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The Prohibitin Protein Complex Promotes Mitochondrial Stabilization And Cell Survival In Hematologic Malignancies

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THE PROHIBITIN PROTEIN COMPLEX PROMOTES MITOCHONDRIAL STABILIZATION AND CELL SURVIVAL IN HEMATOLOGIC MALIGNANCIES

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THE PROHIBITIN PROTEIN COMPLEX PROMOTES MITOCHONDRIAL STABILIZATION AND CELL SURVIVAL IN HEMATOLOGIC MALIGNANCIES

by

ELISA ROBLES ESCAJEDA, B.S.

DISSERTATION

Presented to the Faculty of the Graduate School of The University of Texas at El Paso in Partial Fulfillment of the Requirements for the Degree of

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Abstract

Lymphocyte proliferation and differentiation is coordinated with high precision in healthy humans and is vital to maintaining a normal immune system. Imbalance of these events can result in the development of autoimmune diseases, immunodeficiencies and hematopoietic malignancies. These pathologies, specifically leukemia and lymphoma have a high incidence of relapse and mortality due to limited treatment options. Therefore, there is a critical need to characterize the signal transduction pathways and understand molecular hallmarks that mediate T cell activation in order to develop new strategies for diagnosis and treatments of these diseases.

Prohibitins (PHB1 and PHB2) have been proposed to play important roles in cancer development and disease progression. In this study we used immunoprecipitation coupled to mass spectrometry to identify new forms of PHB regulation. We report four novel interleukin 2 (IL-2) inducible phosphosites in PHB2. To elucidate the potential regulatory role of these sites we generated phosphospecific polyclonal antibodies against one key phosphoresidue Threonine-62, to characterize its function. In addition, evidence is provided for PHB1 and PHB2 upregulation in tumor cell lines and localization mainly at the mitochondria. These proteins were also upregulated during reactive oxygen species (ROS)-mediated apoptosis. Similarity, PHB1 and PHB2 protein levels were significantly higher in tumor cells isolated from leukemia and lymphoma patients and determined to mainly localize to the mitochondria, possibly to maintain mitochondria integrity, which may facilitate the energy requirements of these tumor cells. Therefore, prohibitins may serve not only as biomarkers for cancer, but also act as molecular target for therapeutic intervention in hematopoietic malignancies.
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CHAPTER 1

Introduction
1.1 **T cell-mediated immunity**

The importance of the immune system for human health is regularly exemplified by individuals that suffer from a defective immune system as they are more susceptible to serious and sometimes life-threatening infections, allergy, sepsis, autoimmune diseases, immunodeficiency and cancer. From a functional point of view, the immune system can be divided into the innate and adaptive immunity. The innate immune system is the front line of defense that grants an immediate but not specific response that is mediated mainly by the complement and acute inflammation (neutrophils and macrophages). In contrast, adaptive immunity is a more pronounced and specific response mainly comprised of lymphocytes (T and B cells) that is divided into two general types of reactions: humoral immunity, mediated by antibodies produced by B cells and cell-mediated immunity, mediated by T cells. T lymphocytes are capable of complex biological responses and their activation and clonal expansion is a fundamental event in generating of both humoral and cell-mediated immunity [9].

Naïve mature T cells constantly recirculate between blood, lymphatic system and secondary lymphoid organs until they encounter a specific antigen. As a consequence, T lymphocytes activate, proliferate and differentiate into antigen-specific effector cells capable of modulating the immune response [10]. Complete activation of T cells requires three threshold limited sequential signals. Naïve T cells receive induction signals for activation through engagement of the T cell receptor (TCR) complex via binding to a specific antigen associated with the major histocompatibility complex (MHC) on antigen presenting cells (APC) (signal 1). This signal is then reinforced by CD28 engagement by co-stimulatory molecules such as B7-2 and B7-1 (signal 2). Together, signal 1 and signal 2 initiate a signal transduction cascade that promotes the
synthesis and secretion of T-cell growth factors like interleukin 2 (IL-2). These cytokines (signal 3) are ultimately responsible for driving clonal expansion and functional differentiation [11].

IL-2 was among the first cytokines to be characterized at the molecular level and was originally called T cell growth factor. This T-cell derived cytokine is a secreted glycoprotein with a molecular weight of approximately 15 kDa with a structure comprised of four α-helices folded in a characteristic configuration of the Type I cytokine receptor family. IL-2 is produced mainly by activated helper T (CD4+ T) cells but also in other cells like cytotoxic (CD8+ T), NK, dendritic, and mast cells [12, 13]. IL-2 induces a myriad of effects on the cells of the immune system but the main biological activities are exerted by CD4+ T cells, CD8+ T cells, B cells and NK cells causing expansion of antigen-specific clones, augmenting production of other cytokines, promoting proliferation and enhancing cytolytic activity among other effects [14-16].

The IL-2 signaling pathway is mediated by the IL-2 receptor (IL-2R) that is comprised of three chains: IL2Rα (CD25), IL2Rβ (CD122), and IL2Rγ (CD132) [17, 18]. The IL2Rα chain increases the affinity of ligand, while IL2Rβ and IL2Rγ participate in both ligand binding and cell signaling [19]. The IL-2 signaling pathway is initiated after binding of IL-2 to its receptor, causing Janus Kinases 1 and 3 (JAK1 and JAK3) to become associated with IL-2Rβ and IL-2Rγ chains respectively. This allows for autophosphorylation of the JAK1 and JAK3. Once activated, JAKs phosphorylate the receptor, providing docking sites for Signal Transducer and Activator of Transcription (STATs). Then, phosphorylation of STATs induces their formation of homodimers or heterodimers which translocate to the nucleus, to promote gene transcription that is associated with proliferation and cell survival. [11, 13] (Figure 1.1).

Additionally, IL-2 activates Src homology 2 domain containing transforming protein 1 (SHC) which stimulates the recruitment and activation of class IA phosphatidylinositol-3-kinases
(PI3Ks), which catalyze the conversion of phosphatidylinositol 4,5-biphosphate (PIP2) into phosphatidylinositol 3,4,5-triphosphate (PIP3) [14, 16]. PIP3, as a second messenger, recruits to the cell membrane 3-Phosphoinositide dependent protein kinase-1 (PDK1) to phosphorylate protein kinase B (AKT) [20, 21]. AKT pathway activation ensures T cell growth survival by regulating the transcription of genes like NF-kB as well the expression of IL-2 itself [22] (Figure 1.1).

IL-2 activates a myriad of effectors including SHC that can bind to with the growth factor receptor-bound protein 2 (GRB2). GRB2-associated binding protein (GAB2) activates son of sevenless homologs (SOS) next, and SOS promotes deactivation of Harvey rat sarcoma viral oncogene homolog (Ras) [23]. Activated Ras recruits Raf proto-oncogene serine/threonine-protein kinase (Raf), which in turn, activates mitogen-activated protein kinase kinase 1 and 2 (MEK) and MEK promotes signaling of extracellular signal-regulated kinase 1 and 2 (ERK1/2) [11, 24]. ERK1/2 pathway induces activation of transcription factors that play a critical role in IL-2 gene expression, including AP-1, c-Fos, c-Jun and Elk-1 [15, 25, 26] (Figure 1.1).

The aforementioned transduction signaling pathways are orchestrated by cascades of protein-protein interactions and phosphorylation events. Protein phosphorylation is a post-translational modification that has an essential role in biological homeostasis. As a result, the new phosphorylated group alters the role of the protein: it can be activated “on” or deactivated “off,” causing a change in its function and also, influencing protein sub-cellular localization, interactions with other proteins or their degradation by proteases. Phosphorylation is performed by kinases and occurs on serine, threonine, and tyrosine residues in eukaryotic cells and is reversed by phosphatases. The use of phosphorylation modifications as a regulatory mechanism have the advantage that are rapid, easily reversible and does not require the synthesis of new proteins [27].
Figure 1.1: Schematic representation of IL-2 signal transduction pathways. Binding of IL-2 to the high affinity IL-2R activates JAK1 and JAK3 are via autophosphorylation and then phosphorylate the receptor. This provides docking sites for STAT transcription factors that dimerize and translocate to the nucleus where they promote gene transcription. SHC recruits and activates PI3K that catalyzes the conversion of PIP2 to PIP3, which recruits PDK1 to phosphorylate AKT. SHC also recruits SHC recruits GRB2, GAB2 and SOS to activate Ras, which recruits Raf in order to activate MEK, which in turn activate ERK1/2. Special thanks to SGM for the diagram design [6, 8].
1.2 **Prohibitin Family Proteins**

Even though prohibitins were discovered more than 20 years ago [28], the functions of PHB1 and PHB2 are just beginning to be elucidated. The prohibitin (PHB) family is composed of two members, PHB1 and PHB2. PHB1, formerly known as BAP32 and its homolog PHB2, previously known as BAP37 or REA, are pleiotropic proteins with multiple functions [29]. Genetic deletion of PHB1 and PHB2 is embryonically lethal in mice indicating that these proteins perform an essential role in embryonic development [30, 31] and are known to be evolutionarily conserved with homologues found in organisms from yeast to man [28].

