inflammatory environment due, in part, to *in vivo* reactive oxygen species formation, enhanced levels of low density lipoproteins and serum cholesterols, and the aberrant expression of orphan oxy-sterol receptors such as MSR-A, the mechanism of which is not well understood. *In vivo* and *in vitro* studies have demonstrated that oxLDL alters the lipid-laden macrophage allowing it to persist within the plaque. In these gene expression studies, oxLDL-laden Mφ over-express the Macrophage-Scavenger Receptor (MSR-A) which is associated with resistance to apoptosis signaling. Additionaly, these studies have demonstrated impaired pro-apoptotic signaling in these MPs due to increased Bcl-2 related protein, PIM-1 oncogene, FLICE inhibitory proteins as well as decreased expression of Caspase -1 and -3 (Perlman et al., 1999; Shiffman et al., 2000).

Furthermore, the late atherosclerotic plaque is prone to rupture in part because recruited MΦ's secrete matrix metalloproteases and tissue plasminogen activators. This process results in the thinning of the protective fibrous cap overlying the lesion thus rendering the late plaque susceptible to rupture. This alteration in cellularity has been shown to be directly proportional to MΦ number. Thus in the early phases, the MΦ is

responsible for plaque progression but in late phase plaques, MΦ cell death is the driving force for disease progression (Liao et al., 2000; Liao et al., 1999).

In studying the cellular components in the atherosclerotic lesion and the molecular mechanisms of progression, it emerges that a careful study of the regulation of apoptosis is necessary in order to understand how aberrant apoptosis regulation plays an etiologic role in promoting the establishment and progression of this disease. In the next section, a broad background on apoptosis will be presented with a focus on the regulation of this process.

1.2.3 MECHANISMS OF APOPTOSIS REGULATION

Apoptosis, is a tightly regulated process. This crucial process is necessary from the embryonic environment where vital embryonic structures are formed, then programmed to die at key developmental points. As an organism progresses through its lifetime, normal tissue maintenance relies on apoptosis as older cells outlive their usefulness and are properly replaced with newer cells. In contrast to necrosis, where the cellular morphology is one of osmotic swelling and subsequent release of cellular contents to the surrounding extracellular space, characteristic changes of apoptosis include membrane involution (termed “blebbing”), loss of cell membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation.

In the last twenty years, the field of apoptosis research has revealed more on regulation and the various molecular events related to this process. Apoptosis was first investigated rigorously in 1965 by John Ross Kerr. In his seminal work, Kerr was able to distinguish apoptotic cells and necrotic cells from a morphological standpoint—the first step was in recognizing that cell death was not a passive process. The molecular mechanisms of apoptosis and the involvement of cellular mediators of this process were

later elucidated by Robert Horvitz and John Sulston for which they would receive the 2002 Nobel Prize in Physiology or Medicine.

Apoptosis regulation can be described loosely in terms of an *Extrinsic Pathway* and an *Intrinsic Pathway*. The principle mediators of apoptosis at the molecular level are the cysteine-aspartate proteases, otherwise known as the Caspases. First characterized in *C.* elegans, as CEDs (*C. elegans Death* proteins, CED-3 being the first caspase discovered), these proteases were ultimately linked to the necessary components in apoptosis initiation. Caspases are divided into initiator caspases and executioner caspases. The intiator caspases, Caspase -2, -8, -9, -10 have as their substrates other caspases. Once activated through oligomeric recruitment (DISC recruitment with Caspase 8 and Apoptosome recruitment in the case of Caspase-9), initiator caspases cleave executioner pro-caspases into activated caspases: Caspase -3,-6, and -7. These caspases, in turn, cleave a variety of intracellular substrates including structural proteins involved in focal adhesions, enzymes needed in DNA synthesis, endoplasmic reticulum maintenance, and even DNA itself.

1.2.4 THE EXTRINSIC PATHWAY

The extrinsic pathway is mediated principally in a classic ligand-receptor mechanism. As an example, the extrinsic regulation of apoptosis is seen in immune cells where the Fas/Fas-Ligand system is a typical example. In this mechanism, the Fas-Ligand which is found on immune cells, binds to the receptor Fas after ligation and activation of the death receptor, a series of signaling events leads to the formation of the *Death Inducing Signaling Complex (DISC)* in the recipient cell. This leads to a conformational change in the receptor complex intracellularly and recruits a series of intracellular-membrane associated proteins ultimately forming the *Death Complex*. This complex then

recruits and activates the cysteine-aspartatepro-protease (pro-caspase)-8 enzyme. Caspase 8 cleavage and activation then cleaves and activates pro-caspase-3. In some cell types, caspase 8 cleavage can also lead to cleavage of the pro-apoptotic Bid protein to *t-Bid* (truncated-Bid) which then allows for recruitment of the pro-apoptotic Bad and Bax, Bcl-2 family proteins to homodimerize within the mitochondrial membrane. This alternate activation of apoptosis indeed *feeds* into the *intrinsic apoptosis pathway* to be discussed below.

As examples that the extrinsic pathway is involved in atherosclerosis, studies of apoptosis in MΦ'sand VSMCs have shown that within the oxidizing microenvironment of an atherosclerotic plaque, the MΦ increases expression of Fas-L; concomitantly, MΦ nitric oxide release induces Fas expression on VSMCs increasing their demise and ultimately thinning of the plaque fibrous cap (Fukuo et al., 1997; Geng et al., 1997).

1.2.5 THE INTRINSIC PATHWAY

The intrinsic pathway is known to be activated through actions of the mitochondria, although the mitochondria are not required for intrinsic activation. In this well-understood pathway, cellular stress originating from reactive oxygen species in proximity to the mitochondrial electron transport chain causes rupture of the mitochondrial membrane, termed the mitochondrial transition pore. Alternatively, other signals which stress the cell activate B-cell leukemia (Bcl)-2 family proteins which form pores within the mitochondrial membrane through oligmerization of these Bcl-2 proteins. Once mitochondrial leakage occurs, calcium and cytochrome (Cyt)-C, mitochondrial specific proteins, leak into the cytoplasmic space. In a unique emergent evolutionary property, CytC functions not only as a mixed-function oxidase, but also as a signaling molecule in

regulating apoptosis activation. In this scenario, CytC forms a supramolecular complex with another apoptosis-promoting protein, the Apoptosis activating factor (Apaf)-1. Once this large multimeric complex, the apoptosome, is formed, ATP cleavage occurs thereby cleaving and activating the initiator protease pro-caspase 9 whose substrates include the executioner caspase, caspase-3. As in the Extrinsic Pathway, caspase-3 activation begins the process of apoptosis, with a variety of substrates including other proteins and DNA. Figure 2 illustrates both the extrinsic and intrinsic pathway.

1.2.6 Additional Mechanisms of Apoptosis Activation

An endoplasmic-reticulum mediated apoptosis pathway has been described. In this scenario, cellular stress either mediated through Ca2+ or through direct ER stress (heat shock and toxic inhibitors including glycosylase inhibitors in the ER) leads to activation of the initator caspase pro-caspase 12, however the mechanism of activation is largely unknown. What is known is that ER stress first attempts to increase transcription of heat-shock proteins to stabilize existing transcripts in the ER lumen and at the same time to attenuate transcription of other cellular genes in an effort to reduce global protein translation, the aptly-named unfolded protein response (UPR). Yet chronic (over twelve hours) ER "stress" leads to cleavage and activation of pro-caspase 12 through a UPR-dependent mechanism (Feng et al., 2003; Lin et al., 2007; Zinszner et al., 1998). *In vivo* rodent studies have established a link between ER stress and the development of atherosclerosis. Recent evidence has shown the involvement of the ER Transcription factor C/EBP Homologous Protein (CHOP or GADD153) plays a role in activating ER stress and promoting atherogenesis specifically through aberrant activation of apoptosis (Tsukano et al.). One study showed that mouse double knockouts in CHOP and LDL-Receptor were less

prone to developing atherosclerosis than LDL-Receptor single knockout mice by reducing the apoptosis burden in macrophages (Thorp et al., 2009). The authors proposed the mechanism to be mediated through the reduction of foam cell apoptosis in advanced plaques. Intriguingly, this knockout also reduced the plaque burden showing that early reduction in apoptosis is also athero-protective.

Finally, another mechanism of activation of the intrinsic pathway is through transcriptional activation and regulation of the cell-cycle. These pathways include activation of the tumor-suppressor protein, p53. In this pathway, DNA damage leads to activation of checkpoint kinases (Chk1 or Chk2, depending on the phase of the cell cycle the cell is in). These kinases phosphorylate the p53 inhibitor, MDM2, which in turn is ubiquintinated freeing up p53 to translocate to the nucleus and transcribe genes including p21 and Bcl2 and Bax. If the genetic lesion cannot be repaired, apoptosis signaling commences including the transcriptional activation of Bax, Puma, and Noxa, all pro-apoptotic genes. The full pathway of this p53-mediated apoptosis is little understood, however studies in our group have shown that p53-mediated apoptosis activation in response to DNA damage can be mediated, in part, through an interaction with the translationally controlled tumor protein, TCTP, also identified as fortilin. The underlying principle of this pathway is the stabilization of p53 which in turn activates apoptosis promoting genes. These genes then promote activation of the mitochondrial release of CytC and ultimately the intrinsic pathway is activated.

At the level of the genotoxic stress, the inflammatory environment promotes release of oxidative mediators including nitric oxide and peroxynitrides. These oxidative products cause DNA damage locally which then can activate the p53 mechanism. Rapidly accumulating evidence points to a role for low levels of reactive nitrogen species as a

mechanism for cell survival. One study demonstrated that macrophages within plaques expressed high levels of inducible nitric oxide synthase (iNOS), demonstrating detectable amounts of nitrotyrosine, and that these cells showed signs of DNA synthesis and repair, presumably through a p53-mediated mechanism (Cromheeke et al., 1999; Kockx et al., 1998). Additionally, as a mechanism of apoptosis inhibition, ECs have been shown to utilize the low-output isoform of NOS, ecNOS , which can S-nitrosylate Caspase-1 and Capase 3 rendering these apoptotic proteins inactive and thus deregulating apoptosis signaling (Dimmeler et al., 1997a; Dimmeler et al., 1998b).

1.2.7 Regulation and Inhibition of Apoptosis

Through such studies as the aforementioned p53-mediated pathway, apoptosis signaling and regulation is a complex and tightly controlled process. For instance, only if the genetic lesion cannot be repaired is apoptosis initiated. The steps leading to this initation of apoptosis are intriguing and deserve attention.

While the above mechanisms of apoptosis may seem well defined, another layer of complexity is added when considering the mechanism of p53-mediated apoptosis. It can be deduced that genetic lesions leading to cell arrest and ultimately apoptosis is an important process; other mechanisms do exist which exert some control over apoptosis initation. There are two well-studied and characterized protein families which, through a variety of mechanisms, inhibit apoptosis signaling.

1.2.8 BCL2 Family of Proteins

The B-cell leukemia (Bcl)-2 family of proteins are proteins which share sequence homology with the Bcl-2 protein, termed the Bcl2 Homology (BH) domains. Originally identified in B-cell leukemias, the Bcl2 protein itself is antiapoptic and contains four BH domains, BH1,

BH2, BH3, and BH4 and all these proteins typically possess a transmembrane domain as well. The Bcl2 family of proteins consist of anti-apoptotic and pro-apoptotic proteins. The set of anti-apoptotic Bcl2 Family Proteins are those proteins which possess all four domains (as in the case of Bcl-2 itself). Conversely, pro-apoptotic Bcl-2 Family Proteins are divided into multi-domain BH containing proteins (as in the case of Bax, Bak, and Bok) or BH3-Only proteins, as in the case of Bid, Bim, Bad, Puma, and Noxa. These proteins, in the case of Bax or Bim, directly embed within the mitochondria forming the mitochondrialouter membrane pore (MOMP); other proteins can directly influence the levels of Bax/Bim, affect oligomerization of the proteins comprising the MOMP, or inhibit anti-apoptotic Bcl-2 family proteins thereby favoring the cell towards MOMP formation. Obviously, the transcriptional activation of these proteins is necessary for activation of the intrinsic pathway; although not the only way. Once the MOMP forms, CytC and Ca^{2+} leakage occurs, forming the apoptosome, and ultimately Caspase-3 activation.

Conversely, the anti-apoptotic Bcl-2 Family Protein members possess all four BH domains and include Bcl-2, Bcl-xL, Bcl-w, and Myeloid Cell Leukemia (Mcl)-1 as the principle proteins in this family. While an exact understanding of the mechanism of how anti-apoptotic Bcl-2 Family Proteins function is lacking, what can be deduced from several studies are that these anti-apoptotic Bcl-2 Family Proteins tend to function by inhibiting the oligomerization of the pro-apoptotic proteins thereby preserving mitochondrial membrane integrity (Chipuk et al., 2010). In human atherosclerotic plaques, it was found that Bcl-2 proteins were upregulated in close proximity with NO, although the significance is unclear. Additionally, Bax, Noxa, and Puma (all pro-apoptotic Bcl-2 proteins) are p53 target genes with p53 binding sites at their respective gene promoters. Several studies have provided clues into how altered regulation of BCL-2 proteins can promote plaque formation.

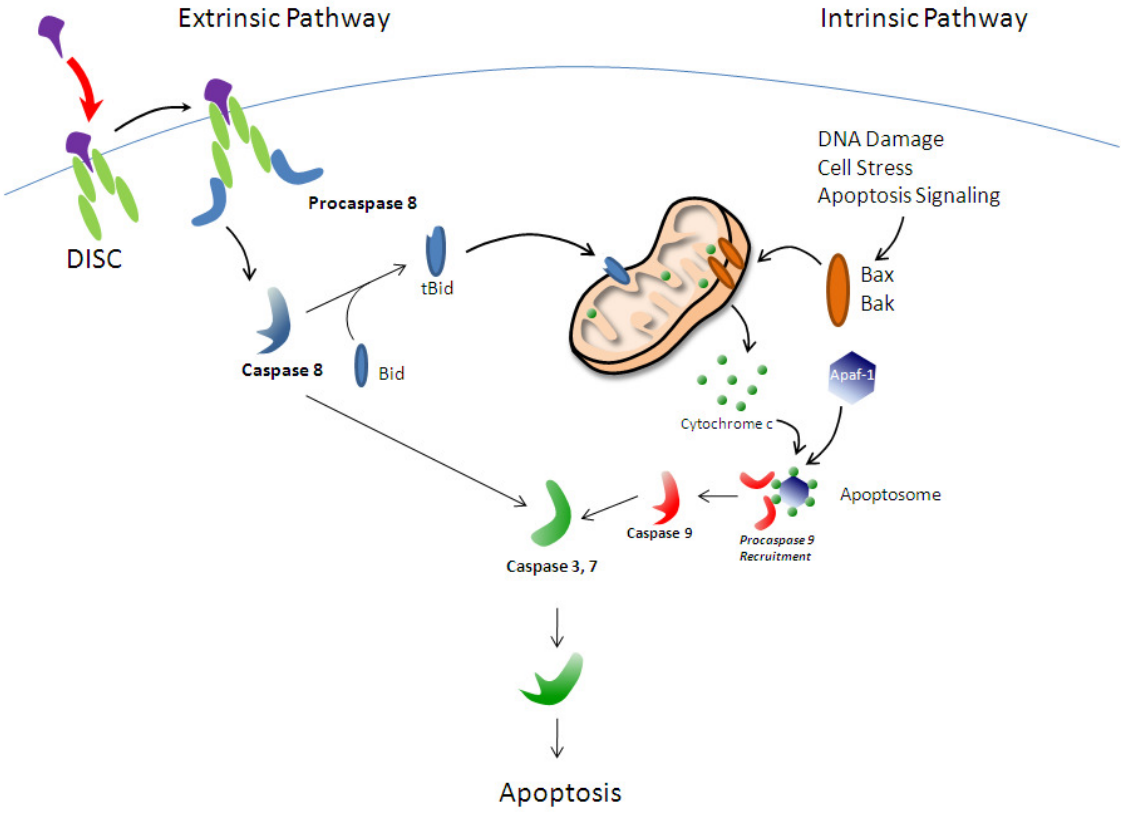


Figure 2: Apoptosis activation through the Extrinsic and Intrinsic Pathway. ***Left***, the extrinsic pathway involves the activation of Death Receptors via a Death Ligand (e.g. Tumor Necrosis Factor or Fas) recruiting the Death Induced Signalling Complex (DISC) intracellularly allowing for the stabilization and self-activation of pro-caspase-8. Activated Caspase-8 directly cleaves pro-caspase 3 to initiate the apoptosis cascade. Caspase-8 also cleaves the pro-apoptotic protein, Bid to tBid, which then embeds within the Mitochondrial membrane feeding into the Intrinsic pathway. ***Right***, the intrinsic pathway is activated at the level of the mitochondria through various intracellular stimuli including cell stress and transcription of pro-apoptotic Bcl-family proteins such as Bax and Bak. Formation of the mitochondrial pore and leakage of calcium and Cytochrome C (CytC) leads to formation of the Apoptosome through formation of a complex with the Apoptosis protein activation factor (Apaf-1). The apoptosome allows for a structural scaffold recruiting pro-Caspase 9. Activated Caspase-9 then cleaves Caspase 3 (or 7) thereby activating apoptosis.

In ECs, increases in Bax, Bak, Bad and a concomitant decrease in Bcl-2 and Bcl-xL (Bcl-2 long isoform) were found within atherosclerotic plaques (Kockx et al., 1998; Saxena et al., 2002 2000). These findings presumably promote cycles of proliferation and apoptosis. VSMC's also expressed increase levels of Bcl-xL promiting the proliferation and intimal migration of this cell type in early plaques (Pollman et al., 1998). Paradoxically in advanced lesions, VSMCs express increased levels of Bax and Bak and concomitantly decreased expression of Bcl-2 and Bcl-xL. Thus apoptosis of smooth muscle cells leads to decreased extracellular matrix deposition destabilizing the plaque. MΦs in advanced plaques show a similar downregulation of Bcl-2 and Bcl-xL with concomitant increased in pro-apoptotic Bax and Bak, lending mechanistic evidence in how accelerated apoptosis in the advanced lesion promotes plaque rupture (Kockx et al., 1998; Saxena et al., 2002). Overall, it is becoming clear that the Bcl proteins collectively regulate apoptosis signaling within atheromas and thus play an important role in disease progression.