Prohibitins have been eliciting multiple functions that may be defined by their cellular localization and cell type. The multiple functions attributed to prohibitins include nuclear transcription, plasma membrane lipid scaffold protein, and in the mitochondria as a regulator of mitochondrial morphogenesis and apoptosis [5, 32-40]. Despite these diverse biological roles, the function of prohibitin proteins in cancer remains poorly understood [41].

PHB1 was originally described as a tumor suppressor due to its ability to inhibit cell proliferation [42]. This effect was later attributed to the 3’-UTR and not the PHB1 protein [43], however, reports indicate PHB1 can inhibit proliferation through interaction with the cell cycle checkpoint molecules E2F [44], p53[45], and Rb [46]. PHB2, a repressor of estrogen receptor activity, directly interacts and inhibits the transcriptional activity of the estrogen receptor [30].

1.2.1 **Biophysical characteristics of the Prohibitin family**

The human prohibitins are encoded by two nuclear genes PHB1 (GeneID:5245) and PHB2 (GeneID: 1331). PHB1 gene is located on chromosome 17q21 with 7 exons [47] and PHB2 gene maps to chromosome 12p13 with ten exons [48]. PHB1 and PHB2 have molecular masses of
approximately 32 and 37 kDa, respectively, and share 47% amino acid sequence identity and 67% similarity [3, 4] (Figure 1.2).

PHB1 and PHB2 are members of a group of membrane proteins containing the SPFH domain (also known as the prohibitin domain), which includes Stomatins, Prohibitins, Flotillins and bacterial HflK/C proteins. This domain is found in divergent species from prokaryotes to eukaryotes and their included proteins share numerous characteristics, including propensity to oligomerize, subcellular sorting and co- and post-translational modifications [49]. All proteins in this group are enriched in detergent-resistant membrane fractions, indicating that they are components of lipid raft membrane [50]. Lipid rafts are membrane microdomains enriched in certain lipids and cholesterol that function as platforms for compartmentalizing cellular processes such as signal transduction, protein sorting, receptor trafficking, membrane fluidity, and regulation of neurotransmission among other functions [51, 52].

Protein members of the SPFH domain group are reported to be anchored in membranes like the membranes in Golgi apparatus, mitochondria, endoplasmic reticulum, lipid droplets, and endosomes [49, 53]. The functions that have been implicated are chaperones and scaffold proteins [54, 55], protein and vesicle trafficking [56], ion channel regulation [53], microdomain formation and the formation of specialized membrane structures related to endocytosis and mechanosensation [50, 57, 58].

Prohibitins are composed of the previously mentioned prohibitin domain, an N-terminal transmembrane region consisting of a hydrophobic membrane-anchoring alpha helix [6, 59], a C-terminal coiled-coil structure that is involved in protein-protein interaction between PHB1 and PHB2 [59, 60], and a nuclear export sequence consisting of a putative leucine-rich nuclear export signal [37], (Figure 1.3). The prohibitin domain spans residues 26-187 in PHB1 and 39-201 in
PHB2, and is the region of highest homology (55% identity and 81% similarity) [3, 4, 59]. The coiled-coil regions of alpha helices are positioned towards the C-terminal region of both proteins that spans amino acids 180-224 and 212-253, respectively, for PHB1 and PHB2 [59]. The C-terminal region of PHB1 also contains a putative nuclear export sequence [37].

While the molecular structure of these proteins has not been solved, Winter et al. were able to produce structural models of PHB1 and PHB2 based on secondary structure prediction and homology modeling using the known nuclear magnetic resonance (NMR) structure of flotillin-2, which belongs to the SPFH domain protein family [60]. In addition to this domain PHB2 also contain an estrogen receptor binding domain near its N-terminus spanning residues 23-27 [61].
Figure 1.2: Protein alignment of human PHB1 and PHB2. Human PHB1 (Accession P35232) (upper sequence) and PHB2 (Accession Q99623) (lower sequence) protein sequences were aligned using the EMBOSS Needleman-Wunsch algorithm. (|), identical residues; (:), strongly similar residues; (.), weakly similar residues [3, 4].
Figure 1.3: Schematic representation of PHB 1 and PHB 2 phosphorylation sites. Identified phosphosites in human prohibitin proteins and the amino acid residues they modify. Yellow and green boxes indicate the prohibitin domain for PHB1 and PHB2 respectively. Numbers indicate the amino acid residues. Serine (S), Threonine (T), Tyrosine (Y). Amino-terminal end (N-) and carboxyl-terminal end (-C). Adapted from [2, 5].
1.2.2 Prohibitin Phosphorylation

Prohibitin phosphorylation has been reported to be important for cell signaling, cell differentiation and protein-protein interactions [54, 62, 63]. The schematic diagram (Figure 1.4) displays the phosphosites that have been identified in PhosphoSitePlus (www.phosphosite.org). This platform is an open, comprehensive, manually curated and interactive resource to compile experimentally observed post-translational modifications. The majority of the phosphorylation events assigned for prohibitins have been obtained via proteomic discovery-mode mass spectrometry with only very few of these sites having been characterized using site-specific methods such as site-directed mutagenesis, modification with site-specific antibodies as subsequent functional assay assessment. Moreover, upstream kinases have not been identified or validated. In other words, four of 14 sites for PHB1 and four of 20 sites for PHB2 have been identified and studied by site-specific methods. The sites characterized are Tyrosine (T)114, Serine (S)218, Threonine (T)258 and T259 for PHB1 and S91, S176, S243 and T248 for PHB2 (shown in red in Figure 1.4).

For instance, Jiang et al. reported that phosphorylation of PHB1 by AKT at Thr258 induces its localization from the nucleus to mitochondria causing cell proliferation in bladder cancer cell lines [64]. Also, the same residue was found to be phosphorylated in pancreatic carcinoma cells by AKT in vivo and in vitro [65]. Likewise, Zhu et al. identified a dual role of PHB1 to transforming growth factor-β (TGF-β) as a downstream effector causing cell survival following serine phosphorylation, in contrast, apoptosis was mediated by serine dephosphorylation in certain prostate cancer cells [66]. Moreover, it has been reported that cell stimulation with insulin leads to phosphorylation of PHB1 at Tyr114 and that residue creates a binding site for the Src homology
region 2 domain-containing phosphatase-1 (Shp1) blocking phosphorylation of AKT of *in vivo* and *in vitro* in myoblast and breast cancer cell lines [63].

In PHB2 Ser91 was demonstrated to be phosphorylated by calcium/calmodulin-dependent protein kinase IV (CaMKIV) causing the blocking of this kinase to repress the transcriptional activity of myocyte enhancer factor 2 (MEF2) [64]. Likewise, our group identified Tyr248 and Serine 248 as being phosphorylated upon T cell activation [7, 67].

### 1.2.3 Prohibitins, Mitochondrial Biology, and Oxidative Stress

Prohibitins in the mitochondria associate with each other to form a ring-shaped complex comprised of 12 to 16 PHB1 and PHB2 heterodimers [39]. This high molecular complex (approximately 1.2 megadaltons), has a diameter of 20 to 25 nanometers and is located in the inner mitochondrial membrane (IMM) [5, 39, 59, 60, 67]. Deletion of one prohibitin gene or siRNA knockdown of PHB1 or PHB2 results in the degradation of the respective prohibitin protein [5, 39, 67]. Similarly, co-immunoprecipitation experiments show an association of PHB1 with PHB2 and vice versa [32, 67]. These findings demonstrate the interdependence between prohibitins in the protein complex formation.

Yeast molecular genetics has played a key role in understanding PHB function and structure. The prohibitin homologues in *Saccharomyces cerevisiae* have been proposed to function as chaperones for newly imported proteins including electron transport enzymes [5, 34, 68]. Studies report that Prohibitins maintain the morphogenesis of cristae and biogenesis of the respiratory chain [5, 39, 69]. Additionally, they protect newly imported proteins from the degradation by m-AAA proteases, serve as scaffold proteins and have chaperone activity [5, 29, 34, 70]. For the first time, our laboratory group demonstrated that prohibitins were highly upregulated upon activation of normal primary human T cells following engagement of the TCR
complex. Moreover, these proteins were phosphorylated which may facilitate T cell survival through stabilization of mitochondrial electron transport enzymes during increased metabolic demand required for T lymphocyte proliferation [67].

Enhanced oxidative stress has been associated with prohibitins expression. In endothelial cells, down-regulation of PHB1 resulted in increased mitochondrial reactive oxygen species (ROS) production and cellular senescence [71], while over-expression of PHB1 in intestinal epithelial cells ameliorated oxidative stress in inflammatory bowel disease [72]. In the mitochondrial environment under physiological conditions, the optimal levels of intracellular reactive oxygen species (ROS) are maintained as byproducts of normal metabolism in eukaryotic cells. These normally low ROS concentrations have important roles in cell signaling and homeostasis [73]. However, oxidative stress can occur when the equilibrium between the generation of ROS and detoxification by antioxidant proteins is disrupted. Oxidative stress disturbs crucial cellular functions and has been related to a wide spectrum of diseases, including chronic inflammation and oncogenesis [74, 75]. Indeed, increased levels of ROS are persistently elevated in several types of cancer cells [74]. Regardless of the evidence that prohibitins are involved in the regulation of ROS and oxidative stress, their correlation with hematopoietic malignancies remains an unexplored area [76-79].
Figure 1.4: Schematic representation of PHB1 and PHB2 phosphorylation sites. Mass spectrometry identified phosphosites in human prohibitin proteins and their amino acid residue. Yellow and green boxes indicate the prohibitin domain for PHB1 and PHB2 respectively. Numbers indicate the amino acid residues. Numbers marked in red indicate residues characterized by site-specific methods. Serine (S), Threonine (T), Tyrosine (Y). Source: PhosphoSitePlus [6, 7].
1.2.4 Prohibitin Proteins in Cancer

The link between prohibitins and cancer is striking because these proteins relate to pathways that induce cell proliferation, resistance and metastasis (Ras/Raf/MEK/ERK, PI3K/AKT, TGF-β, transcription factors) [29, 54, 65, 80]. As a consequence, high levels of prohibitins expression have been demonstrated in several transformed cells and in many primary human cancers, including endometrial adenocarcinoma [81], hepatocellular carcinoma [82], gastric cancer [83], esophageal cancer [84], bladder cancer [85], and breast cancer [86], however, their tumorigenic mechanism have not been fully elucidated. Evidence suggests that cellular localization is a determinant of prohibitin function especially in oncogenic cells [29, 84, 86, 87].

To illustrate, in non-cancerous breast cells prohibitins are confined to the mitochondria, while in breast cancer cells these proteins are located primarily in the nucleus, suggesting that localization of prohibitins is a determinant for oncogenic potential in certain cancer cell types [88]. In breast cancer cell lines, PHB1 interacts with the transcription factor p53 enhancing its activity and during apoptotic induction PHB1 is translocated to the mitochondria [45]. Yoshimaru et al. reported that PHB2 interacts with Brefeldin A-inhibited guanine nucleotide-exchange protein 3 (BIG3). BIG3 has a critical role in the modulation of estrogen receptor signaling and targeting these proteins overcomes tamoxifen resistance in breast cancer cells [45]. PHB1 is a key regulator of retinoblastoma suppressor protein (Rb) that binds to E2F transcription factors causing cell cycle arrest and consequential growth arrest. These effects may be mediated by their interaction with different chromatin remodeling proteins including Rb, heterochromatic protein (HP1), Histone deacetylase 1 (HDAC) and the nucleosome remodeling proteins (Brg1 or Brm) [89]. In neuroblastoma cells, laser confocal microscopy results revealed that prohibitins colocalized with
the proto-oncogenes p53, Rb, c-myc, and c-fos. Also, the expression and localization of prohibitins changed during differentiation with retinoic acid.

Finally, it is of interest to note that some microRNAs (miRs) targeting untranslated regions (UTR) of prohibitins are classified as oncogenic. For instance, the oncomir miR-27a expression has been reported to be altered in several cancers including breast and relapsed leukemias [90, 91].

1.2.5 Prohibitins and metastasis

Many factors are involved in the initiation of cancer, but in the vast majority of these cases, mortality is due to metastatic progression. Metastasis requires a number of genetic or epigenetic modifications to complete the stages of the migratory process [92]. Along with the oncogenic cells’ limitless replicative potential and their increased capacity to survive, cancer cells must be equipped with at least three essential characteristics to cause metastasis. First, to escape the surrounding matrix and become migratory, these cells must undergo an “epithelial-to-mesenchymal transition”. Next, the cell must develop a mechanism to resist apoptosis. Finally, to sustain the supply of oxygen and nutrients angiogenesis is a requirement in order to ensure survival [93, 94]. Indeed, recent studies have shown that although aerobic glycolysis is known to promote metastasis, an aberration in a mitochondrial complex that involves overload of the electron transport chain can also profoundly enhance tumor metastasis [95, 96].

Prohibitin upregulation is positively related to esophageal and squamous cell carcinoma node metastasis and distant metastasis [84, 97]; in bladder cancer, prohibitin upregulation is positively related to multiple tumors, muscle invasion and tyrosine-protein kinase Met (c-MET) proto-oncogene overexpression [98]. Moreover, serum from colorectal patients contains significantly higher levels of these proteins compared to serum from healthy volunteers [99]. Prohibitins were found to be differentially overexpressed in highly invasive metastatic breast
cancer cell lines and in lymph node positive breast cancer patient samples using proteomic analysis [92, 100]. These findings may encourage further studies investigating prohibitin expression as a biomarker and clinicopathologic indicator of systemic malignancies.

1.2.6 Prohibitins as a therapeutic target

Localization of prohibitins in different cellular compartments, their potential to interact with tumor suppressor and transcription factors, their ability to stabilize mitochondrial electron transport and their capacity to act as scaffold proteins in the plasma membrane indicate that these proteins have a potential as therapeutic targets [5, 35, 37]. Furthermore, cellular localization and expression is a determinant of prohibitin function especially in oncogenic cells [29, 84, 86, 87]. This difference in behavior, which is not fully understood, may be exploited to develop therapeutic agents.

The proposed therapeutic target model for prohibitins (Figure 1.5) is based on studies reporting that these proteins are required scaffolds for cRaf localization to the plasma membrane, which is crucial for Ras-cRaf interaction and downstream activation of the ERK signaling pathway causing cell proliferation by activation of transcription factors (c-Myc, CREB, c-Jun, c-Fos) and survival by enhancing the activity of anti-apoptotic proteins (Bcl2, Bcl-xL) [101, 102]. Targeting prohibitins will therefore inhibit the Ras-cRaf interaction and will suppress the proliferative and pro-survival effects of this pathway causing death or growth arrest in cells that are dependent on the Erk pathway directly or indirectly.

Treatment with inhibitors of Ras, Raf, and MEK has shown therapeutic value, unfortunately, tumor resistance counteracts their efficacy. Natural derivative compounds such as Rocaglamide has been shown to target prohibitins and prevent their interaction with cRaf at IC\textsubscript{50} values within nanomolar concentrations [103]. Moreover, this substance blocked metastasis
growth \textit{in vitro} and \textit{in vivo} in cells that depend on to Ras-ERK signaling. More importantly, Rocaglamide was relatively nontoxic in normal cells, suggesting that the scaffold function of prohibitins is dispensable in these cells [80]. In the same context, previous studies have shown that phytochemical flavaglines and the synthetic fluorinated small molecule fluorizoline target prohibitins causing disruption of the Raf-MEK-ERK signaling pathway and induction of apoptosis through the intrinsic mitochondrial pathway, respectively [103-105]. Taken together, the array of prohibitin activity suggests these proteins might be attractive therapeutic targets for a variety of disease states, including inflammation, obesity and cancer, however a better understanding of their cell dependent function appears to be essential [55].
Figure 1.5: Therapeutic target model for Prohibitins proteins. A schematic model showing the mechanisms of prohibitins activation in the Ras/Raf/MEK/ERK pathway. Prohibitins are required scaffolds for the cRaf localization on the plasma membrane, which is crucial for Ras-cRaf interaction and downstream activation of the ERK signaling pathway causing cell proliferation and survival. Targeting prohibitins should inhibit the Ras-cRaf interaction and will suppress the downstream effects of this transduction pathway.
1.3 **SIGNIFICANCE AND HYPOTHESIS**

The importance of the immune system for human health is regularly observed in individuals that suffer from a defective immune system as they are more susceptible to serious and sometimes life-threatening infections, allergy, sepsis, autoimmune diseases, immunodeficiencies and cancer. Specifically, T cell regulation is vital in maintaining immune system homeostasis. An uncontrolled increase in T cell proliferation can lead to the development of hematopoietic malignancies that have a high incidence, resulting in relapse and in patient mortality despite current treatments. According with the American Cancer Society, leukemia is the most common cancer in children. Additionally, Hispanic children are more likely to be diagnosed with leukemia and they have a greater propensity to die from this pathology as compared with other children \[106\]. For this reason, there is a critical need to elucidate the regulatory mechanisms of the cell signaling pathways that mediate T-cell responses to establish a wider molecular foundation for the development of new therapeutic strategies into these diseases.