1.2.9 INHIBITORS OF APOPTOSIS

In addition to the regulatory Bcl-2 Family Proteins which can promote or inlhibit apoptosis, another recognized group of proteins regulating apoptosis are the inhibitors of apoptosis proteins (IAPs). There have been eight mammalian IAPs that have been identified: Survivin, X-linked IAP (XIAP), cytosolic IAP (cIAP) -1, cIAP-2, Ts-IAP, ML-IAP, NIAP, and Apollon. The mechanism of IAP activation in mammals, of which XIAP is the most well-characterized, is through the specific inhibition of Caspase-3, -7, and -9.IAPs typically contain two distinct domains: the zinc binding Baculovirus IAP Repeat (BIR) domain and a RING Domain, typically in the carboxyl terminus. A protein possessing a BIR constitutes membership in the IAPs. The BIR domain has been shown to be essential in the direct

inhibition of caspase activity in IAPs. Furthermore, an additional layer of regulation of apoptosis occurs through regulation of the IAPs. This regulation includes transcriptional events, post-translational modifications (including phosphorylation and ubiquitinylation), and the direct inactivation through protein interactions with IAPs by the Second Mitochondrial Activator of Caspases (Smac) and the Omi proteins. Additionally, in addition to direct inhibition of caspases, IAPs also participate in cell signaling pathways including inhibition of prosurvival pathways involved in TRAF6/NF-kB signaling and TGF-β/JNK pathway and thus through direct inactivation of caspases or through signal transduction pathways, the IAPs can negatively regulate cell death signaling. Table 1 summarizes the protein regulators of apoptosis indicating the various similarities and differences within the Bcl Family Proteins and the IAPs.

1.3 Fortilin as a Novel Regulator of Apoptosis

In investigating novel protein regulators of apoptosis, a yeast two-hybrid screen revealed that the Translationally Controlled Tumor Protein (TCTP) interacts with Mcl-1, an antiapoptotic Bcl-2 protein (Li et al., 2001; Liu et al., 2005; Zhang et al., 2002). In fact, the mechanism of Mcl-1 mediated cytoprotection is not well understood and it is only inferred that Mcl-1 is antiapoptotic through inhibition of Bax dimerization through a physical interaction of the Mcl-1 BH3 domain with that of Bax (Susini et al., 2008). In this study, TCTP was shown to interact with and stabilize MCL-1 intracellularly, Presumably, the cytoprotective mechanism of TCTP is through its interaction and stabilization of Mcl-1 thus rendering a cell more resistant to apoptosis. TCTP was renamed as fortilin and further studies showed that fortilin can regulate apoptosis independent of Mcl-1 presence within the cell (Zhang et al., 2002). Intriguingly, fortilin shares no structural homology to the Bcl-2

family or IAP families (it possesses no BH or BIR domains). Structural homology studies of fortilin have shown that the *physical* structure of fortilin resembles a similar conservation with guanosine nucleotide-free chaperones of the Mss4/Dss4 family however sequence alignments do not reveal this homology (Thaw et al., 2001). The 172 amino acid protein was also identified as P21, P23, or Q23; point mutation analysis has demonstrated that the arginine-21 residue is critical for interaction with Mcl-1 and that abrogation of this interaction resulted in increased susceptibility to apoptosis (Li et al., 2001). Interestingly, fortilin is not only an antiapoptotic protein, it was previously revealed to also be an IgE-dependent Histamine Releasing Factor (another name for fortilin is HRF), an intracellular protein involved in tumor reversion, a protein which may regulate the cell cycle through an interaction with tubulin at the mitotic spindle, and as a calcium binding protein (Bazile et al., 2009; Feng et al., 2007; Gachet et al., 1997; Gachet et al., 1999; Graidist et al., 2007; MacDonald et al., 1995; Tuynder et al., 2004; Tuynder et al., 2002). Because fortilin binds calcium, it is thought that Fortilin-mediated cellular protection is mediated in part through this calcium "scavenging" function of fortilin. It was revealed that Glu^{58} and Glu^{60} mediate the calcium binding activity of fortilin (Graidist et al., 2007). Interestingly, another study showed through solution structure NMR that the putative critical Ca^{2+} binding residues were Asn^{131}, Gln^{133}, and Asp^{150} of human fortilin. In this study, the authors proposed that these residues may form a distorted coordination complex with Ca^{2+} thereby scavenging Ca^{2+} released from leaky mitochondria (Feng et al., 2007).

Additionally it is hypothesized that fortilin protects cells from apoptosis by inhibiting Bax homo-dimerization through an interaction of the fortilin H2-H3 helices with the Bax H5-H6 domains, but little direct evidence has shown this to be so (Susini et al.,

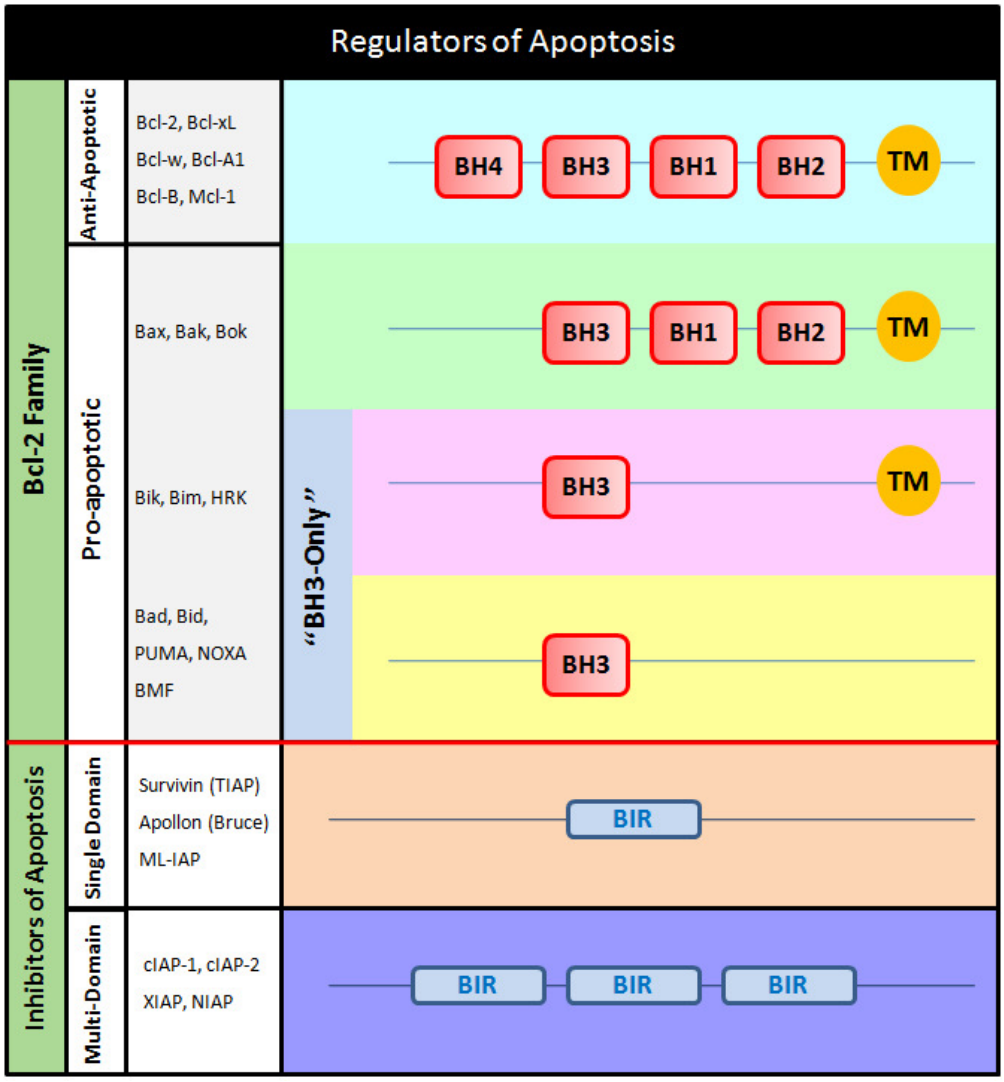


Table 1: Regulators of Apoptosis. Two well characterized families of apoptosis regulatory proteins include the Bcl-2 Family and the Inhibitors of Apoptosis. Bcl-2 Family Proteins all have a Bcl Homology (BH) domain. Anti-apoptotic Bcl-2 protiens have four BH domains and a transmembrane domain. Pro-apoptotic Bcl-2 proteins contain varying BH domains with or without a transmembrane domain. Inhibitors of apoptosis proteins contain the Baculovirus IAP Repeat in either multil-BIR or a single BIR-containing protein. The BIR domains themselves function, in part, through inhibition of the active site of caspases. Additional layers of regulation occur through transcription and there have been reports of IAPs regulating Bcl-2 proteins as well.

2008). Instead the authors were only able to demonstrate that in cells over expressing Fortilin, there is less Bax embedded in mitochondrial extracts, but do little to show direct binding of fortilin to Bax (Susini et al., 2008). In this scenario, the authors proposed that fortilin acts in the *same* manner as Mcl-1 and thus rectifies the Mcl-1 findings with the anti-apoptotic mechanism of fortilin.

In fortilin knockout embryos, the blastocysts do not survive past 3.5 days post coitus; we have shown that this occurs through an over activation of the BMP pathway enhancing DNA fragmentation and overexpression of BMP4 gene Msx1 (Koide et al., 2009). In fact, fortilin embryonic lethality occurs earlier than any organogenesis demonstrating that fortilin regulates embryonic processes where presumably apoptosis plays a significant role. While the mechanism is not clear, in this study Koide and colleagues were able to discern that fortilin negatively regulats BMP4-dependent Msx1 activation, knockdown of which leads to over-activation of the BMP pathway (Koide et al., 2009).

Finally, fortilin has been shown to interact specifically with the tumor suppressor and regulator of apoptosis, p53. In our study, we showed that fortilin binds and inhibits the DNA-binding domain of p53 thereby inhibiting the transcriptional activation of p53 and p53 dependent genes. Furthermore, in a subcutaneous tumor xenogra Ft model, cancer cells that contained a mutant form of fortilin which cannot bind p53 were not able to establish a detectable tumor mass by six weeks after injection whereas wild-type fortilin cells grew to form large tumors (Yanjie et al., 2011). The findings from this study were further confirmed by Rho and colleagues. In this study, the authors further showed that fortilin interacts with but increases the degradation of p53, but the mechanism remains unknown. Our results as well as those of Rho *et al.* showed that fortilin destabilizes p53 thereby preventing

apoptosis activation. This finding is intriguing and adds yet another layer of Fortilin-mediated cytoprotection.

1.3.1 FORTILIN IN CARDIOVASCULAR DISEASES

The role of fortilin in apoptosis regulation is clear from our published data. Additionally, there is an overwhelming body of evidence which shows the importance of apoptosis deregulation in the initation and maintenance of athersoclersosis as well as other cardiovascular conditions including the development of heart failure and restenosis injury (Geng and Libby, 2002; Whelan et al., 2010) .

Considered together with our fortilin studies and those of others, we hypothesize that fortilin mediated inhibition of apoptosis is involved in the maintenance of unwanted cells within the atherosclerotic plaque. On a molecular and mechanistic level, the aberrant deregulation of fortilin leads to the persistence of these cells within the plaque. As such the further study of fortilin biology is warranted.

In an effort to elucidate the clinical significance of fortilin, two pre-clinical studies were initiated. In a small study (N=19), coronary artery disease patients had a statistically significant elevation of fortilin RNA transcripts from whole blood extraction compared to volunteer controls (Figure 3A). The demographics from this study considered the risk factors of coronary artery disease patients including advanced age, chronic hypertension and diabetes compared to control. Future studies will have more groups for comparison and larger numbers. Additionally, in atherosclerotic plaques of endarterectomy samples from limb amputations, fortilin expression was found in atherosclerotic plaques co-localized with the MΦ marker CD-68 (Figure 3B). Close pathologic examination demonstrates that fortilin expression colocalizes with cells of a foam-cell like morphology

but not within the α-SMA cells indicating that fortilin is specific to portions of the atherosclerotic plaque where foam cells reside and not arterial media. This also argues against the notion that VSMCs play an active role in plaque progression. Taken together, the study of FT-mediated apoptosis deregulation may shed light on how apoptosis deregulation can mediate disease progression pointing the way for promising therapeutics for the prevention of atherosclerosis. Figure 4 illustrates a proposed model into how the intracellular mechanism of Fortilin-mediated antiapoptosis could possibly regulate MΦ survival thereby promoting atherogenesis.

1.3.2 FORTILIN AND PROHIBITIN: A NOVEL PROTEIN-PROTEIN INTERACTION

In order to better understand the cellular function of Fortilin, we speculated that fortilin protein interactions would shed light on the anti-apoptotic role of fortilin— in similar manner to the Fortilin-Mcl-1 interaction. In this study we show that fortilin specifically interacts with a well known anti-proliferative protein, Prohibitin (Phb). The aims of this study are to further investigate and describe the significance of this protein interaction. Phb is a ubiquitous and evolutionarily conserved protein of approximately 32 kDa in humans and mice (37 kDa in rats) and was discovered as an anti-proliferative protein (Altus et al., 1995; McClung et al., 1992; Nuell et al., 1991; White et al., 1991). Other proteins in the Phb family include Phb-2, Erlins, and Podocin. Phb was additionally recognized as a mitochondrial specific protein creating homodimers with the related Phb-2 protein (Nijtmans et al., 2000). While early studies of Phb characterize this protein as a mitochondrial chaperone, recent studies have demonstrated a nuclear and cytosolic function of Phb (Ande et al., 2009; Ande and Mishra, 2009; Chander et al., 2010; Emerson et al., 2010; Han et al., 2008; He et al., 2011; Li et al., 2011; Muraguchi et al., 2010). Nuclear

functions of Phb include transcriptional regulation of Retinoblastoma (Rb) target genes and negative transcriptional regulation of p53 (Fusaro et al., 2003; Fusaro et al., 2002; Shi et al., 2009; Wang et al., 2002a; Wang et al., 2002b). These studies also show that this transcriptional activation of Phb is through a novel Phb-Rb or Phb- p53 interaction as demonstrated by co-immunoprecipitations (Fusaro et al., 2002; Shi et al., 2009; Wang et al., 2002a). Additionally, upon apoptosis signaling, Phb is translocated from the nucleus to the cytosol through a CRM1-mediated nuclear export mechanism (Rastogi et al., 2006). While the significance of the nuclear export is unclear, this study points towards the cytosolic importance of Phb and speculates that Phb plays a role in apoptotic signaling. Nuclear involvement of Phb include cell cycle regulation through inhibition of Cyclin E, transcriptional co-repressor of estrogen receptor alpha signaling, but as a transcriptional co-activator of androgen receptor signaling (Choi et al., 2008; Gamble et al., 2007; Gamble et al., 2004; He et al., 2008; Urbanucci et al., 2008; Zhang et al., 2007). Additionally, cytosolic functions of Phb include Phb involvement in Diapause signaling mediated through IGF-1 pathway and TGF-b (Artal-Sanz and Tavernarakis, 2009; Guo et al., 2007; Zhu et al., 2006; Zhu et al., 2010). Other cytosolic roles of Phb signaling include the requirement for Phb in Ras-mediated Raf/MEK/ERK signaling (Rajalingam and Rudel, 2005).

Indeed the involvement of Phb in apoptosis is intriguing and warranted especially in the context of the emerging cytosolic role of Phb coupled with our observed protein interaction with fortilin. The value of conducting this study points to a viable clinical target in the continuing effort to decrease the burden of cardiovascular diseases to society.

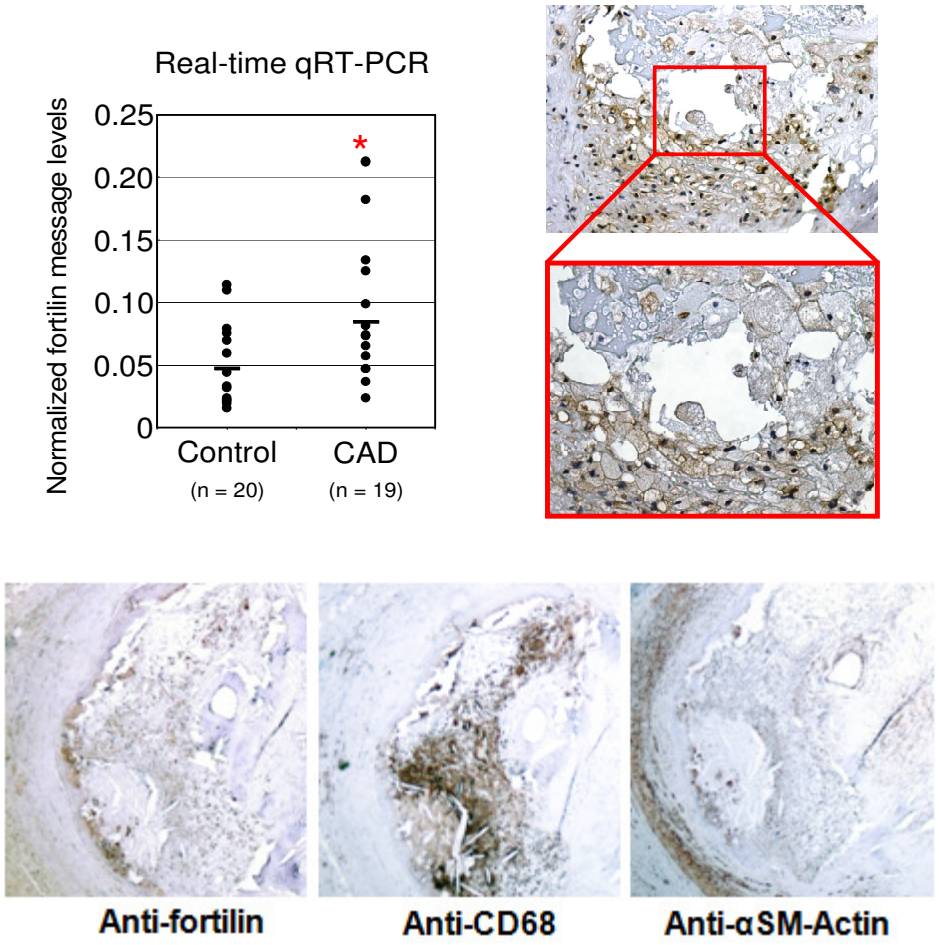


Figure 3. Fortilin is found in atherosclerotic tissues. **A**, Atherosclerotic plaque from human endartertomy tissues demonstrate that fortilin expression is found in the atherosclerotic plaque necrotic core. fortilin expression occurs in cells co-expressing the macrophage marker, CD-68. fortilin expression is minimally present in Smooth Muscle (SM)-Actin staining cells, a marker for Vascular Smooth Muscle Cells. **B**, fortilin expression in human Coronary Artery Disease patients. Total RNA from whole blood of control volunteers or coronary artery disease patients were measured for *TPT1* mRNA levels using Real-Time PCR. **C**, Higher magnification of atherosclerotic plaque stained with anti-Fortilin antibody demonstrating that fortilin is expressed in cells with a foam cell morphology.