The objective of this dissertation was initiated to determine the role of PHB1 and PHB2 in T- and B-cell malignancies. **We hypothesize that prohibitins are cell signaling intermediaries involved in differentiation and proliferation in hematological malignancies.** To test this hypothesis the PHB1 and PHB2 phosphorylation state was examined in the leukemia cell line Kit225. In addition, prohibitin proteins have been proposed to play significant roles in cancer development and progression by regulation of apoptosis and cell proliferation \[5, 32, 34-36, 39, 41, 69\]. Therefore, the second objective of this dissertation was to define the expression of prohibitins in human lymphoid and myeloid tumor cell lines and patient samples. We hypothesized that PHB1 and PHB2 represent anti-apoptotic proteins involved in lymphoid oncogenesis.
We provide novel evidence that PHB1 and PHB2 are upregulated in hematologic tumor cells and possibly serve in the maintenance of mitochondrial integrity to protect against oxidative stress-induced cell death. These findings provide further evidence regarding the importance of PHB1 and PHB2 in lymphocyte function and dysfunction.
CHAPTER 2

Identification of Novel Phosphosites in Prohibitins
2.1 **Introduction**

Since their discovery in 1992 by McClung et al., cellular localization and function of prohibitins in different cell types has remained controversial. Moreover, protein phosphorylation represents a pivotal post-translational modification that regulates protein activity, sub-cellular localization, interactions with other proteins or their degradation by proteases [63, 67]. Dysregulation of normal patterns of protein phosphorylation can lead to a myriad of pathologies including cancer [27]. Previous work from our group showed that prohibitins are phosphorylated and differentially expressed during T cell activation [8]. This data strongly suggested that phosphorylation of these proteins plays an important role in cell differentiation and proliferation. Notably, there are no previous reports regarding the phosphorylation state of either PHB1 or PHB2 in T cells following IL-2 stimulation.

IL-2 induces several effects in immune cells of the but the main biological activities are believed to be exerted by CD4+ T cells, CD8+ T cells, B and NK cells causing expansion of antigen-specific clones, augmenting the production of other cytokines, to promote cell proliferation and to enhance cytolytic activity [14, 15]. The IL-2 signal transduction begins following binding of IL-2 to its receptor, and the subsequent activation of downstream signaling regulates proliferation, survival and apoptosis [11].

In order to understand the phosphorylation role of specific serine, threonine and tyrosine residues in prohibitin proteins and IL-2 signaling, cell culture and mass spectrometry was used. Knowledge about phosphorylation status of the prohibitin complex should have important implications for understanding its function in human T cells and T cell malignancies as these results may represent a possible regulatory point for lymphocyte function which could result in previously unrecognized therapeutic strategies to control T cell mediated diseases.
The strategy employed here consisted of immunoprecipitation (IP) reactions coupled to liquid chromatography and tandem mass spectrometry (LC-MS/MS) analysis. The treatments included: cells that were untreated, stimulated with IL-2 for 20, 45 or 60 min or treated with Calyculin A (CA) for 30 min. The rational to use CA is because this selective protein phosphatase inhibitor might further augment serine/threonine phosphorylation and it has been shown to regulate IL-2 signaling at multiple levels, including the formation of the receptor complex and activation of downstream signaling molecules [107]. To harvest PHB1 and PHB2 from their native environment we used an IL-2 dependent chronic lymphocytic leukemia Kit225 cell line [108, 109] which provided an excellent source for large scale immunoprecipitation, allowing for mass spectrometry to identify phosphorylated peptides at a picomol concentration without the need of extensive purification [110].

2.2 MATERIALS AND METHODS

2.2.1 Cell Culture and Treatments

The human IL-2 dependent Kit225 cell line was maintained in RPMI 1640 (Thermo Scientific Inc.) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals), 2 mM L-glutamine (cellgro), 50 IU/ml penicillin (cellgro), and 50 mg/ml streptomycin (cellgro) (complete RPMI media) plus 10 IU/ml recombinant IL-2 at 37 °C with 5% CO2. Kit225 cells were made quiescent by growing to exhaustion (> 5X10^5 cells/mL) and then stimulated with 10,000 IU of human recombinant IL-2 (NCI Preclinical Repository) for 20, 45 and 60 minutes, or treated with 100 nM Calyculin A (CA) (Sigma-Aldrich) for 30 minutes. Treatments were performed at 37 °C using 50x10^6 cells per treatment. Untreated, unstimulated cells were used as control.
2.2.2 Solubilization of Proteins, Immunoprecipitation, Western Blot and Mass Spectrometry Analysis

Cells were pelleted and solubilized in lysis buffer [10 mM Tris-HCl (pH 7.6), 5 mM EDTA (pH 8.0), 50 mM sodium chloride (NaCl), 30 mM sodium pyrophosphate (Na₄P₂O₇), 50 mM sodium fluoride (NaF), 1 mM sodium ortho-vanadate (Na₃VO₄), 1% Triton X-100] containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 μg/ml pepstatin A, and rotating at 4 °C for 1 h. Whole cell lysates were clarified by centrifugation (15,000x g, 15 min, 4 °C). For immunoprecipitation reactions (IP), supernatants were rotated with 5 μg of PHB2 rabbit monoclonal antibody clone E1Z5A for 2 hours at 4 °C. Immune complexes were captured by incubation with Protein A-Sepharose beads (Rockland Immunochemicals) rotating for 1 hr at 4 °C. The beads were then washed three times with ice cold lysis buffer and eluted by boiling 5 min in 4X SDS sample buffer (0.125 M Tris-HCl (pH 6.8), 10% 2-mercaptoethanol, 4.6% SDS, 0.004% bromophenol blue, 10% glycerol, pH 6.8). Samples were resolved in 12% SDS-PAGE and visualized by Coomassie blue staining (BioRad) after 1 hr. An input control (5%) of each sample was simultaneously run in the gel, transferred to polyvinyl-difluoride (PVDF) membrane and blocked with 1% bovine serum albumin (BSA) for 1 hr at room temperature.

Western blot analysis was performed by incubating the membrane with affinity purified rabbit polyclonal anti-PHB2 [67], monoclonal anti-PHB1 (Abcam, II-14-10), rabbit monoclonal anti-Phospho-Stat5 (cell signaling, D47E7) overnight at 4 °C. Assays were developed with horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse IgG (heavy plus light chains; KPL) and visualized by using enhanced chemiluminescence and radiographic film. Bands corresponding to PHB1 and PHB2 were excised from Coomassie blue stained gel and sent to the
Taplin Biological Mass Spectrometry Facility (Harvard University) for analysis through liquid chromatography-tandem mass spectrometry (LC-MS/MS). Any protein with three or more unique peptide matches was considered confidently identified. Spectra analysis using the SEQUEST search algorithm, manual examination of the spectra as well as the probability-based score Ascore where used to determine phosphorylation site localization [111-113].

2.3 RESULTS

2.3.1 Large-scale Immunoprecipitation of PHB1 and PHB2 and Subsequent Phosphosite Mapping

A large-scale immunoprecipitation of PHB2 was performed in order to obtain the necessary protein amount required for LC-MS/MS analysis. For each sample, 50x10^6 quiescent Kit225 cells per treatment were used for a single immunoprecipitation reaction. Cells were left untreated, stimulated with IL-2 (10^4 IU) for 20, 45 or 60 min or treated with CA (100nM) for 30 min. PHB2 was immunoprecipitated and the samples separated by 12% SDS-PAGE and visualized by Coomassie blue staining. The bands corresponding of PHB1 (≈35 kDa) and PHB2 (≈32 kDa) from non-stimulated, IL2-stimulated, and CA-treated were excised, subjected to trypsin digestion, and analyzed by liquid chromatography-tandem mass spectrometry (Figure 2.1 A). An input control of the sample (5%) was evaluated by Western blot for total PHB1 and PHB2 protein and phospho-STAT5 to ensure IL-2 induced activation (Figure 2.1 B). Representative data of several experiments is shown. The location of the novel phosphorylation sites in the prohibitins is shown in (Figure 2.1 C). The diagram includes the novel phosphorylation sites found in the present study (green) as well as residues previously identified and characterized using site-specific methods (red) and their localization in relation with the prohibitin domain. Spectra analysis using the SEQUEST
search algorithm revealed a protein coverage fluctuating from 77% to 92% for the analyzed samples, and the identification of several phosphorylated peptides for both proteins including four novel phosphosites for PHB2 (Table 2.1 and Figure 2.1 C). Table 2.1 shows a summary of treatments, protein coverage by mass spectrometry, and phosphosites identified for both proteins, novel phosphosites are underlined.
Figure 2.1: Identification and mapping of novel phosphorylation sites in human PHB1 and PHB2. Quiescent Kit225 cells were left untreated (-), stimulated with IL-2 for 20, 45, 30 min or treated with CA (100 nM) for 15 min. Cell lysates were immunoprecipitated (IP) with anti-PHB2 and separated by SDS-PAGE. One set was Coomassie blue-stained (A), and the other was immunoblotted (B) with anti-PHB1 and anti-PHB2, or anti-phospho-STAT5 antibodies. (C) Schematic representation showing the novel phosphorylation sites found with IL-2 (green) as well as residues previously identified and characterized using site-specific methods (red) and their localization in relation with the prohibitin domain for PHB1 and PHB2.
Table 2.1: LC-MS/MS identification of phosphorylation sites in PHB1 and PHB2.