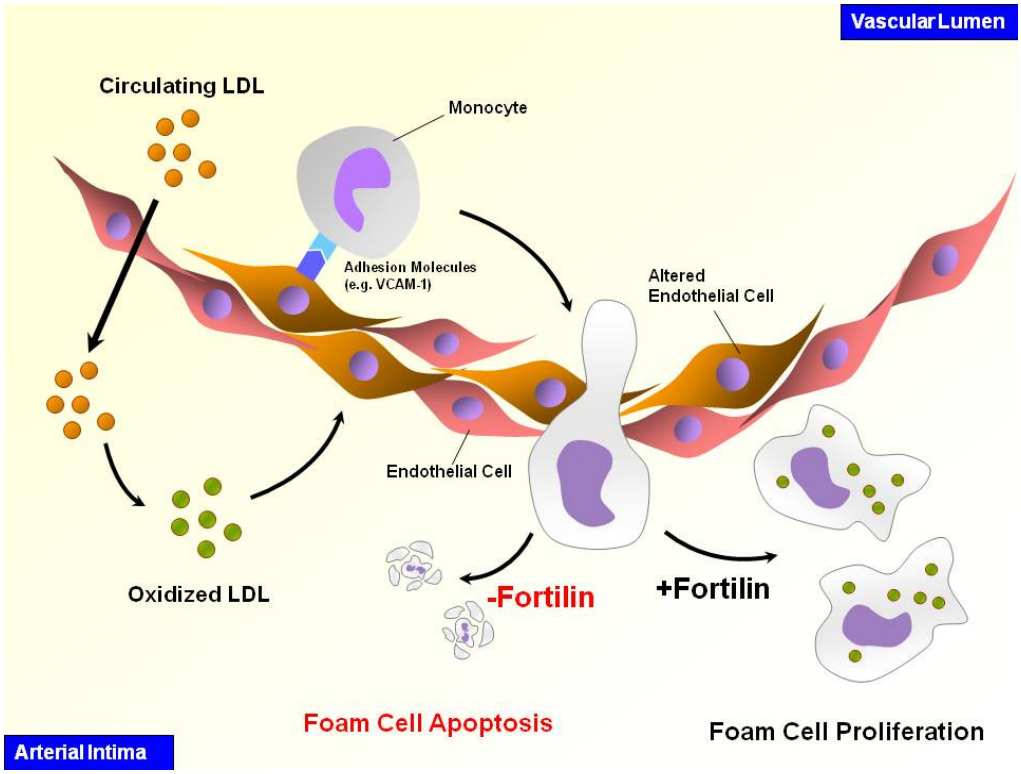


Figure 4. Potential mechanism into how fortilin allows for the persistence of macrophage-derived foam cells. As described in the text, atherogenesis involves the retention of LDL within the arterial intima leading to LDL oxidative modification (OxLDL). OxLDL creates an altered oxidative microenvironment affecting the endothelium, increasing the expression of adhesion molecules in the vascular luminal side, and allowing for the recruitment and retention of circulating monoctyes. Macrophage differentiation is activated with the OxLDL through expression of orphan Macrophage Scavenger Receptors and leads to the formation and persistence of foam cells as a result of OxLDL being incompletely processed within the Macrophage.

1.4 Rationale for this Study

Our group has extensively characterized fortilin (Ft) as an anti-apoptotic protein. Fortilin, through a protein interaction with Mcl-1 enhances cytoprotection (Li et al., 2001; Liu et al., 2005; Zhang et al., 2002). Additionally, Ft overexpression in cells alone was sufficient to protect cells from 5-fluoruracil-induced cytotoxicity as well as UV-induced apoptosis activation (Graidist et al., 2004). Ft also binds calcium and in-turn modulates Ca^{2+} induced apoptosis (Graidist et al., 2007). Finally, our preclinical study and data derived from human atherosclerotic tissues truly show the novel role of Ft in regulating processes which promote disease progression.

In addition to other functions ascribed to this novel protein, Ft protein interactions are very important in understanding the cellular context of protein function. It has been proposed that fortilin may function as a protein chaperone due in part to the small size of this protein and due to the myriad described protein interactions (Thaw et al., 2001; Zhang et al., 2002). As such the goal of this study is to further elucidate novel

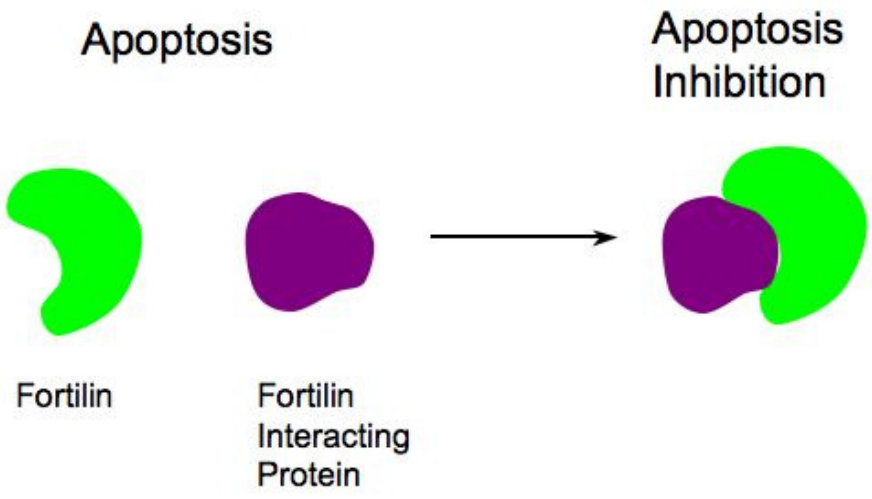


Figure 5: Potential mechanism for fortilin regulation of apoptosis. Previous work and work performed in this laboratory has identified fortilin interacing proteins such as prohibitin. We hypothesize that fortilin interactions with other protein partners allows for the mutual stabilization of this protein thereby enabling a cell to be resistant to apoptotic stimuli.

Ft protein interactions to shed light on a possible novel mechanism of Ft antiapoptotic activity. We seek to test the hypothesis of whether and how these novel protein-interactions modulate cell survival after a stress. A rigorous understanding of fortilin protein interactions is lacking which this study aims to provide. Furthermore, the results of this study will shed light on a better understanding of apoptosis regulation that can affect various cells and in turn, target tissues. By understanding novel mechanisms into how apoptosis is modulated, this will lead to better hypotheses and models into atherosclerosis initiation and maintenance. Additionally, such studies can provide a platform for further models into the investigation or development of novel therapeutics. As the rise, popularity, and success of targeted inhibition and molecular mimetics has demonstrated, starting with a sufficient cellular model and then investigating novel mechanisms of inhibiting those processes can lead to better clinical outcomes in various diseases as cancer and chronic inflammatory diseases. Such studies as ours will provide fundamental knowledge towards the potential design of targeted molecular therapeutics for atherosclerosis which is desperately lacking in comparable success stories.

In order to pursue this study, we sought to first identify novel Ft-interacting proteins (FIPs) using a co-immunoprecipitation screening method. After identification of putative FIPs through Matrix Assisted Laser Desorption Ionization Time- of- flight (MALDI-TOF/TOF) Mass Spectrometry we were able to identify Phb as a novel FIP. The purpose of this study is to verify this interaction and to test how this protein-protein interaction facilitates or inhibits fortilin antiapoptosis function as illustrated in Figure 5. In order to accomplish this, we seek to characterize the Ft-Phb interaction through two specific aims. Aim one will further verify this interaction as novel and specific to fortilin. We seek to test this interaction through the design of a FLAG-epitope-tagged Phb construct which will then

be used in a reciprocal co-immunoprecipitation to test that this interaction is specific and can be performed *in vivo*. We will then evaluate if Ft and Phb are found in the same cellular locations through a subcellular fractionation study and by co-staining using immunofluorescence techniques.

In Specific Aim 2, we seek to test the functional significance of the Ft-Phb interaction by evaluating the relative stabilities of Ft and Phb by performing a chaperone assay to determine if Ft and Phb protein degradation is changed with the targeted deletion of the cognate binding partner through short-interfering RNA techniques and overexpression of the proteins in the presence or absence of the binding partner.

Additionally, we will test the functional consequence of this interaction by utilizing cells that over-express Ft and Phb and their effects on apoptosis activation after cellular stress. Cells will over-express Ft, Phb, or both and subjected to UV-induced cell stress and evaluated for caspase 3 activity and DNA fragmentation. Cells will also be tested and evaluated for Caspase 3 activity and DNA fragmentation to detect levels of apoptosis activation after the targeted deletion of FT, PHB, or both. These studies are necessary to establish and evaluate the significance of this novel FT-PHB protein interaction. Shedding light on the significance of this novel protein interaction is the primary focus of this dissertation.

CHAPTER 2: PROTEOMIC IDENTIFICATION AND VERIFICATION OF PROHIBITIN AS A NOVEL FORTILIN INTERACTING PROTEIN

2.1 ABSTRACT

Fortilin, a 172 amino acid nuclear-cytosolic shuttle protein is a novel regulator of apoptosis. Dysregulation of apoptosis has been shown to be involved in a variety of human diseases such as neurodegenerative diseases, neoplasia, autoimmune, and cardiovascular disorders. Previous studies have shown that fortilin is an antiapoptotic protein and protects from 5-fluorouracil-mediated apoptosis. Additional studies have indicated that fortilin has a wide variety of cellular roles including an extracellular histamine releasing activity, a regulator of polo-like kinase activity, and as a calcium binding protein. While such studies have shed light on the intracellular and extracellular roles of Fortilin, sequence alignment and structural studies reveal that fortilin has no enzymatic activity and has no sequence homology to know regulators of apoptosis. Thus, studies on the biological context of Fortilin-mediated apoptosis regulation are still lacking. The strength of previous studies show that fortilin participates in a variety of biological phenomena, but what is lacking is a more detailed understanding of the biological interactions of fortilin. In this study, we shed light on a novel fortilin protein interaction with the anti-proliferative protein, Prohibitin (PHB). The goal of this study was to better elucidate a potential mechanism for Fortilin-mediated apoptosis regulation. Our studies in this chapter demonstrate that fortilin and Prohibitin interact with one another, that this interaction is specific, and colocalization studies demonstrate that this interaction is demonstrated *in vivo.* This findings of this first study will set the

stage for future functional studies into the importance and biological significance of this FT-PHB interaction.

2.2 INTRODUCTION

Fortilin (Ft) is a novel regulator of apoptosis (Li et al., 2001). It is proposed that Ft accomplishes this through three distinct mechanisms. 1) Binding and inhibiting released Ca2+ thereby inhibiting calcium-mediated apoptosis (Arcuri et al., 2004; Arcuri et al., 2005; Feng et al., 2007; Graidist et al., 2007) 2) directly inhibiting Bax dimerization thereby inhibiting Bax-mediated apoptosis signaling (Susini et al., 2008); and 3) similar to (2), directly binding to and stabilizing Mcl-1, which in turn inhibits Bax (Li et al., 2001; Liu et al., 2005; Zhang et al., 2002). There is direct evidence for all three mechanisms working distinctly and concurrently. Graidist *et al.* showed that while Ft can bind calcium and scavenge some released calcium, the affinity of Ft for calcium is modest and at physiologic concentrations may be sufficient to maintain modest increases in calcium. Other studies have verified the importance of the calcium-binding activity of Ft in normal and cancerous tissues. These studies further verify that in a proteomic screen, Ft was the most recognized as a calcium-binding protein and was overexpressed in hyperplastic and cancerous prostate tissue. Ft was identified as being up-regulated in cancer tissues and down-regulated in normal cells and revertant cancer-cells. The possible mechanism of which could be in-part, due to the higher levels of fortilin in cancer cells rendering these cells more prone to survival and spared from calcium-mediated cell-death.

Other evidence points to how Ft may inhibit Bax-dimeriztion within the mitochondrial membranes. Direct evidence from Susini's studies shed light on how fortilin is found anchored to mitochondrial fractions after cell stress and protects mitochondria from Bax-induced mitochondrial leakage (Susini et al., 2008). Along these lines, Li *et al* showed that fortilin binds to and stabilizes Mcl-1, an anti-apoptotic protein of the Bcl-2 family (Li et al., 2001). Susini suggests that FT-MCL-1 heterodimers can then form self-oligomerize indirectly inhibiting Bax embedding within the mitochondrial outer membrane. While no structural or biochemical data exist to support this hypothesis, this study showed that increasing amounts of fortilin affected the amount of dimerized Bax inserted into mitochondria but not monomeric Bax. To further add insight to those studies, Li's data in conjunction with Zhang *et al.* and Graidist *et al.* propose that fortilin alone is sufficient to protect cells from apoptotic stimuli (Graidist et al., 2007; Zhang et al., 2002). Taken together, Ft is a *bona fide* regulator of apoptosis, yet the mechanism of this antiapoptotic activity remains unknown.

Structural studies of fortilin have shown a similarity to a chaperone, and still other studies have shown the significance of the Ft H2-H3 helices to be similar to the Bax H2-H3 helices, lending credence to Susini's argument that fortilin may self-dimerize to inhibit Bax dimerization. Considering these studies and published functional studies of Ft-mediated apoptosis protection, Ft has no known enzymatic activity and potentially acts as a co-regulator of cell survival. Previous studies from this group have shown that protein interactions with Ft reveal novel biological roles for Ft allowing us to infer on novel functions of Ft in the context of its interacting partner. The purpose of this study was to determine novel Ft interacting proteins to

better understand the complement of fortilin protein partners which would shed more light on the cellular regulators which fortilin may act upon or in conjunction with in order to exert its anti-apoptotic effects.

2.3 METHODS

Reagents, antibodies, and buffers:

Immunoprecipitation lysis buffer consisted of 30mM NaCl, 20mM HEPES, pH 7.4, 0.001% Igepal non-ionic detergent (Calbiochem) supplemented with protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN), and 1mM PMSF. Immunoprecipitation wash buffer consists of 150mM NaCl, 20mM kHEPES, pH 7.4, and 0.01% Igepal. Elution buffer was 50mM Tris-Cl, 0.1% glycerol, 1% SDS, 8µg/mL bromophenol blue.

For immunoblot analysis rabbit anti-fortilin antibody (MBL International), mouse anti-TPT1 antibody (Abnova), rabbit anti-prohibitin (Santa Cruz), mouse anti-GAPDH antibody (Fitzgerald Scientific), mouse anti-FLAG M2 antibody and anti-FLAG M2 agarose beads (Sigma-Aldrich, Saint Louis, MO), rabbit anti-hemagluttinin antibody (Bethyl Laboratories, Montogomery, TX), rat anti-HA matrix beads and mouse anti-Protein C matrix beads (Roche Applied Science, Indianapolis, IN). Immunofluorescence required the use of AlexaFluor 488 and AlexaFluor 568 (Invitrogen). Vectors for epitope tagged constructs included pcDNA4 HisMax and pcDNA6 HisMax designed with a hemagluttinin epitope tag in-frame with the *TPT1* gene of human origin. For FLAG-epitope constructs, pFLAG-CMV-14 vector (Invitrogen) was used.

Immunoprecipitation:

U2OS osteosarcoma cells were collected in phosphate buffered saline (PBS) and pelleted. Cells were resuspended in lysis buffer consisting of 35mM NaCl, 20mM HEPES, pH 7.4, 0.001% Igepal non-ionic detergent (Calbiochem) and supplemented with protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) and 1mM PMSF and lysed using sonication three times. Lysates were clarified by centrifugation at 4°C for 20 minutes. For hemagluttinin (HA) epitope immunoprecipitation, rat HA-specific matrix beads (clone 3F10, Roche Applied Science) was added to the clarified lysates with mouse Protein-C matrix beads serving as a negative control. For FLAG-epitope immunoprecipitation, FLAG-M2 agarose beads (Sigma-Aldrich, Saint Louis, MO) were collected and washed three times in lysis buffer as above to remove glycerol and resuspended in the same bed volume in a 1:1 mixture. This mixture was then added to the prepared lysates. Sepharose 6B beads (Sigma) served as a negative control when used. Immunoprecipitations were performed either at 4°C overnight in an end-over-end rotator, or for 2 hours at 37°C using the Precipitor advanced immuneprecipitation system (Abnova Corporation, Taipei, Taiwan).

Western immunoblot analysis:

Whole-cell lysates or immunoprecipitates were prepared as previously described. Radioimmunoprecipitation assay (RIPA) buffer buffer containing 1 mM PMSF and protein inhibitors cocktail (Roche Applied Science) was used to extract total protein. The protein concentrations were quantified using Bio-Rad Protein Assay kit. Equivalent amounts of protein (30-100 μg) were electrophoresed on 10%

Bis-Tris gels and electro-transferred to nitrocellulose membranes (Bio-Rad), and probed with primary rabbit, mouse or goat anti-human antibodies (1:200-1000 dilutions) for overnight at 4°C. The membranes were washed and incubated with secondary antibodies (1:10,000 dilution) conjugated to AlexaFLuor 680, IRDye 680, or IRDye 800 (Licor Corp, Lincoln, NE). Immune complexes were visualized using the Licor near-infrared imaging system. Equal protein loading was confirmed by blotting the same membrane with glyceraldehydes-3-phosphate dehydrogenase antibody.

Proteomic Screening of fortilin Interacting Proteins

U2OS cells transfected with the Empty pcDNA 4 vector or the HA-Fortilin vector were lysed. 10mg of clarified lysates were immunoprecipitated with HA-agarose beads overnight at 4°C. Immunoprecipitates were then washed with IP washing buffer three times and eluted directly with SDS Loading Buffer. Eluates were resolved on an 8% SDS gel. Gel was stained using SYPRO Ruby (Invitrogen) and bands visualized under UV light. Bands were excised and submitted to the UTMB Biomolecular Resource Facility (BRF) and analyzed using Matrix Assisted Laser Desorption Time-of-Flight (MALDI-TOF/TOF) Mass Spectrometry to identify potential protein interactions.

Immunofluorescence and Confocal Microscopy

U2OS cells were plated at 103 cells on a glass coverslip and fixed in a 1% paraformaldehyde-PBS solution (USB Labs) and permeabilized in a 0.1% Triton-X solution. Fixed cells were incubated overnight in a humidifying chamber at 4°C with primary antibody against Fortilin, Prohibitin, and both. Coverslips were washed and incubated with donkey-anti-mouse AlexaFluor 488 and donkey anti-rabbit

AlexaFluor 596 fluorescent secondary antibodies. Confocal microscopy was performed on a Zeiss LSM 510 UV Meta Laser Scanning Confocal Microscope and images obtained using the Zeiss LSM so Ftware.

Subcellular Fractionation

Nuclear and cytoplasmic extraction was performed on U2OS cells using the NE-PER nuclear extraction kit (Pierce, Rockford, IL) and following the manufacturer's instructions. Mitochondrial enrichment was performed using the Mitochondrial Isolation Kit from Pierce. Endoplasmic reticulum enrichment was performed on U2OS cells using the Imgenix Endoplasmic Reticulum Enrichment Kit. 10µg of purified enriched fractions were analyzed by SDS-PAGE and immunoblots stained with antibodies against Fortilin, Prohibitin, nuclear marker Topoisomerase IIb (Santa Cruz), mitochondrial marker Succinate Dehydrogenase subunit A (SDHA, Santa Cruz), endoplasmic reticulum marker Calnexin (Santa Cruz), and cytosolic marker GAPDH (Santa Cruz).

2.4 RESULTS

2.3.1 Immunoprecipitation of FT

In order to study the complement of proteins which interact with fortilin a co-immunoprecipitation strategy was employed. The gene for Ft, *TPT1*, was cloned from human lymphocyte cDNA library using reverse transcription PCR to isolate the gene and directionally cloned into the pcDNA6 vector. The N-terminus was cloned in-frame with the influenza hemagluttinin epitope. The HA- Ft plasmid was then transfected into U2OS cells, which contain a minimal level of detectable fortilin

(Figure 6). Figure 6B shows that HA- Ft is shifted compared to endogenous Ft, indicating that the Ft antibody recognizes both tagged and untagged protein, rendering this construct a useful biological tool for our subsequent studies. In order to optimize HA-Ft immunoprecipitations, we optimized both the salt and the NP-40 concentration to those as described in my Materials and Methods section. I chose to pursue all subsequent immunoprecipitations with a modification of previous buffers used (See Li *et al,* 2001): 150mM NaCl, 20mM HEPES, 0.001% NP-40, pH 7.4. Control cells were those which were transfected with the empty vector and immunoprecipitated in the same manner. Figure 7 shows the differential immunoprecipitation of HA- Ft using anti-HA agarose beads. This verifies that HA-Ft was correctly cloned, expressed, and able to be immunoprecipitated.

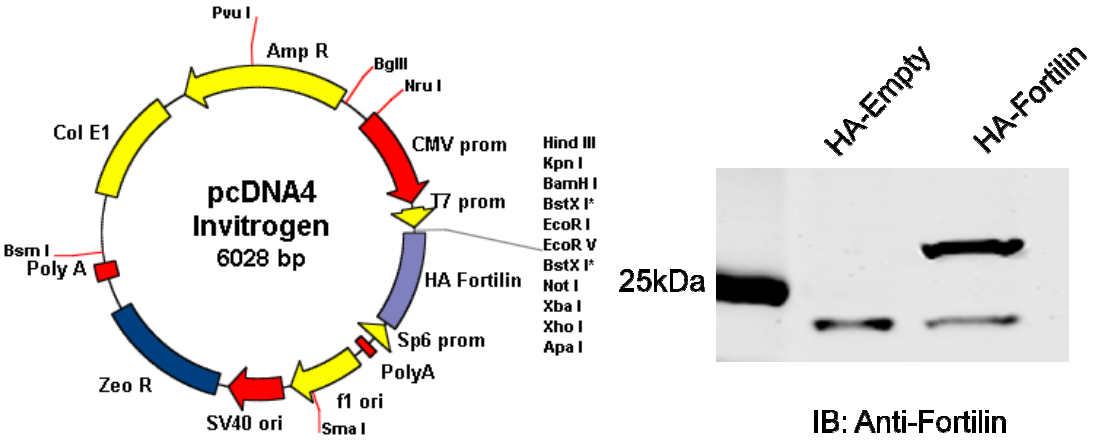


Figure 6. Molecular cloning of Hemagluttinin-tagged fortilin. A. Vector utilized in constructing the overexpression system for fortilin. fortilin was cloned in frame with influenza hemagluttinin into the pcDNA3 vector, as previously described. The vector was then transfected into the U2OS cell line and stably grown under selection antibiotic, Zeocin at a final concentration of 400µg/mL. **B.** Confirmatory western blot demonstrates that endogenous fortilin and HA- Ft were both detectable by the same antibody and that the molecular weight shi Ft was consistent with the poly-histidine tag as well as the HA epitope in frame with the fortilin protein.

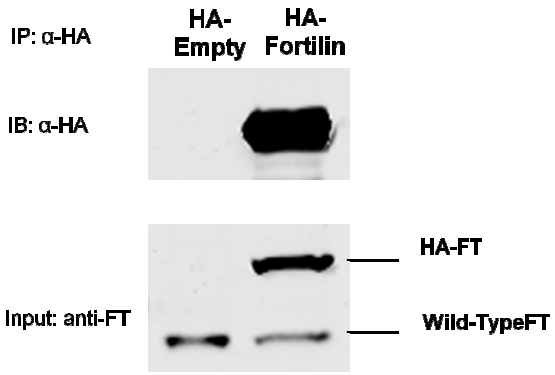


Figure 7: Fortilin immunoprecipitation. Fortilin was immunoprecipiated and confirmatory western blot was performed demonstrating that specific immunoprecipitation of HA- Ft (right lane) but not wild-type fortilin was successful. The input blots show both endogenous and HA-tagged Ft confirming the appropriate expression of our construct.

2.3.2 Co-Immunopreciptiation of HA- Ft and Proteomic Screen of Interacting Partners

Cells containing either the empty vector (HA-Empty) or HA- Ft vector were lysed and immunoprecipitated as above. Immunoprecipitates were subjected to a large format SDS-PAGE and resolved. The gel was then stained with Sypro Ruby and imaged under UV. Bands were excised and submitted to the Proteomics Section of Biomolecular Research Facility for Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF/TOF) Mass Spectrometry. Spectroscopic data were then analyzed using the Mascot Proteomics Server. As shown in figure 8 of the original gel, bands were excised and identified as putative fortilin interacting proteins the proteomic results are summarized in Table 2. Prohibitin was identified as a putative fortilin interacting protein. In addition, Table 2 demonstrates other well-characterized fortilin interacting proteins including tubulin and actin. Thus these

results recapitulate established fortilin interacting partners lending further evidence that these identified proteins are probable interacting partners of Ft.

2.3.3 Verification of Prohibitin as a fortilin Interacting Protein

Prohibitin, Phb, is a well characterized mitochondrial protein which functions in cristae morphogenesis and as a mitochondrial chaperone which facilitates the folding of mitochondrial m-AAA proteases (Nijtmans et al., 2000). In addition, other studies have shown a transcriptional regulatory role for Prohibitin (Fusaro et al., 2003; Fusaro et al., 2002). In our studies, Prohibitin is shown to interact with Ft, possibly facilitating the cytoprotective function of fortilin through this novel protein interaction. In order to verify the results from our proteomic screen, U2OS cells were transfected with HA Empty, HA- Ft, or another HA construct of the antioxidant protein NQO2, HA-NQO2. The cell lysates were subjected to immunopreciptation as before. Immunoprecipitates were resolved on SDS-PAGE and western blots were stained against the HA epitope. As my results indicate, endogenous Prohibitin was co-immunoprecipitated specifically with HA- Ft suggesting that Phb interacts with Ft. To further evaluate whether this interaction was specific to HA- Ft and not due to adventitious binding, immunoprecipitations were carried out using HA-specific agarose beads or Protein C-specific agarose beads. Indeed, as shown in Figure 10B, Phb was pulled down specifically with HA- Ft but not with Protein-C antibody. Taken together, the results from figures 8 and 9 suggest that fortilin interacts specifically with Phb. In order to characterize this interaction further, the Prohibitin gene, *PHB1*, was cloned from human cDNA library using the primers (GCGGCGGAATTCCGATGGCTGCCAAAG) and (GGCGGATCC

GCGCGGCTGGGGCAGCTGG) and directionally cloned into the pFLAG-CMV-14 vector which has a 3xFLAG epitope (DYKDHDGDYKDHDIDYK DDDDK). Sequencing results verified our clones to be full-length PHB1 with the 3xFLAG tag (Figure 9).

Next, cells were co-transfected with the HA- Ft and the FLAG-Phb vectors and subjected to co-immunoprecipitation with either anti-HA agarose beads, anti-FLAG agarose beads or sepharose beads alone. As shown in Figure 10, reciprocal co-immunoprecipitations occurred clearly showing that HA- Ft and FLAG-Phb specifically interact. These results further suggest that fortilin and prohibitin do indeed interact in *in vitro* immunoprecipitation studies. All data up to this point suggest that Ft-Phb interact, however, such studies were performed on cell lysates. In order to determine the cellular localization of the Ft and Phb and to determine their *in vivo* interaction, immunofluorescence microscopy was performed on fixed U2OS cells and stained stained using specific antibodies against Ft, Phb, or negative control. Confocal microscopy demonstrates a perinuclear distribution of FT-PHB colocalization, as seen in Figure 11A and 11C. These Ft-Phb interaction studies suggest a novel *in vivo* interaction between Ft and Phb further suggesting a physiologic function of this novel protein interaction. Based on our confocal results, we sought to determine the subcellular compartmentalization of Ft with Phb. Previous studies

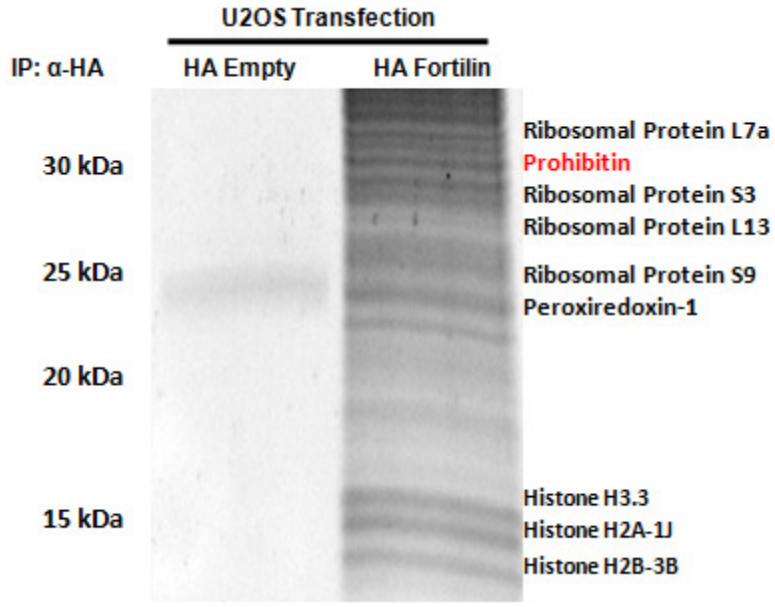


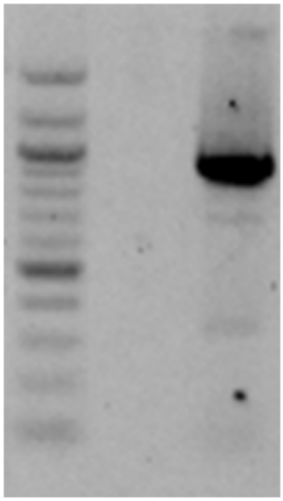
Figure 8: Electrophoresis and protein identification of putative fortilin Interacting Proteins. Whole cell lysates from U2OS cells overexpressing the hemagluttinin epitope-tagged fortilin (HA- Ft) or Empty vector were collected and immunoprecipitated as described. Eluates were electrophoresed and the gel stained with Sypro Ruby (Invitrogen) to fluorescently stain proteins. Bands were excised and analyzed by MALDI-TOF/TOF Mass Spectrometry and protein ID's assigned; anti-proliferative prohibitin (PHB) was identified as a putative Fortilin-Interacting Protein.

Table 2: Putative Fortilin Interacting Proteins

Protein	Access.	M.W.	Statistical Scores	
			95 % CI	Expectation
Histone H4	H4_HUMAN	11360.4	100%	9.97e-34
Histone H2A-1J	H2A1J_HUMAN	13927.8	100%	1.99e-25
Histone H2B-3B	H2B3B_HUMAN	13899.5	100%	9.97e-19
Ribosomal Protein S9	RS9_HUMAN	22577.6	100%	2.50e-21
Ribosomal Protein L7a	RL7A_HUMAN	29997	100%	3.14e-11
Prohibitin	PHB_HUMAN	29785.9	100%	5.0e-31
Peroxiredoxin	PRDX1_HUMAN	22096.3	100%	5.0e-20
Actin	ACTING_HUMAN	41765.8	100%	9.98e-24
Tubulin	TBA1C_HUMAN	49863.5	100%	1.55e-25

Table 2: Proteomic results of fortilin interacting proteins with predicted molecular weights and expectation values. Prohibitin was identified as a possible fortilin interacting protein and further studies were performed to evaluate this novel interaction.

A



B

ATGGCTGCCAAAGTGTTTGAGTCCATTGGCAAGTTTGGCCTGGCCTTAGCTGT
TGCAGGAGGCGTGGTGAACTCTGCCTTATATAATGTGGATGCTGGGCACAGAG
CTGTCATCTTTGACCGATTCCGTGGAGTGCAGGACATTGTGGTAGGGGAAGGG
ACTCATTTTCTCATCCCGTGGGTACAGAAACCAATTATCTTTGACTGCCGTTC
TCGACCACGTAATGTGCCAGTCATCACTGGTAGCAAAGATTTACAGAATGTCA
ACATCACACTGCGCATCCTCTTCCGGCCTGTCGCCAGCCAGCTTCCTCGCATC
TTCACCAGCATCGGAGAGGACTATGATGAGCGTGTGCTGCCGTCCATCACAAC
TGAGATCCTCAAGTCAGTGGTGGCTCGCTTTGATGCTGGAGAACTAATCACCC
AGAGAGAGCTGGTCTCCAGGCAGGTGAGCGACGACCTTACAGAGCGAGCCGCC
ACCTTTGGGCTCATCCTGGATGACGTGCCCTTGACACATCTGACCTTCGGGAA
GGAGTTCACTGAAGCGGTGGAAGCCAAACAGGTGGCTCAGCAGGAAGCAGAGA
GGGCCAGATTTGTGGTGGAAAAGGCTGAGCAACAGAAAAAGGCGGCCATCATC
TCTGCTGAGGGCGACTCCAAGGCAGCTGAGCTGATTGCCAACTCACTGGCCAC
TGCAGGGGATGGCCTGATCGAGCTGCGCAAGCTGGAAGCTGCAGAGGACATCG
CGTACCAGCTCTCACGCTCTCGGAACATCACCTACCTGCCAGCGGGGCAGTCC
GTGCTCCTCCAGCTGCCCCAGCGCGGATCCCGGGCT**GACTACAAAGACCATGA**
CGGTGATTATAAAGATCATGACATCGACTACAAGGATGACGATGACAAGTAGT
GAT

C

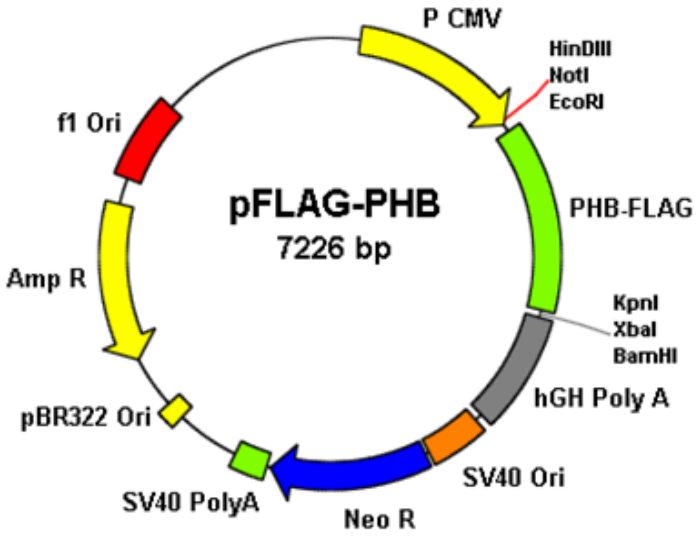


Figure 9: Cloning and verification of FLAG-prohibitin construct . The prohibitin, *PHB1*, gene was cloned from cDNA generated from U2OS cells. GCGGCGGAATTCCGATGGCTGCC AAAG was used as the forward primer and GGCGGATCCGCGCGGCTGGGGCAGCTGG was used as the reverse primer. **A,** PCR was performed as described and the fragment was excised and cloned into pFLAG-CMV. **B**, the sequence verification of FLAG-Phb, bolded text indicates the FLAG epitope **C,** Vector schematic of cloned Phb in the pFLAG-CMV vector.