| Treatment | PHB1 | Phospho residues | Coverage | PHB2 | Phospho residues |
|-----------|------|------------------|----------|------|------------------|
| None      |      | S101,Y259        | 85%      |      | S91,S293         |
| IL-2 20 min | 79%  | S101             |          | 83%  | S293             |
| IL-2 45 min | 83%  | Y259             |          | 84%  | S91,T62,T155,T169,S293 |
| IL-2 60 min | 80%  | S101,Y259        |          | 78%  | T21,S293         |
| CA 30min  | 77%  | S101             |          | 79%  | S91,S293         |

2.3.2 Mass Spectra, Conservation and Putative Kinases of Two Novel IL-2 inducible phosphorylation sites

The strategy employed in section 2.3.1 lead to the identification of 8 residues that become phosphorylated in PHB1 and PHB2, four of them with IL-2 stimulation (Table 2.1). In PHB1 the residues identified were S101 and T259. Both were constitutively phosphorylated and have already been reported in the literature [7, 114, 115]. In PHB2, T21, T62, S91, T155, T169 and S293 were identified to be phosphorylated. The sites S91 and S293 were constitutively phosphorylated and have already been characterized [7, 62, 116]. Here we report T21, T62, T155 and T169 as novel IL-2 inducible PHB2 phosphorylation sites. T21 and T155 were previously reported in to be
phosphorylated but not with the induction of IL-2, moreover, their identification was through proteomic discovery-mode mass spectrometry, meaning that no site-specific methods were employed in order to characterize them [7, 117, 118]. Of special interest to us were T62 and T169.

Threonine-62 was found to be a novel IL-2 inducible phosphosite. In addition, this threonine is localized in the prohibitin domain of PHB2 and the described [E/D][pS/pT]XXX sequence motif is a consensus β-Adrenergic Receptor kinase substrate (βARK) [119, 120]. Also, using the logarithmic kinase-specific prediction computational analysis T62 was estimated to be modified by Glycogen synthase kinase 3 (GSK3), Protein kinase C (PKC) and Protein kinase A (PKA) [121, 122]. Tandem mass spectrum for the peptide IGGVQDTILAEGLHFR and GGVQDTEILAEGLHFR with the phosphorylated PHB2 T62 residue (underlined) from IL-2 stimulated samples is shown in Figure 2.2 and 2.3 respectively. This phospho-threonine was identified in Kit225 cells that were IL-2 stimulated at 45 min. The Ascore (measures the probability of correct phosphorylation site localization based on the presence and intensity of site-determining ions in LC-MS/MS spectra for this phosphorylated residue) was 1000 indicating unequivocal localization of the phospho-residue [113]. PHB2 T62 is not conserved in its homolog PHB1 protein (Figure 1.1). To determine the extent of T62 conservation, human PHB2 protein sequence was aligned with PHB2 from other species using Clustal Omega program [1]. T62 as well as the surrounding amino acid sequence was found to be well conserved among eleven different species, mainly primates, bovine, rat and mouse (Figure 2.4). These evidence suggests that phosphorylation of T62 could be a result of an evolutionary recent gain-of-function mutation related to a specialized activity of the more complex immune system in higher organisms.

The second site of interest Threonine-169 was found also to be phosphorylated in PHB2. This phosphosite localized in the prohibitin domain and the reported [M/I/L/V][R/K]XX[pS/pT]
motif is a known Checkpoint kinase 1 substrate (CHEK1). Also, [M/I/L/V/F/Y]XRXX[pS/pT] and 
XX[pS/pT]E motifs have been reported to be Calmodulin-dependent protein kinase IV (CaMKII) 
and G protein-coupled receptor kinase 1 (GRK1) substrates respectively [7, 119, 123, 124]. 
Tandem mass spectrum for the peptide RRELERERA, which contains the phosphorylated PHB2 
T169 residue (underlined) from IL-2 treated samples at 45 min is shown in Figure 2.5. The Ascore 
for this phosphorylated site was 14 indicating that the phospho-residue is confidently assigned. 
PHB2 T169 is conserved in its homolog PHB1 protein (Figure 1.1). To determine the extent of 
T169 conservation, human PHB2 protein sequence was aligned with PHB2 from other species 
using Clustal Omega program [1]. T169 as well as the surrounding amino acid sequence was found 
to be well conserved among thirteen different species, including primates, bovine, rat, mouse, 
fungus, and plants (Figure 2.6).
Figure 2.2: Tandem mass spectra of T62 containing PHB2 peptide. (A) Schematic representation of peptide fragment ions of the peptide covering T62. (B) Complete tandem mass spectra on IL-2 stimulated Kit225 cells at 45 min. (C) Spectra escalation on the mass-to-charge ratio (m/z) axis, asterisks are used to designate the monophosphorylated peptides. Serine (S), Threonine (T), Tyrosine (Y).
A

G\textsuperscript{G}{\textsuperscript{N}{\textsuperscript{Q}{\textsuperscript{Q}{\textsuperscript{O}{\textsuperscript{D}{\textsuperscript{T}{\textsuperscript{I}{\textsuperscript{L}{\textsuperscript{A}{\textsuperscript{E}{\textsuperscript{G}{\textsuperscript{L}{\textsuperscript{H}{\textsuperscript{F}{\textsuperscript{R}{\textsuperscript{1}}}}}}}}}}}}}}}}\textsubscript{16} \textsubscript{15} \textsubscript{14} \textsubscript{13} \textsubscript{12} \textsubscript{11} \textsubscript{10} \textsubscript{9} \textsubscript{8} \textsubscript{7} \textsubscript{6} \textsubscript{5} \textsubscript{4} \textsubscript{3} \textsubscript{2} \textsubscript{1}

B

![Mass spectrometry diagram](image-url)
Figure 2.3: Tandem mass spectra of T62 containing PHB2 peptide. (A) Schematic representation of peptide fragment ions of the peptide covering T62. (B) Complete tandem mass spectra on IL-2 stimulated Kit225 cells at 45 min. (C) Spectra escalation on the mass-to-charge ratio (m/z) axis, asterisks are used to designate the monophosphorylated peptides. Serine (S), Threonine (T), Tyrosine (Y).
**Figure 2.4: Sequence conservation of PHB2 T62.** Amino acid sequence alignment of the region surrounding T62 (asterisk) from different organisms using Clustal Omega program [1].
Figure 2.5: Tandem mass spectra of T169 containing PHB2 peptide. (A) Schematic representation of peptide fragment ions of the peptide covering T62. (B) Complete tandem mass spectra on IL-2 stimulated Kit225 cells at 45 min. (C) Spectra escalation on the mass-to-charge ratio (m/z) axis, asterisks are used to designate the monophosphorylated peptides. Serine (S), Threonine (T), Tyrosine (Y).
Figure 2.6: Sequence conservation of PHB2 T169. Amino acid sequence alignment of the region surrounding T62 (asterisk) from different organisms using Clustal Omega program [1, 2].
2.4 **DISCUSSION**

In an effort to gain comprehension into the molecular mechanism of action of PHB1 and PHB2 during T cell proliferation and differentiation, phosphorylation status of these proteins was evaluated. Phosphorylation of proteins is post-translational modification employed by cells as a primary protein regulatory mechanism. As a result, the new phosphorylated group alters the role of the protein: it can be activated “on” or deactivated “off”, causing a dramatic change in its function like altering substrate binding affinity, sub-cellular localization, interaction with other proteins or their degradation by proteases. These actions regulate cell vital processes like differentiation, proliferation and apoptosis. As mentioned before, several proteins involved in T cell activation are phosphorylated forming a highly coordinated system of signal transduction pathways induced by the T cell receptor, co-stimulatory molecules, and IL-2 receptor. Through phosphoamino acid analysis, our group has reported that prohibitins are phosphorylated and upregulated at the messenger RNA (mRNA) level during T cell induction in response to diverse activating agents like phytohemaglutinin (PHA), anti-CD3, concanavalin A (ConA) and phorbol 12-myristate 13-acetate (PMA)/ionomycin [67].