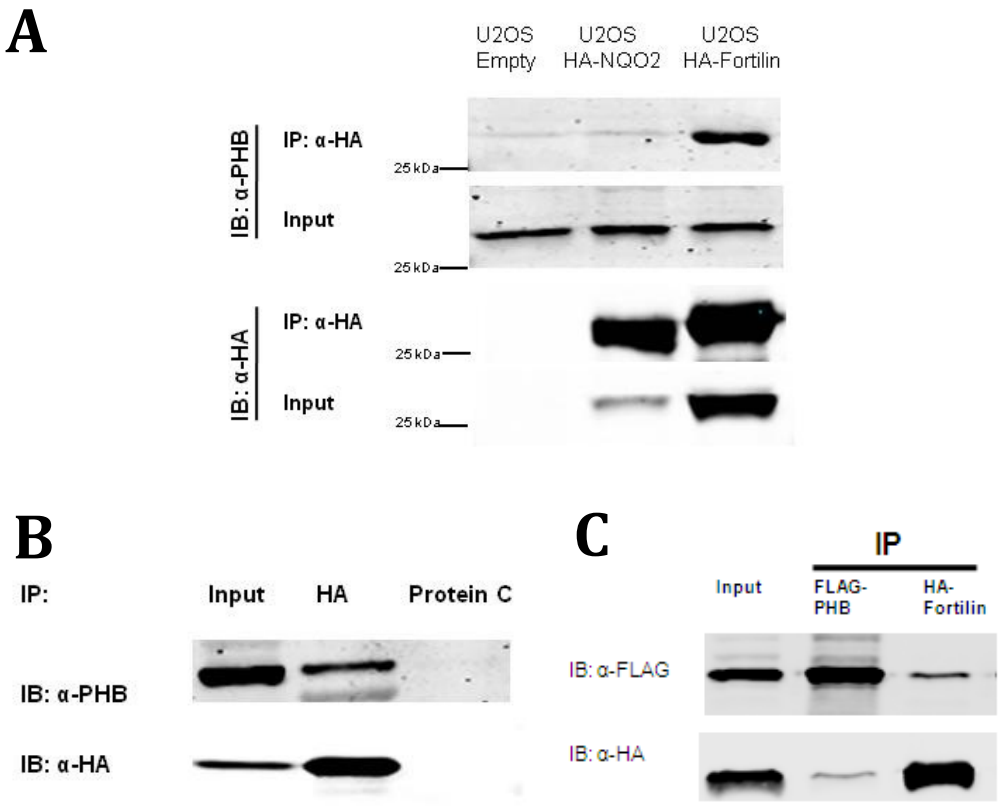


Figure 10. Verification of fortilin-prohibitin interaction *in vivo*. A. Cells were transfected with the HA-Empty vector, the HA-NQO2 vector, or the HA-Fortilin vector. HA-NQO2 was cloned from the *NQO2* gene in the same manner as HA-Fortilin. Cells were lysed and immunoprecipitated against the HA-epitope and western blot performed on eluates as described. **B,** IP was performed on U2OS HA-Fortilin lysates using the anti-HA-agarose matrix or the anti-Protein-C epitope agarose matrix as a negative control. **C,** FLAG-Prohibitin and HA-Fortilin co-immunoprecipitate reciprocally. Cells were transfected with both HA-Fortilin and FLAG-Prohibitin and lysates immunoprecipitated using FLAG antibody and HA antibody and analyzed by western blot. HA, hemagluttinin; NQO2, NADPH dehydrogenase, quinone 2; PHB, prohibitin.

have shown a cystosolic and nuclear localizations with some evidence of mitochondrial localization for Ft. Phb has been studied as a nuclear, cytosolic, and mitochondrial protein as well. In order to elucidate if these two proteinsreasonably occur within the same compartment, a purification of the subcellular compartments was performed. Figure 12 demonstrates the results of the subcellular purifications. As expected, Phb was found in the nuclear fraction, cytosolic, and mitochondrial enriched fractions (Figure 12, Phb panel). Surprisingly, there is a detectable level of Prohibitin in the endoplasmic reticulum enriched fraction. Furthermore, Ft levels were detectable in nuclear, cytosolic, and endoplasmic reticulum fractions as well (Figure 12, Ft panel). Taken together, these findings suggest that Ft and Phb interact in the cytosol or ER.

2.5 DISCUSSION

Fortilin is a known anti-apoptotic protein yet there is debate as to the mechanism of how this protein can exert this phenotype. In order to shed more light on the anti-apoptotic function of Ft, I performed a proteomic screen of fortilin Interacting proteins using an overexpression co-immunoprecipitation methodology. We have identified a list of putative Ft interacting proteins. Our screen revealed a list which included nuclear proteins, mitochondrial proteins, and verified *bona fide* interactions such as tubulin and actin. In reference to the nuclear proteins, we hypothesized that this interaction could be possibly due to electrostatic nonspecific interactions. Of note are the Histones which have a high pI (approximately pI=11.27). Considering the predicted pI of Ft as being in the range of pH 6, there is a high likelihood that the finding of histone proteins in our screen would be of a

nonspecific nature at neutral pH. Futher experiments would need to be performed to elucidate the specificity of those nuclear proteins.

Previous studies from this laboratory and the present results from immunefluorescence indeed demonstrate that Ft is a nuclear protein. fortilin was shown to regulate cell cycle progression and found to be at the metaphase plate in a protein interaction with actin. The tubulin interaction has been described but not verified by co-immunoprecipitations. Based on our results we can lend further evidence that these interactions are likely to occur, especially since the pI values of actin and tubulin are more acidic than fortilin (pI =5.29 for actin and pi=4.94 for tubulin). Of the possible Ft protein interactions, interaction with Phb was the most suggestive in elucidating the anti-apoptotic mechanism of Ft. Initially identified as an anti-proliferative protein, Phb was studied and shown to be up-regulated in breast cancers. Subsequent studies showed that the 3' UTR of the Phb transcript conferred its anti-proliferative function. Recent studies have shown that Phb can also promote proliferation in rodents and plants, although the significance of this effect is not well characterized. Prohibitin has been extensively studied as a mitochondrial protein where its localization inside the mitochondrial inner membrane allows it to dimerize with the closely related Prohibitin 2 and form a supramolecular pore complex which allows for the proper folding of mitochondrial proteins and protection from mitochondrial m-AAA proteases. Additionally , studies also indicate that Phb can possibly interact with OPA1, a novel mitochondrial protein that is involved in apoptosis. In these studies, the addition of the novel cytotoxic small molecule, alluride, bound to Phb thus freeing OPA1 for proteolytic

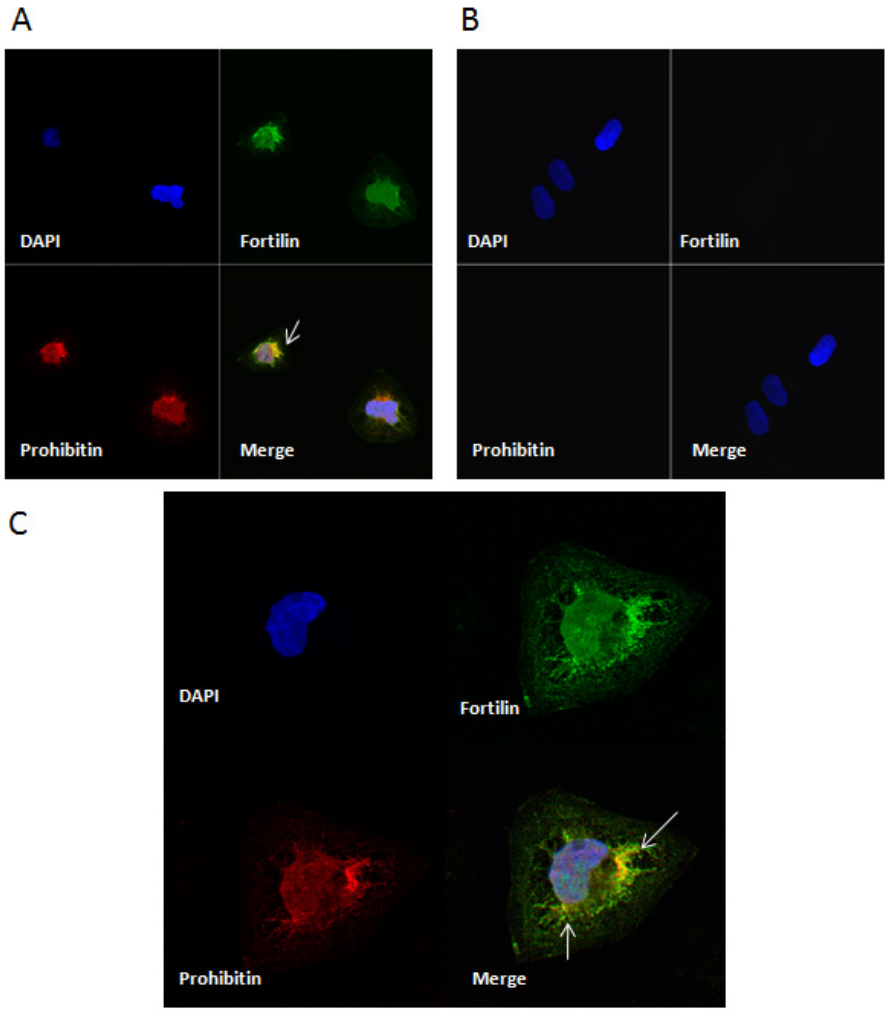
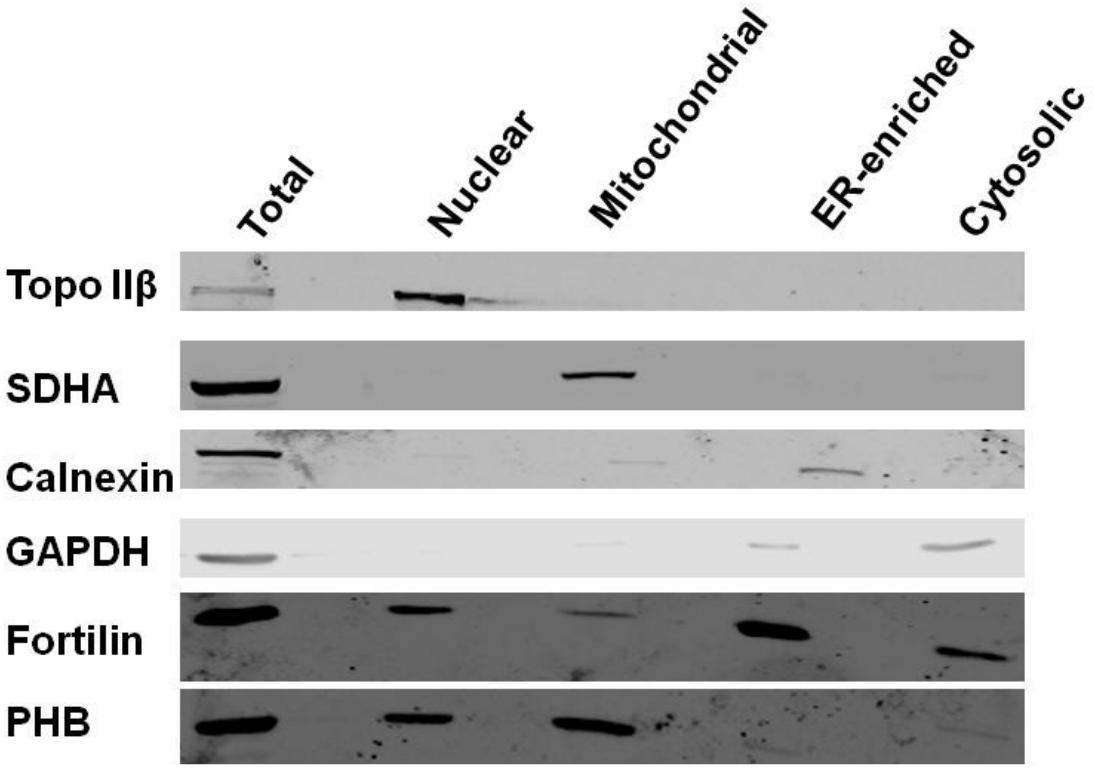


Figure 11: Fortilin co-localizes with prohibitin *in vivo*. A, confocal fluorescence **imaging** of U2OS cells at 60X magnification with fortilin in green, prohibitin in red, and nuclei stained with DAPI. Arrows point towards area of co-localization. **B,** DAPI only shows the specificity of the antibodies. **C,** 100X magnification showing discrete areas of colocalization in a perinuclear distribution.



Fitgure 12: Fortilin and prohibitin expression in enriched subcellular compartments. Cells were lysed and enriched for subcellular compartments as in the materials and method and each subcellular compartment was evaluated for prohibitin and fortilin expression. Topo IIβ, topoisomerase IIβ subunit; SDHA, succinate dehydrogenase A; GAPDH, glyceraldehyde phosphate dehydrogenase; PHB, prohibitin.

processing. The mechanism of how Phb can facilitate OPA-1 in protecting cells from mitochondrial-mediated apoptosis remains unanswered. In other studies, Phb downregulation lead to a concomitant increase in cell death after staurosporine stress. These studies indicate that Prohibitin is necessary for maintaining mitochondria. In the present study, I focused on the Ft-Phb interaction and demonstrate that this interaction is novel. I showed that Ft and Phb co-localize through confocal imaging establishing that this interaction does occur *in vivo*. Furthermore, the immunofluorescence data indicated that this novel interaction occurred in a perinuclear or cytosolic distribution. Previous studies have clearly demonstrated Phb distributes in a perinuclear fashion that is indicative of the mitochondria (Gregory-Bass et al., 2008). While my results corroborate those of Gregory-Bass and colleagues, my subcellular findings indicate that Ft and Phb fractions co-express in the nucleus, cytosol, and the endoplasmic reticulum with a large fraction of Phb but not Ft being found in the mitochondria-enriched fraction. These results are the first to our knowledge of Phb occurring in the endoplasmic reticulum. Further studies would be needed to evaluate the significance of this finding in resting cells. Based on the colocalization and subcellular results, I can reasonably propose that fortilin and Prohibitin interactions occur in the cytosol or the ER.

Prohibitin also has nuclear functions as a transcriptional regulator of Rb and p53 signaling wherein Phb inhibits Rb and p53. Other studies which have evaluated Phb nuclear function note its regulatory role in cell cycle progression (i.e. anti-proliferation in a cyclin-dependent manner). The Rb studies demonstrated how Phb could regulate cell cycle progression. Concomitant with these studies, Phb

interactions with p53 demonstrate that Prohibitin can participate in p53-mediated signaling including regulating p53-mediated cell death. As such, our studies into the Ft-Phb protein interaction are novel and consonant with the previously described roles of Phb as being involved in maintaining mitochondrial homeostasis and participating in transcriptional regulation of known regulators of apoptosis such as Rb and p53.

In the above studies we established a biological tool for studying Ft function within living cells and screened for novel interactions. Our results verified this interaction *in vitro* and an *in vivo*. Additionally, our studies shed light on the cellular distribution of this interaction and our subcellular studies further validate the feasibility of this novel interaction as being specific. In performing these studies, I soughtto further shed light on the role of Ft in cellular homeostasis and in so doing discovered that fortilin specifically interacts with Phb. My results indicate a possible therapeutic target towards this interaction either through promoting or disrupting this novel interaction. In order to do so, further studies would need to evaluate how Prohibitin modulates Ft-mediated antiapoptotic function. The studies in the next chapter will evaluate how this protein interaction can possibly modulate cellular homeostasis and whether these proteins stabilize one another.

CHAPTER 3: PROHIBITIN AND FORTILIN ARE STABILIZED AND CONTRIBUTE TO PROTECTING CELLS FROM UV-MEDIATED CELL DEATH

3.1 ABSTRACT

Fortilin and prohibitin were shown to be interacting partners. Previous studies have established that prohibitin is involved in apoptosis but a unifying mechanism remains elusive. While some studies have shown that prohibitin is necessary to maintain mitochondrial integrity, others have shown that prohibitin regulates cell signaling pathways known to increase the expression of proteins involved in apoptosis signaling. In evaluating this novel fortilin-prohibitin protein interaction, the goals of this study are to evaluate the role of prohibitin involvement in apoptosis in the context of fortilin expression. In this set of studies we evaluated the functional consequences of exogenous administration of prohibitin on the cellular homeostasis in the presence and absence of fortilin and showed that fortilin stabilizes prohibitin and that this occurs reciprocally. In the previous chapter I showed that fortilin and prohibitin interact *in vitro* and *in vivo*; given those findings, we tested whether overexpression of either or both proteins would be sufficient to modulate UV-induced apoptosis. My results indicate that co-expression of fortilin and Prohibitin was sufficient to reduce the level of UV-induced apoptosis in cells whereas overexpression of either construct alone was modestly beneficial to cells. These results in conjunction with those of the previous chapter establish that prohibitin is involved in apoptosis signaling in the context of fortilin-mediated cytoprotection and these findings provide further support for the role of Prohibitin as an anti-apoptotic protein.