In this work, with the strategy of large-scale immunoprecipitation of PHB2 followed by identification of phosphopeptides through LC-MS/MS we were able to identify novel phosphorylated residues in response to IL-2 stimuli. These findings provide a novel understanding into the function of prohibitins during this specific immune response. We found eight residues that are phosphorylated in PHB2, four of them were constitutively phosphorylated, and for the first time, PHB2 T21, T62, T155 and T169 were identified to become differentially phosphorylated in response to IL-2 induction (Figures 2.1-2.6 and Table 2.1). Moreover, T62 and T169 are phosphorylated residues that never have been reported before.
T62 and T169 amino acid sequence alignment of the region surrounding each of the phosphosites from different organisms revealed conservation among different species. Interestingly, T169 was found to be more highly conserved than T62. Based on the analysis of the LC-MS/MS spectra and the Ascore T62 was chosen for further examination.

In conclusion, using immunoprecipitation coupled to mass spectrometry four novel phosphosites in PHB2 were identified. In order to elucidate the potential regulatory role of the T62 in IL-2 signaling, phosphospecific polyclonal antibodies were generated against this phosphoresidue. The characterization of such antibodies is the focus of the next chapter.
CHAPTER 3

Characterization of PHB2 Phospho-Threonine 62 Antibodies
3.1 **INTRODUCTION**

Threonine phosphorylation of proteins is a vital component in regulating cell signaling. As shown earlier (Table 2.1) PHB2 T62 becomes phosphorylated in response to IL-2 stimulation of Kit225 cells. In order to verify phosphorylation of T62 and to elucidate its role in IL-2 signaling, we developed phosphospecific polyclonal antibodies against that site. This chapter is focused on the characterization of the anti-phospho-PHB2-T62 (anti-pT62) antibodies by testing their efficacy by ELISA, specificity using dot blot analysis and peptide competition assays, protein characterization via confocal immunofluorescence and analysis through immunoprecipitation and Western blot studies. Overall, these antibodies represent a new tool that could more accurately define T62 phosphorylation and its function in regulating PHB2 activity.

3.2 **MATERIALS AND METHODS**

3.2.1 **Generation of Phosphospecific Antibodies**

The anti-pT62 PHB2 rabbit polyclonal antibody was custom-generated by GenScript (Piscataway, NJ) using the immunogen GVQQD(pT)ILAGLHFS (where pT indicates phosphothreonine) coupled to keyhole limpet hemocyanin. Four rabbits were immunized per phosphopeptide (designated 17629, 17630, 17631 and 17632). The efficacy of whole antiserum from final bleeds was tested through enzyme-linked immunosorbent assay (ELISA) by the company. According to their protocol, wells were coated with 100 µl of either phosphopeptide or non-phosphopeptide at 4 µg/ml. Peroxidase conjugated anti-rabbit IgG (H&L) goat antibody was used as the secondary antibody.

3.2.2 **Dot Blot Analysis, Peptide Competition and Antiserum Purification**
PHB2 pT62 phosphopeptide (GVQQD(pT)ILAGLHFS) and non-phosphopeptide (GVQQDTILAGLHFS) were diluted in PBS and blotted in decreasing concentration (100 ng, 10 ng, 1 ng) into methanol activated PVDF membranes. The membranes were allowed to dry, then re-activated and blocked in 1% BSA for 1 hr at room temperature followed by incubation with phosphospecific antisera (1:5000) for 2 hrs. For peptide competition assays, a 1:5000 dilution was performed by incubating the phosphospecific antibodies with 10 μg of either PHB2 pT62 non-phosphopeptide (pre-clear) or phosphopeptide (pre-block) rotating for 2 hr at room temperature before probing dot blotted membranes. All antisera were affinity purified using a commercially available kit (SulfoLink, ThermoFisher) a using 2-step phosphospecific purification method. The fractions containing the protein of interest were dialyzed overnight at 4 °C in phosphate-buffered saline (PBS).

3.2.2 Cell Culture and Treatments

The human IL-2 dependent Kit225 cell line was maintained in RPMI 1640 (Thermo Scientific Inc.) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals), 2 mM L-glutamine (cellgro), 50 IU/ml penicillin (cellgro), and 50 mg/ml streptomycin (cellgro) (complete RPMI media) plus 10 IU/ml recombinant IL-2 at 37 °C with 5% CO₂. Kit225 cells were made quiescent by growing to exhaustion (> 5X10⁵ cells/mL) and then stimulated with 10,000 IU of human recombinant IL-2 (NCI Preclinical Repository) for 5, 15, 30, 45 and 60 minutes. Treatments were performed at 37 °C using 50x10⁶ cells per treatment. Untreated, unstimulated cells were used as control.

3.2.3 Solubilization of Proteins, Immunoprecipitation and Western blot analysis
Cells were pelleted, solubilized, and clarified as described in section 2.2.2. For immunoprecipitation reactions, supernatants were rotated with 5 μg of anti-PHB2 Rabbit monoclonal antibody clone E1Z5A (Cell Signaling technology) for 2 hr at 4 °C. Immune complexes were captured by incubation with Protein A-Sepharose beads (Rockland Immunochemicals) rotating for 1 h at 4 °C. The beads were then washed three times with ice-cold lysis buffer and eluted by boiling 5 min in 2 x SDS sample buffer (50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 0.02% bromophenol blue, 10% glycerol, pH 6.8). Samples were resolved in 12% SDS-PAGE, transferred to PVDF membranes and blocked with 1% bovine serum albumin (BSA) for 1 hr at room temperature. Western blot analysis was performed by incubating the membrane with the four custom generated anti-pT62 PHB2 rabbit polyclonal antibodies (17629, 17630, 17631 and, 17632) overnight at 4 °C. Assays were developed with horseradish peroxidase-conjugated goat anti-rabbit IgG (heavy plus light chains; KPL) and visualized by using enhanced chemiluminescence and radiographic film. For reprobing, PVDF membranes were incubated with stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) at 55 °C for 30 min, blocked, and then reprobed with anti-PHB2 Rabbit monoclonal antibody clone E1Z5A (Cell Signaling technology) overnight at 4 °C.

For reverse-immunoprecipitation, supernatants were rotated with 5 μg of each custom generated anti-pT62 PHB2 rabbit polyclonal antibodies (17629, 17630, 17631 and, 17632) 2 hr at 4 °C. Immune complexes were captured by incubation with Protein A-Sepharose beads (Rockland Immunochemicals) rotating for 1 h at 4 °C. The beads were then washed three times with ice-cold lysis buffer and eluted by boiling 5 min in 2 x SDS sample buffer. Samples were resolved in 12% SDS-PAGE, transferred to PVDF membrane and blocked with 1% bovine serum albumin (BSA)
for 1 hr at room temperature. Western blot analysis was performed by incubating the membrane with anti-PHB2 Rabbit monoclonal antibody clone E1Z5A overnight at 4 ºC.

For lysates cell pellets were solubilized in 1% Triton X-100 containing lysis buffer and Western blot analysis was performed using 20 µg of protein for each sample as previously described using the custom generated anti-pT62 PHB2 rabbit polyclonal antibodies (17629, 17630, 17631 and, 17632) overnight at 4 ºC [125]. After developing, membranes were stripped, blocked, and then reprobed with anti-PHB2 Rabbit monoclonal antibody clone E1Z5A (Cell Signaling technology) overnight at 4 ºC. For all samples, total protein was determined by the bicinchoninic acid method (Pierce Biotech).

**3.2.4 Immunofluorescent confocal microscopy**

Kit225 cells were cytocentrifuged onto glass slides, fixed with cold methanol and permeabilized with 0.2% Triton X-100. The cell staining procedures were performed using the following antibodies: custom made affinity purified anti-PHB2 pT62 (17632) rabbit polyclonal antibody, mouse monoclonal anti-PHB1 (abcam, II-14-10), rat monoclonal anti-PHB2 (Millipore, MABC953). To reduce photobleaching effects, all staining steps were executed in the dark. High-resolution digital fluorescent images were captured from the stained cells using an LSM 700 confocal laser scanning microscope equipped with a 40x and 63x immersion oil objective (Zeiss, New York, NY). Image acquisition was performed in the multitrack scanning mode with the excitation wavelength at 405, 488 and 555, 639 nm, corresponding to the blue, green, red and magenta fluorescence signals, respectively. Consistently, the confocal images were acquired with the same settings and analyzed using the ZEN 2009 software (Zeiss). Collected images were exported in a 12-bit TIFF RGB format. The LSM 700 confocal microscope system is located in
the Cytometry, Screening and Imaging Core Facility, a component of the Border Biomedical Research Center (BBRC-UTEP).