3.2 INTRODUCTION

Previous studies in this laboratory have shown that fortilin, in conjunction with MCL-1 or alone was sufficient in protecting cells from cytotoxic and genotoxic stress. Furthermore, our group has demonstrated that fortilin-mediated cytoprotection was abolished when fortilin was inhibited from interacting with MCL-1 through mutagenesis studies. To this end, the goals of this study were to test the importance of fortilin and Prohibitin in cellular homeostasis and after cellular stress.

Few studies exist establishing the role of prohibitin as a cytoprotective molecule. While prohibitin is shown to regulate cell cycle progression, which in turn could exert a protective effect during genotoxic stress through a p53→p21 dependent manner, few studies have looked at the mechanism of Prohibitin in stressed cells.

The studies in this chapter seek to investigate how the novel interaction of fortilin and prohibitin functions in cells to promote survival after stress. My hypothesis is that prohibitin interacts with and facilitates the anti-apoptotic function of fortilin within cells. In order to test this hypothesi stability studies were employed to evaluate the *in vivo* nature of the prohibitin-fortilin protein interaction as well as stressing cells with UV, as in our previously published studies (Graidist et al., 2004; Li et al., 2001). The cellular stress studies employed both over-expression type studies as well as short interfering (si)RNA studies in order to elucidate the functional significance of this interaction and the consequences after cellular stress.

3.3 METHODS

3.3.1 Cell Culture

U2OS cells were cultured in DMEM with high glucose and supplemented with 10% FBS, nonessential amino acids, and glutamine (all from Thermo/Hyclone) with 5% CO2 in a humidified Sanyo incubator (Sanyo Corp., Tokyo, Japan).

4.3.2 Reagents and chemicals

HA-Fortilin and FLAG-Prohibitin plasmid constructs were generated as previously described. U2OS cells were transfected with either the HA-Fortilin, FLAG-Prohibitin, or both using the FuGene6 reagent (Roche Applied Sciences, Indianapolis, IN) at a ratio of 6:1 and stable transfectants were selected using Zeocin at 400μg/mL for HA-Fortilin constructs and 400μg/mL of G418. Monoclonal cells were selected using the limited dilution technique.

Rabbit anti-HA antibody was purchased from Bethyl Laboratories (Montgomery, TX); mouse anti-HA antibody was purchased from Santa Cruz Biotechnology. Cycloheximide, Mouse anti-FLAG antibody and M2 agarose beads were purchased from Sigma-Aldrich (St Louis, MO).

Cells were lysed in Radioimmunoprecipitation assay buffer (RIPA, 50mM Triss, 150mM NaCl, 0.1% SDS, 0.5% Deoxycholate,0.1% Triton X-100), protein measured using the Bio-Rad Protein Assay (Hercules, CA) and equal amounts of protein loaded for western blot analyses.

3.3.3 Cycloheximide stability assay

U2OS HA-Fortilin or HA-Empty cells were plated at 3x105 cells in a 6 well dish overnight. After cells became adherent, CHX treatment at 100µg/mL was initiated and collected at 0 (no treatment), 1, 3, 6, 12, and 24 hours after treatment. For collection, cells were trypsinized and pelleted. Pellets were washed 1X in PBS to remove any remaining media and lysed in RIPA buffer supplemented with Protease Inhibitor Cocktail (Sigma) and 1mM PMSF (Sigma). Proteins were measured using the Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA). Equal amounts of protein were utilized for western blot analysis. Densitometry measurements were made using the LiCor Imaging Densitometry Tool.

3.3.4 Western blot analysis

Equal amounts of protein were boiled in 4X SDS loading buffer for 5 minutes at 95°C. loaded onto 10% SDS polyacrylamide gels. Gels were run for 1 hour at 100V and then transferred to nitrocellulose membranes for 2 hours at 300mA. Membranes were blocked for one hour in TBS-T (50mM Tris, 150mM NaCl, 0.1% Tween, pH 7.4) supplemented with 2% non-fat milk and then incubated with the appropriate antibody overnight at 4°C. The membranes were then washed three times in TBS-T for 5 minutes each and then stained with the appropriate fluorescent secondary antibody (AlexaFluor 688 or 800; Invitrogen, Carlsbad, CA) for one hour. Membranes were washed three times in TBS-T to wash off excess secondary antibody and blots were imaged using the Odyssey Infrared Imaging System (LiCor, Lincoln, NE).

3.3.5 DNA Fragmentation

U2OS Empty, HA-Fortilin, FLAG-Prohibitin, or HA-Fortilin/FLAG-Prohibitin cells were seeded onto 6 well plates at 3 x 105 cells/well. After adherence, cells were either not treated or treated with 20mJ/cm2 of UV in triplicate and incubated for an additional 16 hours. Supernatant was collected and adhered cells were trypsinized and combined with the supernatant. Cell pellets were then washed 1X with PBS to remove any remaining media. Cell pellets were then lysed at 4°C using the Cell Death Plus DNA Fragmentation ELISA (Roche) for 30 minutes. After which, cell pellets were collected at 200 x g and supernatant containing fragmented DNA-histone complexes were added to immunoreagent and incubated at room temperature for 2 hours in the included ELISA plate. After washing three times with incubation buffer, ABTS substrate was added to the wells and absorbance measured at 405nm on a SpectraMax M2 spectrophotometer (Molecular Devices, Sunnyvale, CA). Increased absorbance at 405 indicates increased measured apoptosis.

3.3.6 Caspase 3 Assay

U2OS Empty, HA-Fortilin, FLAG-Prohibitin, or HA-Fortilin/FLAG-Prohibitin cells were seeded onto 6 well plates at 3 x 105 cells/well. After adherence, cells were either not treated or treated with 20mJ/cm2 of UV in triplicate and incubated for an additional 16 hours. Supernatant was collected and adhered cells were trypsinized and combined with the supernatant. Cell pellets were then washed 1X with PBS to remove any remaining media. Cell pellets were lysed in Triton-X lysis buffer (10mM Tris, pH 7.5, 100mM NaCl, 1mM EDTA, 0.01% Triton X-100) for 30

minutes at 4°C. Cells were centrifuged at 5000rpm for 5 minutes and supernatants were added to R-DEVD-containing solution. Caspase 3 activity was measured fluorescently at 496nm/520nm excitation/emission wavelengths using the SpectraMax M2 device for 18 hours. The initial velocity of each treatment group was measured and activity was normalized to initial velocity/μg total protein.

3.3.7 Statistical Analyses

One-way ANOVA analysis was performed using SigmaPlot and statistical significance for individual pairwise analyses were also determined using Fisher's exact T-Test. Statistical significance was set at $p<0.05$.

3.4 RESULTS

3.4.1 Fortilin and Prohibitin are mutually stabilized

In an effort to shed more light on the nature of Fortilin-Prohibitin protein interaction we sought to perform stability assays. From our previous studies, if fortilin and prohibitin do indeed interact, then a perturbation in the endogenous levels of one protein would conveivably affect its complementary interacting protein. In these studies, we employed the protein-synthesis inhibitor cycloheximide (CHX). As synthesis is decreased, then the stability of a protein can be determined from western blotting. HA Empty cells, which have a low to absent level of endogenous fortilin, and HA-fortilin cells were transiently transfected with FLAG-prohibitin and CHX added at different timepoints. FLAG expression was monitored over time via western blot analysis. Figure 13A shows degradation of FLAG-prohibitin of in HA-Empty cells or in HA-fortilin cells. The apparent half-life of

FLAG-prohibitin in a relatively high background of fortilin is dramatically increased compared to FLAG-prohibitin transfected into HA-Empty cells indicating that fortilin stabilizes prohibitin and that FLAG-prohibitin degrades at a more rapid rate in the absence of abundant fortilin (Figure 13A, dotted line). The predicted half-life is increased from approximately ten hours in the absence of fortilin to almost twenty hours when cells contain exogenous administered fortilin.

Next, I tested the hypothesis that prohibitin stabilizes fortilin *in vivo*. To test this, I utilized the HA-fortilin cells transfected with FLAG-PHB. This time, the expression of exogenously administered HA-fortilin was evaluated. Figure 14 demonstrates that HA-fortilin is stabilized when in the presence of exogenously administered prohibitin (Figure 14A, solid line compared to dashed line) and that the cells expressed the correct constructs and were equally loaded (Figure 14B). While endogenous prohibitin levels are detectable in the HA-fortilin cells, additional administration of prohibitin further stabilizes HA-fortilin increasing the half-life of HA-fortilin from approximately ten hours to over twenty-five hours.

In an adjunct study, I investigated if knocking down fortilin would influence Prohibitin expression in an inverse manner relative to the above results. U2OS cells do indeed express detectable levels of fortilin, thus in order to knockdown endogenous fortilin, short-hairpin (sh)RNA against fortilin (shFortilin) was used to stably decrease the levels of endogenous fortilin to practically undetectable levels (Figure 15A, right panel). Figure 15 shows that after 100µg/mL CHX treatment at various timepoints, shControl cells contained relatively stable levels of prohibitin whereas shFortilin cells demonstrated enhanced prohibitin degradation after protein synthesis inhibition (Figure 15A, dashed line compared to solid line).

Interestingly, Figure 15B suggests that there could be relatively more prohibitin in the shFortilin cells, however, these results indicate that based on fortilin levels within the cell prohibitin levels declined sharply over longer CHX treatment times.

Taken together, these data show a functional significance of the prohibitin - fortilin interaction. These results suggest a novel reciprocal stabilization of fortilin and prohibitin. Furthermore, the knockdown studies suggest that prohibitin stability is at least partially reliant on the levels of fortilin within the cell. The next set of studies will evaluate the relative abundance of these proteins and their contribution to cell survival after a stress.

3.4.2 Prohibitin attenuates the anti-apoptotic effects of Fortilin

Published studies on fortilin and prohibitin show that these proteins individually participate in a cytoprotective manner, with fortilin overexpression being better characterized. Next we posed the question if prohibitin and fortilin interact, could the concomitant over-expression of these proteins protect cells from UV-mediated cell stress. Few studies have evaluated the role of prohibitin in genotoxic stress. Genotoxic stress can halt cell-cycle progression (a p21ARF pathway) or activate the endogenous apoptosis machinery (p53 transcriptional signaling as well as activation of intracellular kinases which mediate cell death). Few studies, however, have actually evaluated the significance of prohibitin in protecting cells or promoting cell death. I hypothesized that the dual presence of these proteins with each other could further protect cells from UV-mediated cell death. I reasoned that since the proteins mutually stabilize each other, that the persistence and relative upregulation of both proteins is protective over the up-

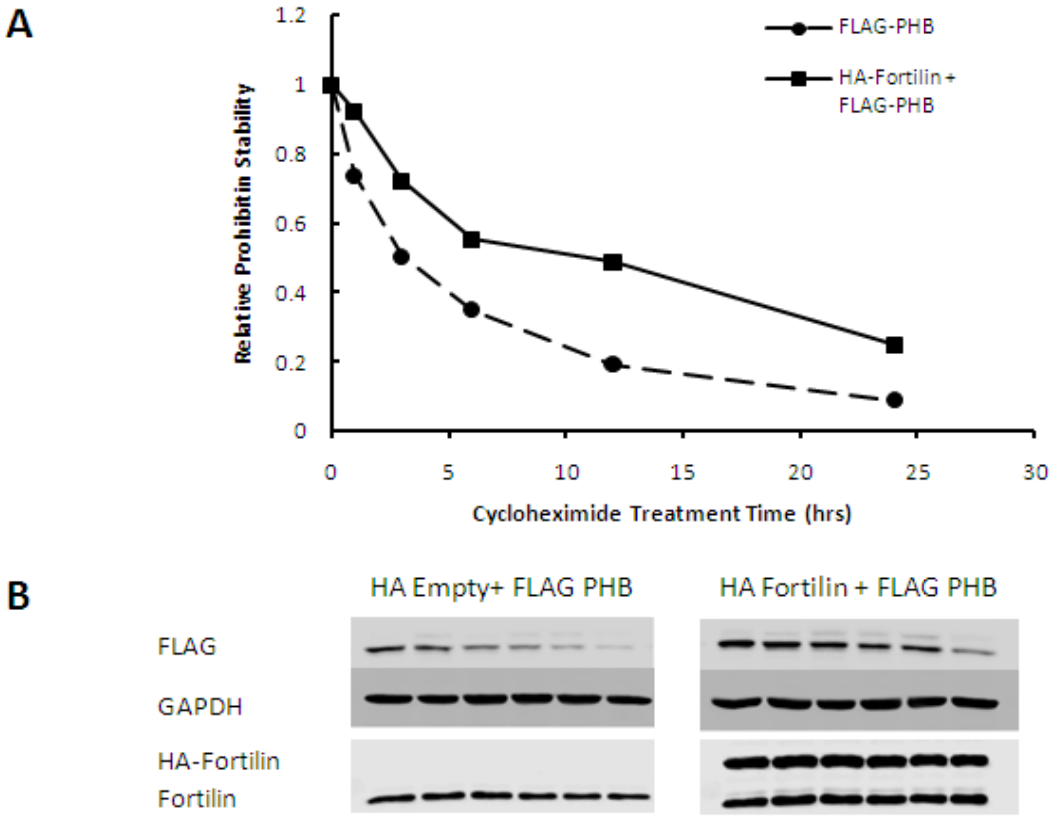


Figure 13. Fortilin maintains Prohibitin stability intracellularly. A, cycloheximide study. U2OS HA-Empty or U2OS HA-Fortilin cells were transfected with 1ug of FLAG-Prohibitin plasmid for 24 hours. Cells were then treated at various timepoints with 100ug/mL of the protein synthesis inhibitor, Cycloheximide. After treatment, cells were lysed and equal protein was loaded for western immunoblot analysis. FLAG stability decreases with increasing incubation time in CHX. The presence of HA-Fortilin increases the relative half-life of FLAG-Prohibitin. B, western immunoblots of CHX-treated HA-Empty or HA-Fortilin cells with FLAG-Prohibitin. Densitometry was measured using the Licor system. GAPDH densitometry was used as an internal loading control.

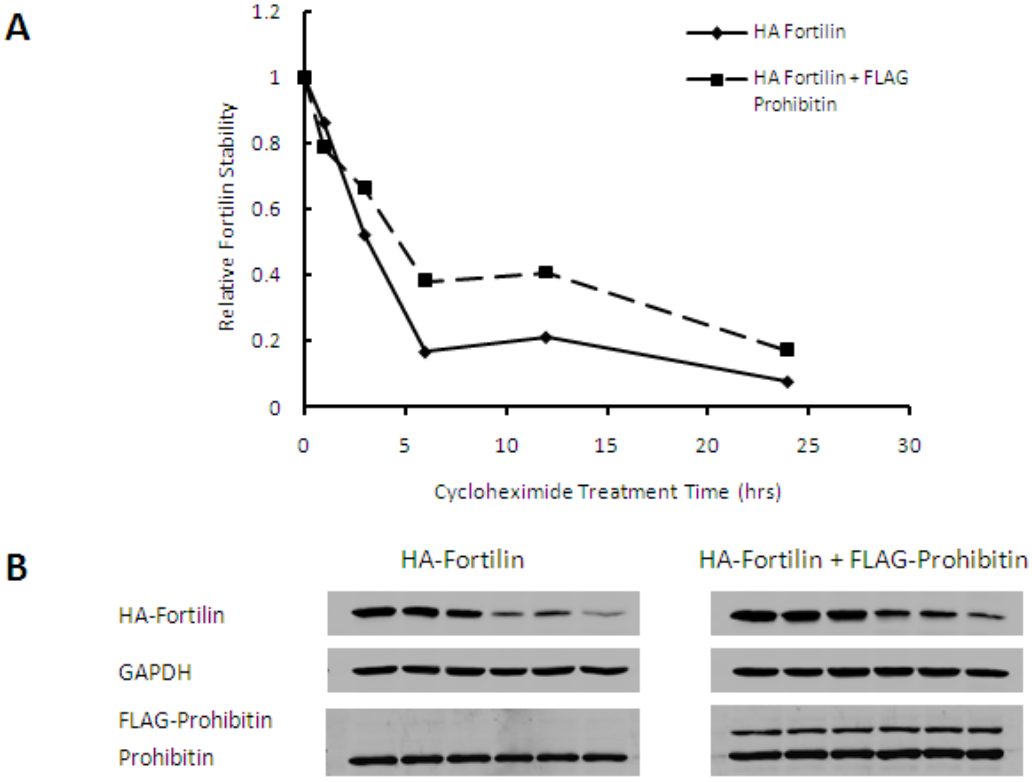


Figure 14. Prohibitin maintains fortilin stability intracellularly A, cycloheximide study. U2OS or U2OS FLAG-Prohibitin cells were transfected with 1ug of HA-Fortilin plasmid for 24 hours. Cells were then treated at various timepoints with 100ug/mL of the protein synthesis inhibitor, Cycloheximide. After treatment, cells were lysed and equal protein was loaded for western immunoblot analysis. HA-Fortilin stability decreases with increasing incubation time in CHX. The presence of HA-Fortilin increases the relative half-life of FLAG-Prohibitin. B, western immunoblots of CHX-treated U2OS or U2OS FLAG-Prohibitin cells with HA-Fortilin. Densitometry was measured using the Licor system. GAPDH densitometry was used as an internal loading control.

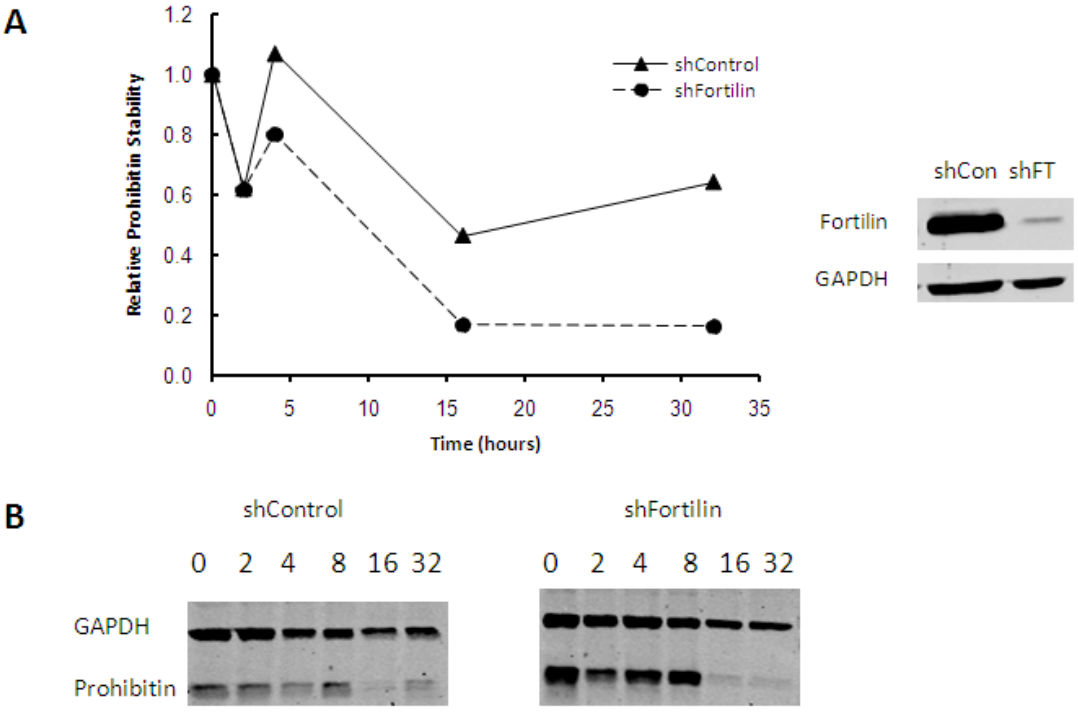


Figure 15. Fortilin knockdown modulates prohibitin expression. A, U2OS shControl (solid line) or U2OS shFortilin cells (dashed line) were treated with CHX at various time points. After treatment, cells were lysed and equal protein was loaded for western immunoblot analysis. A, Prohibitin levels were analyzed relative to controls and plotted. B, western immunoblots of CHX-treated cells. Densitometry was measured using the Licor system. GAPDH densitometry was used as an internal loading control. Con, control; FT, fortilin.

reglation of either protein alone. To test this hypothesis, U2OS cells lines were generated overexpressing the Empty vector, HA-fortilin, FLAG-prohibitin, or both proteins (Ft+Phb). Cells were treated with 20mJ/cm^2 UV and cell death was assessed using DNA Fragmentation ELISA. As seen in Figure 16, negative control cells showed a high level of cell death (Figure 16, far left bars). Cells overexpressing HA-fortilin showed a reduced cell death response, in agreement with previously published reports (Figure 16A, bars labeled "Fortilin"). Cells overexpressing FLAG-prohibitin also showed a reduced death response that was similar in response to that of HA-fortilin cells; however, the reduction in cell death was modest (Figure 16A, bars labeled "Prohibitin"). Interestingly, cells which over-expressed both HA-fortilin and FLAG-prohibitin exhibited an enhanced level of cytoprotection—an approximately 40% fold reduction in cell death (Figure 16A, far right bars). Indeed, the relative reduction in double-overexpressing cells as compared to each individual over-expressing construct indicates that this interaction showed a synergistic effect.

In addition to DNA fragmentation as an apoptotic parameter, caspase 3 activity is another *in vitro* test to evaluate apoptosis. This follow-up was performed identically to the DNA fragmentation study and caspase 3 activity was assessed. Figure 17 shows that UV treatment increased caspase 3 activity, verifying that UV treatment leads to the increased activation of the effector caspase 3, which is upstream of DNA Fragmentation. As in the DNA Fragmentation results caspase 3 activation was blunted in cells over-expressing FLAG-prohibitin and HA-fortilin (Figure 17A, far right bars). Indeed, the results were reproducible and comparable to those of our DNA Fragmentation results with an approximately 30% reduction in cell death compared to Empty controls cells.

In summary, these data demonstrate the functional consequence of over-expression of both fortilin and prohibitin simultaneously protecting cells from UV-induced death compared to either protein alone.

3.5. DISCUSSION

In these studies, I sought to investigate the functional significance and consequence of fortilin interacting with prohibitin. Fortilin is a novel anti-apoptotic protein yet the mechanism of fortilin regulating apoptosis is yet to be fully elucidated. I showed that prohibitin stabilizes fortilin and that this stability was reciprocal (Figures 3 and 4). Additionally, I showed that prohibitin stability was modulated when endogenous levels of fortilin were knocked out via shRNA, indicating that this *in* vivo interaction was necessary for prohibitin stability. Additionally, these results further complement the binding results from Chapter 2. Finally, in an over-expression system of fortilin, prohibitin, or both, I was able to demonstrate that the combination of fortilin and prohibitin within the cell further protects cells from UV induced DNA Fragmentation and Caspase 3 activity. Because I have extensively showed that these proteins interact and further confirmed through cycloheximide stability assays, it is feasible that this additional cytoprotection is due to the fortilin-prohibitin protein interaction. In fact, Figure 6B shows that fortilin and prohibitin are similarly upregulated in the double over-expression cells relative to those levels from fortilin-only and prohibitin-only cells. Based on this observation the additional decrease in DNA Fragmentation and Caspase 3 activity is reasonably due to fortilin interacting with prohibitin. Prohibitin was characterized as a mitochondrial chaperone and later discovered to have other cellular functions in the nucleus and

cytosol. While several studies have summarily shown that fortilin and Prohibitin interact with various protein partners, no studies have evaluated fortilin-prohibitin protein interaction. In fact, functional studies of prohibitin have focused largely on prohibitin mediated transcriptional regulation of target genes, through nuclear interactions with Rb, p53, or nuclear receptor in various cell type. Given these studies, intriguing results obtained in this chapter establish that this protein interaction is functional and raise some questions as to the mechanism of how this protein interaction protects cells. In the previous chapter I demonstrated that this interaction was novel and specific. Building on that, in this chapter I was able to show the functional consequences of this interaction through stability and overexpression studies. I reasoned that since fortilin and prohibitin interact specificially *in vivo*, then the consequences of cellular stress in an over-expression background could be due, in part, to this protein interaction. While these studies are not exhaustive they do indeed shed light on fortilin as an antiapoptotic protein, in part, through a protein interaction with prohibitin.

Several structural studies have demonstrated that prohibitin acts as a chaperone and that the SPFH domain (the "Prohibitin domain") contains hydrophobic residues which facilitate folding. It is possible this domain allows for a stabilization of fortilin. Fortilin itself is speculated to be a chaperone perhaps mediating the myriad observed protein interactions of with other described cellular components ranging from structural proteins (e.g. actin, tubulin), to metabolic proteins and enzymes. Previous studies have established that fortilin protein interactions with MCL-1 or p53 mediate a cytoprotective effect. Our findings showing that fortilin could protect from UV-mediated cell death in part through

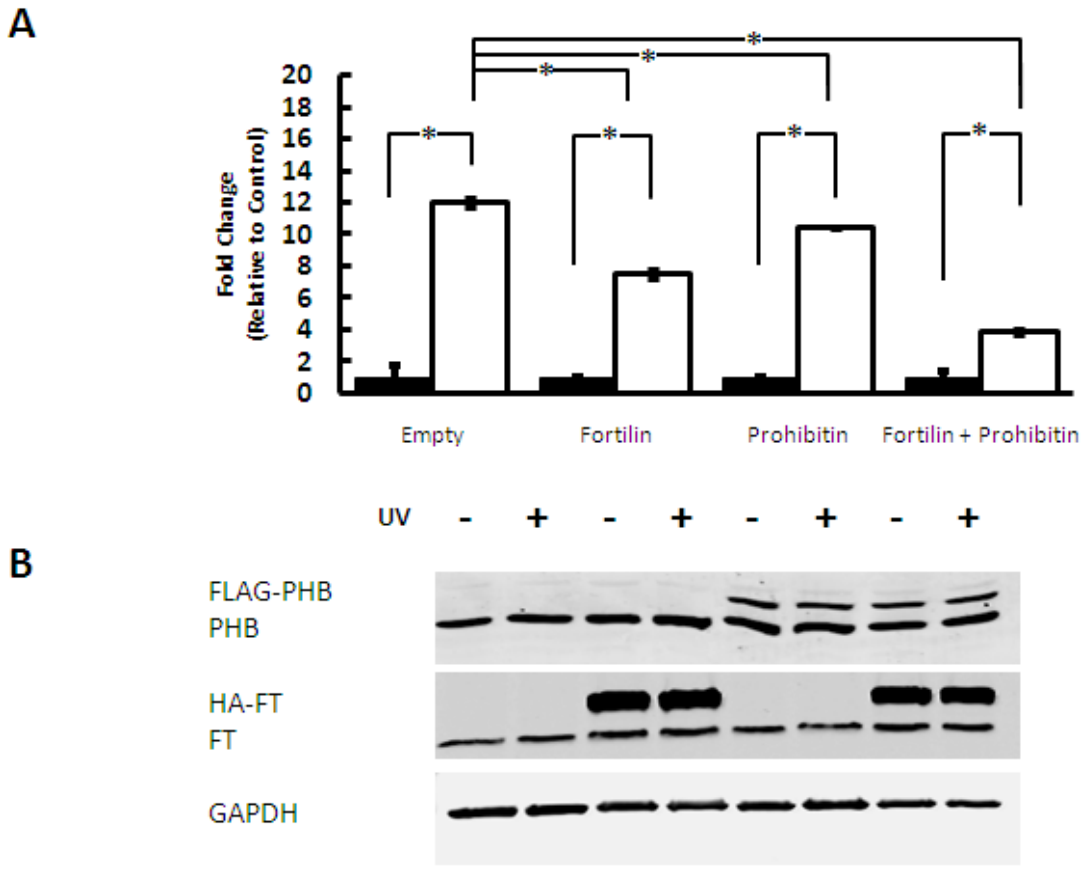


Figure 16. Fortilin and prohibitin over-expressing cells are further protected from UV-induced cell death. A, U2OS cells transfected with the Empty vector, HA-fortilin, FLAG-prohibitin, or HA-Fortilin/FLAG-Prohibitin vectors were subjected to UV at 20mJ/cm2 and then incubated for an additional 16 hours. Cell death was measured using the DNA-Fragmentation ELISA and compared to non-treatment negative controls. B, Confirmatory western blot for the cell lines to demonstrate overexpression levels of either construct. PHB, prohibitin; FT, fortilin; * indicates $p<0.05$.

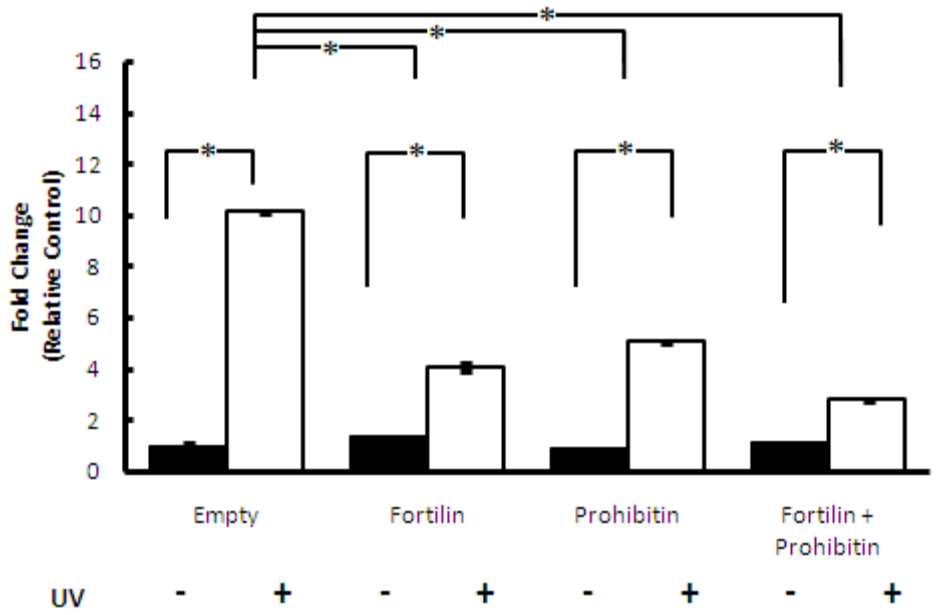


Figure 17. Caspase 3 Activation is reduced in cells over-expressing fortilin and prohibitin. A, U2OS cells transfected with the Empty vector, HA-fortilin, FLAG-prohibitin, or HA-fortilin/FLAG-rohibitin vectors were subjected to UV at 20mJ/cm2 and then incubated for an additional 16 hours. Cell lysates were analyzed for Caspase 3 activity. PHB, prohibitin; FT, fortilin. * indicates $p<0.05$.

a protein interaction with prohibitin further complements the already established studies on fortilin and apoptosis. Given these results, however, the question remains whether this interaction is enough to protect cells. My results indicate that overexpression of prohibitin and fortilin was sufficient to further rescue cells from UV-mediated DNA fragmentation and Caspase 3 activation. However, UV-mediated apoptosis activates other stress genes including Bax, Noxa, and PUMA in a p53 dependent manner and some studies even suggest that UV-mediated stress can signal to the ER to either upregulate the unfolded protein response or increase cytosolic Ca2+ levels. Considering the results from Chapter 2, evaluating ER stress may shed some light on this novel interaction as both these proteins have not been reported to be found in the ER. Future work would additionally be needed to further tease out how this interaction mediates or is mediated by genotoxic stress in the context of other mediators genotoxic stress such as p53 dependent genes and ER stress genes.

My results showing that cells over-expressing both proteins further protect cells from UV-induced apoptosis are also consistent with previous reports showing that a protein-interaction can protect cells from cell death. Early studies on fortilin protein interactions demonstrated that fortilin and Mcl-1 interact to protect cells, however a mechanism remains elusive. Later results showed fortilin to be cytoprotective independent of Mcl-1 levels in the cell. The relative abundance and interaction of both proteins in cells could be coincidental, however, the quantitative data from the DNA-fragmentation experiment demonstrates a biological synergy. This synergy is, in part, attributed to the mutual stability of both proteins. Previous studies on prohibitin reveal very little on how it is an anti-apoptotic protein in and

of itself. Two studies have shown that prohibitin can protect cells from staurosporine-induced cell death.

Once again, the nature of the stress agent plays an important role. Fortilin is a known modulator of p53 signaling, and as discussed above, the stressor I chose is genotoxic stress which activates p53. Other studies have looked more at oxidative stressors or mitochondrial poisons which may mimic the intrinsic pathway but the pattern is not the same. Published results from our laboratory group have shown a significant p53-Fortilin interaction; this interaction specifically decreases Bax signaling; a similar study in another laboratory group has verified that this interaction dampens apoptotic signaling (Rho et al., 2010; Yanjie et al., 2011). Thus, our study is significant in demonstrating that genotoxic-stress mediated apoptosis signaling is specifically modulated by the double expression of fortilin and prohibitin in a cell. Taken together with my results from Chapter 2, the protein interaction between fortilin and prohibitin is a possible nexus of this stress signaling.

Of the studies which have evaluated prohibitin in stressed cells, prohibitin could potentially signal apoptosis signaling in a mitochondrial-dependent manner and in an extra-mitochondrial manner. In the mitochondria, prohibitin is suggested to be protective of the mitochondria, maintaining cristae morphogology. The resulting mitochondrial dysfunction can then increase cellular susceptibility to cytotoxic drugs (Muraguchi et al., 2010; Sato et al., 2011). Additionally, in cancer cells, prohibitin knockdown via siRNA can alter the expression of mitochondrial OPA-1, which in turn would render mitochondria more susceptible to cytotoxic drugs (Gregory-Bass et al., 2008).

Studies which have evaluated an extra-mitochondrial role for Prohibitin have shown that nuclear export after campothecin treatment leads to an increase in apoptosis signaling. Campothecin is a topoisomerase inhibitor leading to single stranded DNA knicks. These studies showed that prohibitin is exported after a genotoxic stress yet the mechanism of that signaling remains unclear. Furthermore, the significance of prohibitin export could be a cause or an effect of apoptosis signaling, and is yet to be elucidated. Considering those, the findings of this present study could potentially complement the nuclear export study. That is, after genotoxic stress, through a yet-to-be-determined mechanism, prohibitin is exported to the cytoplasm where it stabilizes endogenous fortilin. In so doing, the two proteins work in concert to promote cell survival.

Additionally, prohibitin is known to directly interact with prohibitin and to modulate the ERK pathway. An interesting model would be that fortilin modulates p53 and ERK and through an interaction with prohibitin, fortilin stability is increased thereby maintaining this modulation. Could this direct protein complex be the modulator of these cellular processes when under cell stress? Whether prohibitin could act as a type of protein scaffold or whether the fortilin-prohibitin interacts directly or in a complex remains to be answered.

In summary, this chapter explored the significance of fortilin interacting with prohibitin. I showed that fortilin and prohibitin mutually stabilize one another, that prohibitin expression levels change when fortilin levels are altered. Finally, I showed that co-expression of fortilin and prohibitin allowed for cytoprotection suggesting that cellular amounts of fortilin and prohibitin are stabilized and influence the cellular response to UV-induced apoptosis. These findings suggest that this novel

protein interaction would be an interesting therapeutic target for inhibition of diseased cells including cancer cells and atherosclerosis associated MΦ's.

CHAPTER 4: GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

GENERAL CONCLUSIONS

Fortilin is a unique protein associated with a variety of cellular processes from cancer reversion and apoptosis to transcription initation and associating with the tubulin and actin regulating cell shape (Bazile et al., 2009; Gachet et al., 1999; Langdon et al., 2004; Tuynder et al., 2002). Initially described as a *translationally controlled tumor protein*, this protein is well characterized as being expressed in a wide variety of cancer cells and normal cells (Bommer et al., 2010; Tuynder et al., 2004). fortilin is also known to be a histamine releasing factor – parasitic homologs of fortilin have been shown to lead to human allergic rhinitis-like symptoms (MacDonald et al., 2001). Within the cell, fortilin is shown to be a putative scaffolding protein during cell division and could possibly function as a chaperone (Gnanasekar and Ramaswamy, 2007; Thaw et al., 2001; Zhang et al., 2002). Finally, in our studies, fortilin is known to be a protein which protects cells from apoptosis. Considering the wide array of fortilin cellular and extracellular function, how does fortilin modulate cellular homeostasis and human disease?