3.2.5 Phosphoamino Acid Analysis

Kit 225 cells were metabolically labeled with 1 mCi/ml $^{32}$P-orthophosphate (PerkinElmer Life Sciences) overnight at 37 °C. The cells were lysed and PHB2 was immunoprecipitated as described above. The corresponding proteins were visualized by Coomassie blue R-250 stain (Bio-Rad) and autoradiography, excised, and subjected to limited hydrolysis in 6 N HCl at 100 °C for 30 min. The samples were then dried and resuspended in pH 1.9 buffer (formic acid:acetic acid:water at 50:156:1794 ratio) containing 1 μg of phosphoamino acid standards. The samples were spotted on a thin layer cellulose-acetate gel and electrophoresis was performed in the first dimension at 1500 V for 30 min in pH 1.9 buffer and in the second dimension at 1300 V for 15 min in pH 3.9 buffer (pyridine:acetic acid:water at 10:100: 1890 ratio) using the Hunter Thin Layer Electrophoresis apparatus. Standards were visualized with ninhydrin and radiolabeled samples detected by autoradiography [67].

3.3 RESULTS

3.3.1 Anti-PHB2 pT62 Antiserum Preferentially Recognizes Phosphorylated Peptide

Phosphorylation of PHB2 pT62 was identified through mass spectrometry analysis and may represent a novel mechanism of regulation of IL-2 signaling. To verify that IL2Rβ is phosphorylated at threonine 62 and to investigate the regulatory role of this phosphorylation site, phosphospecific polyclonal antibodies were generated. Four rabbits were immunized with the phosphopeptides and are designated 17629, 17630, 17631, and 17632. Final bleed antisera were
analyzed by ELISA and specificity to phosphopeptide (GVQQD(pT)ILAGLHFS) in blue versus non-phosphopeptide (GVQQDILAGLHFS) in red was confirmed for all four rabbits (Figure 3.1).

To determine whether the PHB2 phosphospecific antisera cross-reacts with the non-phosphorylated form of the peptide, dot blot analysis with the immunizing phosphopeptide and the corresponding non-phosphorylated peptide was performed. Increasing amounts of pT62 or T62 peptides were spotted onto PVDF membranes and immunoblotted with PHB2 anti-pT62 antisera at a 1:5000 dilution. The PHB2 anti-pT62 antisera primarily recognized the phosphorylated peptide but not its non-phosphorylated counterpart, indicating that these phosphospecific antisera do not cross-react significantly with the non-phosphorylated peptide (Figure 3.2 top panel). In addition, a peptide competition assay was performed to confirm the specificity of the antisera. Dot blotted membranes were probed using anti-pT62 antisera pre-incubated with 10 µg the non-phosphopeptide (pre-clear) or phosphopeptide (pre-block) as indicated. The PHB2 anti-pT62 antisera signal was specifically blocked by the phosphopeptide, but not the non-phosphopeptide (Figure 3.2, middle and bottom panel).
Figure 3.1: PHB2 pT62 antisera has specificity towards phosphopeptide. Four rabbits, 17629, 17630, 17631, and 17632, were immunized with phosphopeptide. Increasing dilutions of each antiserum were analyzed through ELISA. Specificity of the antisera to phosphopeptide (blue) and non-phosphopeptide (red) is shown.
Figure 3.2: PHB2 pT62 recognizes phosphorylated peptide and is specific. Antisera were tested by dot blot (DB) analysis using decreasing concentrations of PHB2 T62 and pT62 peptides spotted on to PVDF membranes and incubated at a 1:5000 dilution of the anti-serum as indicated (top panel). For peptide competition analysis, the PHB2 pT62 antisera was incubated for 2 hr at room temperature with 10 µg of the non-phosphopeptide (pre-clear) or phosphopeptide (pre-block) as indicated (middle and bottom panel).
3.3.2 Affinity Purified 17632 Phospho-antibody recognize the T62 Phosphorylated form of PHB2 in Western blot analysis.

Antisera were affinity purified using a commercially available kit (SulfoLink, ThermoFisher) using 2-step phosphospecific purification method. First, antisera were purified through negative selection using a nonphosphopeptide column to remove the non-phosphospecific antibodies, and to collect the phosphospecific antibodies in the flowthrough. Then, this fraction positively purified using a phosphopeptide column to purify the phosphospecific antibodies from the other immunoglobulins.

To test the affinity of purified phosphospecific antibodies in whole cell lysate, quiescent Kit225 cells were treated with IL-2 for 5, 15, 30, 45 and 60 min. Untreated, unstimulated cells were used as a control. Samples were analyzed by Western blot analysis of total cell lysate using the four purified specific antibodies. The membranes were stripped and reprobed for total PHB2 to discriminate for protein size with other non-specific bands. As shown by arrows in Figure 3.3, the antibodies do not recognize PHB2 T62 during IL-2 induction since we observe signal in all treatments including untreated control in three antibodies (A, B, and C) and a very weak signal for one antibody (D). Also, non-specific proteins bands are detected by all antibodies. Representative data of six independent experiments is shown.

To further characterize these phosphospecific antibodies, quiescent Kit225 cells were treated with IL-2 for 5, 15, 30, 45 and 60 min. Untreated, unstimulated cells were used as a control. PHB2 was immunoprecipitated, resolved in SDS-PAGE, transferred to PVDF membrane and examined for phosphorylation of T62 by Western blot. Reprobing these blots with total PHB2 antibody confirmed similar PHB2 expression. As presented if Figure 3.4, the 17632 purified antibody recognizes IL-2 inducible pT62, however, we observed a variation in reproducibility in

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the level of pT62 detection between experiments (Figure 3.4 D and E, two independent results displayed inside a gray box). Representative data of six independent experiments is shown. Furthermore, pT62 was analyzed through reverse-immunoprecipitation. In other words, we performed an IP reaction with each purified anti-pT62 antibody, resolved in SDS-PAGE, transferred to PVDF membrane and examined for total PHB2 by Western blot. In agreement with the previously discussed result, pT62 17632 antibody was able to detect pT62 and hence immunoprecipitate PHB2 with IL-2 induction (Figure 3.5). The same variation in reproducibility was faced with these experiments (Figure 3.5 D and E), two independent results displayed inside a gray box). Representative data of six independent experiments is shown. Following immunoprecipitation or reverse-immunoprecipitation antibodies 17629, 17630 and 17631 were not able to detect pT62. Taken together the data suggest that phosphospecific antibody (17632) recognizes the T62 phosphorylated form of PHB2 in Western blot analysis.
Figure 3.3: Affinity purified phosphospecific antibodies do not recognize pT62 in whole cell lysate. Kit225 cells were grown to exhaustion and then stimulated with IL-2 (10⁴ IU) for 5, 15, 30, 45 and 60 min. Untreated, unstimulated cells were used as negative control. Cell lysates (20 µg) were separated by 12% SDS-PAGE and subjected to Western blot (WB) analysis with purified PHB2 anti-pT62 antibodies (A, B, C and D). The membranes were stripped and reprobed for total PHB2. Arrows indicate location of PHB2.
Figure 3.4: Affinity purified 17632 Phospho-antibody recognizes the T62 phosphorylated form of PHB2 in Western blot analysis. Kit225 cells were grown to exhaustion and then stimulated with IL-2 (10^4 IU) for the indicating times. PHB2 was immunoprecipitated, resolved in SDS-PAGE, transferred to PVDF membrane and examined for phosphorylation of T62 by Western blot (WB) using the purified PHB2 anti-pT62 antibodies (A, B, C, D and E). The membranes were stripped with total PHB2 antibody. Gray box displays two independent results for 17632 antibody (D and E).
Figure 3.5: Affinity purified 17632 Phospho-antibody recognizes the T62 phosphorylated form of PHB2 in Western blot analysis. Quiescent Kit225 cells were stimulated with IL-2 (10^4 IU) for the indicating times. PHB2 was immunoprecipitated, resolved in SDS-PAGE, transferred to PVDF membrane and examined for phosphorylation of T62 by Western blot using the purified PHB2 anti-pT62 antibodies (A, B, C, D and E). The membranes were stripped with total PHB2 antibody. Gray box displays two independent results for 17632 antibody (D and E).
3.3.3 **Affinity Purified 17632 Phospho-antibody recognize the T62 Phosphorylated form of PHB2 in Immunofluorescent Confocal Microscopy.**

To continue with the characterization of the PHB2 anti-T62 (17632) triple labeled immunofluorescent confocal microscopy was utilized. For Kit225 cells that have been treated with IL-2 for 45 and 60 min. Untreated, unstimulated cells were used as control. In all treatments, the nucleus was identified with the DNA binding fluorescent stain DAPI and total PHB1 and PHB2 co-localized primarily to peri-nuclear regions. There were higher fluorescence levels of PHB2 anti-pT62 at 45 min compared with untreated cells and 60 min (Figure 3.6). Likewise with this technique, we observe a variation in reproducibility in the level of pT62 detection between experiments based on two independent results (Figure 3.6, top and bottom panels). Representative data of six independent experiments is shown. Taken together, this data confirms the specificity of the phosphospecific antibody (17632) towards the T62 phosphorylated form of PHB2 in immunofluorescent confocal microscopy.