In the context of protecting cells from apoptosis, fortilin has been shown to be cytoprotective through a wide variety of proposed mechanisms from oxidative stress, calcium-mediated ER-stress, and UV stress (Bommer et al., 2010; Gnanasekar and Ramaswamy, 2007; Graidist et al., 2007; Nagano-Ito et al., 2009; Yanjie et al., 2011). Given all these, fortilin plays a central role in modulating diseases associated with these stress processes; as a result, fortilin has wide implications from cancer

and aging to mechanisms involved in cardiovascular disease. Since fortilin has no sequence homology to known regulators of apoptosis and has no known enzymatic function, studying fortilin protein interactions provides a greater understanding into the molecular mechanism of fortilin cellular function.

In this present study, I sought to evaluate the protein partners of fortilin in an effort to better understand and possibly elucidate the biological significance of fortilin. Through our screening, I showed that fortilin specifically interacts with the anti-proliferative prohibitin protein *in vivo* and that this interaction represents a novel mechanism of how fortilin can protect cells from apoptosis. Specifically, I have shown that the fortilin-prohibitin interaction is a *bona fide* novel protein interaction. In aim one, I developed a methodology to probe possible protein interactors of fortilin using an epitope-tagged fortilin construct. I was able to elucidate a set of putative fortilin protein interactions and focused on evaluating the possible fortilin-prohibitin protein interaction. In the remainder of my First Chapter, I demonstrated that fortilin specifically interacts with prohibitin both *in vitro* and *in vivo*. That this interaction is specific to fortilin and not due to non-specific interactions, and that prohibitin reciprocally binds fortilin. Furthermore, in my *in vivo* study where we evaluated fortilin and prohibitin using immunofluorescence, we were able to find that prohibitin and fortilin do exist in nuclear and cytosolic compartments. More interestingly, my results showed fortilin interacts with prohibitin focally in a perinuclear and cytosolic distribution.. This suggests that only a subset of fortilin and prohibitin interact at any given time. This finding is consistent with previous reports where fortilin is found in nuclear and cytosolic fractions and is considered a nuclear-cytosolic shuttle protein due, in part,

to its size. What is more interesting is that prohibitin is well characterized as a mitochondrial protein. Many published reports have demonstrated that prohibitin exists in nuclear and cytosolic fractions. Indeed our results corroborate those previously published results, but add to that and show that prohibitin interacts with a protein partner at least within the cytosolic compartment.

Future studies to evaluate this interaction in a compartment-specific manner would include performing co-immunoprecipitation after subcellular fractionation. Such studies would elucidate which subcellular compartment the fortilin-prohibitin protein interaction occurs. The results would then shed light on a possible mechanism of the protein interaction; that is, we can postulate how a physical protein interaction would either enhance or modulate apoptosis signaling. One study showed that after apoptosis signaling, prohibitin is exported to the cytosol from the nucleus, the significance of which was not evaluated. The only difficulty to such studies would be the identification of immunoprecipitating antibodies. The immunoprecipitations performed in this study were performed using epitope-tagged overexpression systems, which perturbs endogenous levels of both proteins. Utilization of such systems may not allow for a physiologically relevant evaluation of interactions in a compartment-specific manner. Also, these CMV-overexpression systems are not compartment-specific which would render such overexpression constructs difficult to study were subcellular co-immunoprecipitations pursued.

In Chapter 3, I demonstrated that fortilin and prohibitin mutually stabilize each other, the significance of which was shown that the mutual up-regulation of both proteins protects cells from UV-induced apoptosis in a synergistic manner. I

demonstrated that fortilin stabilizes prohibitin *in vivo* using a cycloheximide stability assay. These data complement data from Chapter 2 which further corroborates my binding results further showing a functional significance to fortilin binding to prohibitin. Thus this mutual protein stability allows for the persistence of fortilin and, based on the DNA fragmentation and Caspase-3 functional assays, enhances fortilin-mediated cellular protection. Additionally, Caspase-3 cleavage is the point of no return for apoptosis. The observation that fortilin-prohibitin overexpressing cells lead to decreased Caspase-3 cleavage raises two possibilities: 1) a mutal inhibition of mitochondrial-mediated apoptosis or 2) a direct inhibition of Caspase-3 cleavage. While further studies would need to be performed to evaluate if there is a direct inhibition of Caspase-3 enzymatic activity, it is conceivable that Fortilin-Prohibitin could inhibit cell death at the level of the mitochondria or before mitochondrial-mediated membrane pore (MOMP) formation occurs – which is precisely the mechanism of how the anti-apoptotic Bcl2 Family Proteins function. Based on these findings, a question to ask is: could fortilin-prohibitin interact to promote cellular protection in the manner of the Bcl2 Proteins? Based on the results of Susini *et al.* (2008) and the results of this study, it is possible that prohibitin interacts with fortilin in the cytosol thus

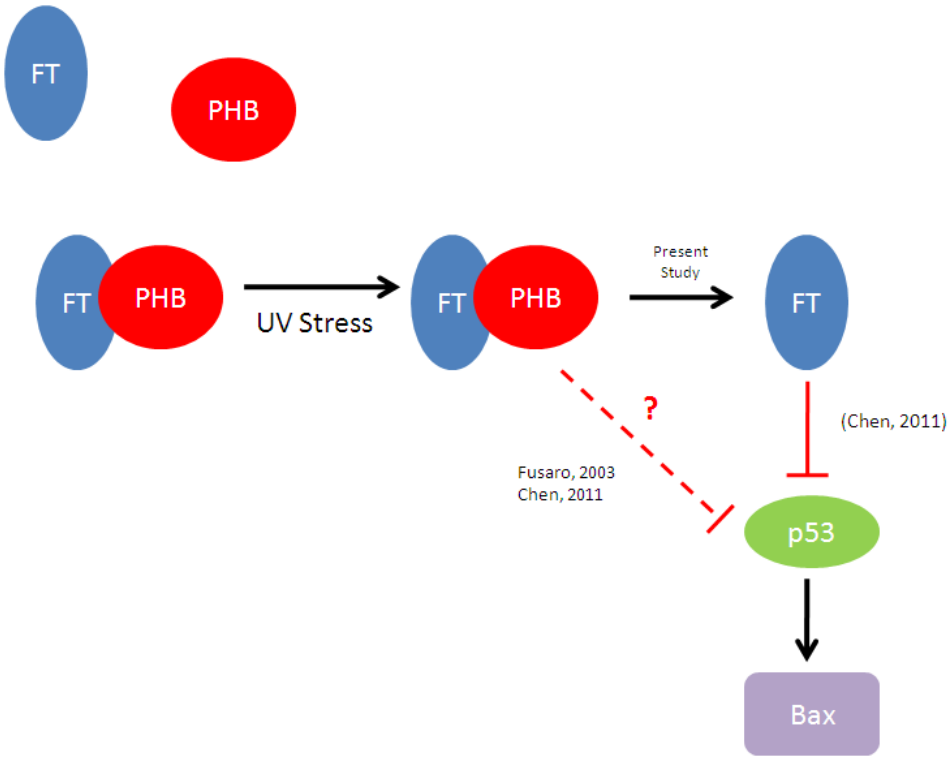


Figure 18. Proposed model for fortilin interacting with prohibitin and modulating apoptosis. In this schematic, fortilin and Prohibitin interact with one another allowing for the stabilization of fortilin in turn exerting an inhibitory function on cellular Bax levels (as published by Yanjie *et al.*, 2011 and Susini *et al*, 2008). Additionally, there is mounting evidence that fortilin and Prohibitin independently modulate p53 signaling, thus a conceivable mechanism is that this protein interaction can modulate p53 signaling together thereby modulating Bax transcriptional activation. Future work would evaluate the significance of this novel protein interaction in modulating p53 dependent transcriptional activation.

allowing for the persistence of fortilin. This suggests that a feasible model of Fortilin-mediated apoptosis inhibition would be that fortilin interacts with and is stabilized by prohibitin in the cytosol and consequently, fortilin stability leads to enhanced cytoprotection in the manner which Susini and colleagues suggest—that fortilin embeds within the mitochondrial membrane (Figure 18). Based on those data and the results from this study, it is conceivable that prohibitin and fortilin functionally complement each other at the mitochondria during cellular stress. Thus this mutual stability allows for fortilin-mediated inhibition of Bax dimerization. Future studies to evaluate this would include generation of a mitochondrial-targeted fortilin construct and evaluate if this construct interacts with prohibitin at the mitochondrial. My subcellular results were performed in non-stressed conditions. Perhaps inducing apoptosis using other stimuli in addition to ionizing radiation to specifically induce mitochondrial-mediated apoptosis would shed more light on the contribution of prohibitin to fortilin-prohibitin mediated inhibition of apoptosis.

FURTHER QUESTIONS AND FUTURE DIRECTIONS

While the results of this study establish a novel protein interaction between fortilin and prohibitin and show that this interaction provides a useful mechanism for a cell to live past its prime, many questions still remain. In Chapter 3, I show that cells overexpressing both prohibitin and fortilin have a more dramatic decrease in cell death as compared to cells expressing either protein alone. Two issues are raised concerning this study. One is that, we did not take into consideration the endogenous levels of either protein. Could there be a more dramatic decrease in

cells that have *no* prohibitin or fortilin? An approach to take would be to construct shRNA cells to fortilin or prohibitin and then concurrently overexpress the complementary interacting partner (prohibitin or fortilin respectively) and evaluate the response to cell stress. Additionally, one could also obtain mouse embryonic fibroblasts from pure fortilin or prohibitin knockouts and perform the above study. One previous study proposed that fortilin inhibits apoptosis by embedding within the mitochondrial membrane to inhibit Bax dimerization (recall that Bax is a pro-apoptotic Bcl-2 family protein). The authors showed using purified mitochondria, the amount of Bax dimers within the mitochondria did not change in the presence of fortilin but that the amount of Bax monomers increased (Susini, 2008). Interestingly, the authors were not able to demonstrate a specific interaction of fortilin with Bax and the experiments were performed in a cell-free system without the need to induce mitochondrial stress.

Other studies have demonstrated that prohibitin alone was cytoprotective, down-regulation of which lead to spontaneous cell death. What has been lacking in several of these studies is an evaluation of the fortilin status of the cells. This study showed that prohibitin is cyto-protective but in ovarian cancer cells and primary cultures of normal granulose cells. Perhaps the levels of fortilin influence whether prohibitin is antiapoptotic or not. Our data seem to indicate that prohibitin over-expression was modestly antiapoptotic. In previous work performed in our lab, fortilin expression was quite high in several cancer lines such as HeLa cervical cancer cells, MCF-7 breast cancer cells, and A549 airway epithelial cells and was particularly low in U2OS osteosarcoma cells. Given these observations and the fact that fortilin is upregulated in various cancers, perhaps the cytoprotective effect

seen in these other cells after prohibitin overexpression could be due to a contribution from fortilin-prohibitin interactions.

Another consideration about this novel interaction is the role of proliferation in affecting tissue turnover and the interplay of proliferation with apoptosis. In previous studies from our laboratory group, fortilin overexpression is associated with a decreased level of apoptosis but not with an increase in cellular proliferation as measured by Ki67 immunohistochemistry in a subcutaneous xenogra Ft model (Yanjie et al., 2011). This is consistent with other Bcl-2 pro-survival proteins; that is, such proteins allow a cell to survive longer but not necessarily participiating in cellular proliferation or transformation. Contrast that with Prohibitin, which is itself anti-proliferative. It has been shown that prohibitin knockdown promotes more cells to enter S-phase, the combination of prohibitin and fortilin knockdown could possibly allow a cell to abnormally persist and be susceptible to more cellular stress perhaps promoting carcinogenesis given the right microenvironment (Kim et al., 2007). Thus the presence of prohibitin in a cell along with fortilin would then promote cell survival allowing the cell to persist abnormally past its usefulness, a trait seen in many diseases including cancer and cardiovascular diseases, especially when macrophages of the atheroma outlive their usefulness but cannot commit to cell death pathways.

In Summary, the present study provides additional insight into fortilin cellular function and investigated a novel protein interaction with prohibitin. Indeed, the goals of this study were to investigate fortilin function by evaluating novel fortilin interactions and stressing cells to evaluate how this interaction may protect cells. The original hypothesis of this study considered if prohibitin

expression modulates fortilin mediated apoptosis activation. In view of the functional consequences of fortilin and prohibitin co-expression within the cell in conjunction with the results of my cycloheximide stability studies, prohibitin does indeed modulate fortilin antiapoptotic activity. Considering the role of apoptosis in various disease states it would be interesting to investigate the co-expression of prohibitin within these diseases and to test whether inhibition of fortilin and prohibitin can modulate disease progression.

In the realm of cardiovascular disease, much more emphasis has been placed on the discovery of novel diagnostics and therapeutics. The role of apoptosis is very integral to the development of atherosclerosis, yet how does apoptosis inhibition affect the progression of the atheromatous plaque? Can interruption of foam cell apoptosis also promote plaque regression? Does apoptosis have a role in plaque regression as it does in plaque progression? Indeed, because fortilin is expressed in atheromas and fortilin and prohibitin interact, future studies would need to investigate if prohibitin is expressed in atheromatous plaques. Furthermore, since prohibitin is known to be involved in the C-Raf/Ras/MAPK pathway, contemporary drugs that involve this pathway may be exploited to address cardiovascular conditions. Interestingly, novel inhibitors of this pathway function through inhibition of PHB (Chiu et al., 2012).

Indeed prohibitin is known to be involved in the development and progression of hepatocellular carcinoma, breast cancer, esophageal cancer, and found in pancreatic islets implicating prohibitin dysfunction in the development of diabetes (Mishra et al., 2005). Given the involvement of both of these proteins in

human disease, the finding of this novel protein interaction can pave the way for future investigations into novel therapeutics.

HOW AND IN WHAT CONTEXT DOES FORTILIN AND PROHIBITIN INTERACT?

The lingering question is how would we tease out the significance of the protein interaction from the functional interaction? Fortilin and prohibitin have other attributed cellular functions (PHB has mitochondrial roles and fortilin is a nuclear-cytosolic shuttle) yet which cellular processes are regulated *de facto* by the interaction? One study would be to actually determine the binding sites of these two proteins and mutate residues within the binding site of either proteins using alanine-scanning mutagenesis and determine the level of interaction of these mutant proteins. After which we can perform functional assays of this "non-interactor." A more robust study would be to perform the mutant analysis and then, again, using site-directed mutagenesis, mutate the other protein so that two mutants can then interact again and perform the same studies. These types of experiments will truly shed light on the nature and the significance of the physical protein interaction in how fortilin and prohibitin can regulate cell death. Of course, even the physical protein interaction is not enough, the mechanism of how a protein interaction can modulate such processes as DNA fragmentation and Caspase 3 activation would need to be further investigated.

Additionally, the question remains as to the mechanism of how fortilin-prohibitin inhibit apoptosis. Our laboratory group has published that fortilin interacts with p53 and prohibitin is known to interact with and regulate p53

transcriptional activation. In our proteomic screen, p53 was absent yet both fortilin and prohibitin are known to interact with this protein. Since p53 is typically activated after DNA-damage, and my functional assays employed UV-mediated DNA damage, a reasonable hypothesis is that fortilin and prohibitin interact in the nucleus and together regulate p53 genes. To test this hypothesis, one experiment would be to purify nuclei after UV induction and perform an immunoprecipitation of p53 and evaluate if fortilin and prohibitin are co-immunoprecipiated. Based on the immunofluorescence and subcellular results (Figures 12 and 13), fortilin and prohibitin are expressed in the nucleus but were not co-localized to an appreciable degree. Because the present study did not purify subcellular compartments after cell stress, this experiment would further shed light on the subcellular distribution of fortilin and prohibitin after a stress. Could there be an as yet-determined mechanism where fortilin and prohibitin are recruited in proximity after a stress?

Another lingering question is that fortilin is rarely found in the mitochondria and prohibitin is found predominantly in the mitochondria with pools found in the cytosol and nucleus. In surveying the literature, prohibitin has been shown to be involved in apoptosis signaling presumably through its stabilization of mitochondria, but other studies have shown a nuclear-cytoplasmic role of prohibitin in regulating apoptosis (Fusaro et al., 2003; Nijtmans et al., 2000). Is there a significance to the subcellular localization of Prohibitin in regulating apoptosis? A question that has not been addressed to an appreciable degree is the possibility of multiple independent pools of prohibitin. Mitochondrial prohibitin is found within the mitochondrial inner membrane, thus would mitochondrial-based prohibitin be able to be exported from the mitochondria or exert an extra-mitochondrial function? Our studies of

prohibitin were from plasmids, which would mean that we have artificially created a pool of cytoplasmic and/or nuclear prohibitin and through those studies, I have shown a functional advantage for this upregulation of extra-mitochondrial prohibitin. Could that mean that cytoplasmic/nuclear prohibitin has a different function compared to mitochondrial prohibitin? An experiment to test this would be to construct a prohibitin plasmid with a specific mitochondrial localization motif coupled to a GFP tag to determine the localization of this construct and to study the apoptosis regulatory role of this construct. The cells would then be stressed and the location of GFP-PHB fluorescently visualized thereby establishing the sequence of steps after cell stress would affect prohibitin localization. Additionally we can ask if this construct would still interact *in vivo* with fortilin. A provocative study could be to construct a fortilin- prohibitin combined polypeptide with the MLS and GFP to determine if this construct could reasonably protect cells from cell death, although the complications arising from the size of this construct and the biological significance of this construct would be called into question. Nonetheless such studies are needed to evaluate the coupling of fortilin- prohibitin and separately whether mitochondrial prohibitin has a role in the fortilin- prohibitin interaction and the findings of the present study.

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