3.3.4 **PHB2 is Serine phosphorylated in response to IL-2 stimulation based on induction in phosphoamino acid analysis.**

To determine the global phosphorylation state of prohibitin proteins, phosphoamino acid analysis of $[^{32}\text{P}]$-labeled immunopurified PHB1 and PHB2 from Kit225 cells was performed. PHB2 was immunoprecipitated from cells radiolabeled overnight with 1 millicurie (mCi) of $[^{32}\text{P}]$-orthophosphate after IL-2 stimulation (untreated, 45 and 60 min) and subjected to separation by 12% SDS-PAGE and transferred to PVDF membrane. PHB1 and PHB2 were visualized by Coomassie blue stain before the membrane was subjected to autoradiography. Autoradiography showed both PHB1 and PHB2 were phosphorylated (Figure 3.7 A). PHB1 and PHB2 bands were
excised and subjected to phospoamino acid analysis. Under these conditions, PHB1 was determined not to be phosphorylated, PHB2 was phosphorylated on serine after 45 min with IL-2 stimulation at but in at very low levels (Figure 3.7 B, dotted line). Representative data of five independent experiments is shown.

This finding suggests evidence that the phosphorylation rate of prohibitins during IL-2 stimulation is low perhaps with a slow rate of phosphate turnover but strong enough to be detected by mass spectrometry (section 2.3.1).
Figure 3.6: Affinity Purified 17632 phospho-antibody recognize the T62 phosphorylated form of PHB2 in immunofluorescent confocal microscopy. Quiescent Kit225 cells were stimulated with IL-2 (10^4 IU) for the times indicated and then cytocentrifuged onto glass slides and subjected to analysis by immunofluorescent confocal microscopy. Nuclear staining was determined with DAPI. anti-pT62, PHB1 and PHB2 were conjugated with Cy3, Alexa and Alexa647, respectively. Overlay panel is displayed. Two independent experiments are shown (top and bottom panels).
Figure 3.7: PHB2 is Serine phosphorylated with IL-2 induction in phosphoamino acid analysis. Kit225 cells were \[^{32}\text{P}\]-orthophosphate labeled overnight under normal culturing conditions, cells were stimulated with IL-2 for the times indicated. PHB2 was immunoprecipitated, separated by SDS-PAGE, and subjected to Coomassie blue staining, autoradiography of the membrane after 14 days exposure is presented (D). Phosphoamino acid analysis was performed on both PHB1 and PHB2 (B), Phospho standards were detected by ninhydrin (left panel), and migration of PHB phosphoamino acids by autoradiography (right panel) is shown for PHB2. Arrows denote locations of PHB1 and PHB2, dotted line indicates serine phosphorylation. pS, pT and Yp denotes phospho-serine, phospho-threonine, phospho-tyrosine, respectively, that corresponds to the migration of the standards.
3.4 DISCUSSION

In order to study the importance of the novel PHB2 phosphorylation sites identified through mass spectrometry T62, rabbit polyclonal phosphospecific antibodies were generated. The strategy for characterization of the antibodies included ELISA, dot blot, Western blot and immunofluorescent confocal microscopy. Western blots were performed on whole cell lysate and immunoprecipitated PHB2 or anti-pT62. These strategies revealed the specificity of one of our antibodies (17632) to be used in Western blot analysis in immunoprecipitated protein and in immunofluorescent microscopy.

All four rabbit antisera preferentially recognized their corresponding phosphorylated peptide based on ELISA and dot blots. Moreover, in peptide competition assays, they were also specifically blocked by the phospho- but not non-phosphopeptide. In addition, the purified fraction of the PHB2 anti-pT62 (17632) recognized phosphorylated T62 form of PHB2 in Western blot analysis following immunoprecipitation but not in whole cell lysates where we observed a very weak signal. The previous result suggests that the phosphorylation rate of this specific amino acid is limited. This assumption was supported when we performed phosphoamino acid analysis and we could only detect a low level of phosphorylation. Likewise, in immunofluorescent confocal microscopy, anti-pT62 (17632) recognized phosphorylated T62 form of PHB2 but with high levels of variability and intensity. With these findings, we can assume that phosphorylation rate in prohibitins specifically phospho-threonine is low and probably very dynamic.

Taken together, this data confirms the specificity of one phosphospecific antibody (17632) towards the T62 phosphorylated form of PHB2 in Western blot analysis and in immunofluorescent confocal microscopy.
CHAPTER 4

The Prohibitin Protein Complex Promotes Mitochondrial Stabilization and Cell Survival in Hematologic Malignancies
4.1 INTRODUCTION

Prohibitins (PHB1 and PHB2) have been proposed to play important roles in cancer development and progression, however their oncogenic mechanism of action has not been fully elucidated. Previously, we showed that the PHB1 and PHB2 protein complex is required for mitochondrial homeostasis and survival of normal human lymphocytes. In this study, novel evidence is provided that indicates mitochondrial prohibitins are overexpressed in hematologic tumor cells and promote cell survival under conditions of oxidative stress. Immunofluorescent confocal microscopy revealed both proteins to be mainly confined to mitochondria in primary patient lymphoid and myeloid tumor cells and tumor cell lines, including Kit225 cells. Subsequently, siRNA-mediated knockdown of PHB1 and PHB2 in Kit225 cells significantly enhanced sensitivity to H2O2-induced cell death, suggesting a protective or anti-apoptotic function in hematologic malignancies. Indeed, PHB1 and PHB2 protein levels were significantly higher in tumor cells isolated from leukemia and lymphoma patients compared to PBMCs from healthy donors. These findings suggest that PHB1 and PHB2 are upregulated during tumorigenesis to maintain the mitochondrial integrity and therefore may serve as novel biomarkers and molecular targets for therapeutic intervention in certain types of hematologic malignancies.

4.2 MATERIALS AND METHODS

4.2.1 Cell culture and patient samples

Kit225 cells were maintained in RPMI 1640 supplemented with 10% FCS (Atlanta Biologicals), 2 mM L-glutamine, 50 IU/ml penicillin, and 50 mg/ml streptomycin (complete RPMI) plus 10 IU/ml recombinant IL-2. Jurkat, MT-2, Hut102, HH, H9, YT, KCL-22, Raji, Ramos, BJAB, Nalm-6 and CCRF-CEM cells were maintained in complete RPMI without IL-2.
Human peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors, purified by isocentrifugation, and activated with phytohemagglutinin (PHA) (1 μg/ml) for 72 hr, as previously described [67]. Primary patient leukemia and lymphoma cells were obtained from de-identified excess diagnostic material (peripheral blood, bone marrow or lymph node biopsies) through a The University of Texas at El Paso Institutional Review Board approved study. All clinical materials were obtained with the patients’ consent and approval from the local ethics committee.

4.2.2 Cell lysis and Western blot analysis

Cell pellets were solubilized in 1% Triton X-100 containing lysis buffer and Western blot analysis performed as previously described using the following antibodies [125]: affinity purified rabbit polyclonal anti-PHB2 [67], monoclonal anti-PHB1 (abcam, II-14-10), rabbit monoclonal anti-PHB2 (cell signaling, E1Z5A), mouse monoclonal caspase-8 (cell signaling, 1C12), mouse monoclonal caspase-9 (cell signaling, C9), rabbit monoclonal caspase-3 (cell signaling, D3R6Y), monoclonal anti-actin (Sigma-Aldrich), and monoclonal anti-GAPDH (Fitzgerald). For all samples, total protein was determined by the bicinchoninic acid method (Pierce Biotech).

4.2.3 Reactive Oxygen Species (ROS)-induced Apoptosis

For induction of ROS-mediated apoptosis, Kit225 cells were treated with hydrogen peroxide (H₂O₂) (500μM) (Sigma-Aldrich) at 37 °C at the time points indicated and cellular viability evaluated. Additionally, caspase activation was determined by detection of PARP cleavage by Western blot analysis with rabbit polyclonal anti-PARP (Cell Signaling), which recognizes full length PARP1 (116 kDa) fragment.
4.2.4 Viability assay

Cell viability was assessed by using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) reagent (Promega), following the manufacturer’s instructions. Cells were seeded in flat-bottom tissue culture 96-well plates at a density of 100,000 per well in a 100 µl of culture media. After the indicated times of incubation, 20 µl of the MTS reagent were added to each well containing the cells, followed by an additional incubation for 30 min. To stop the reaction we add 25 ul 10% SDS. The purple water-soluble formazan product was measured by absorbance at 490 nm utilizing a microplate reader (VERSAmax tunable microplate reader), as previously described [126]. Two independent experiments, each performed in triplicate, were performed. Data are shown as a normalized percentage of cell viability and are consistently reported as the average with the corresponding standard deviations.

4.2.5 Immunofluorescent confocal microscopy

The cell staining procedures were performed using the following antibodies as previously described [67]: mouse monoclonal anti-PHB1 (abcam), affinity purified rabbit polyclonal anti-PHB2 [67], mouse monoclonal anti-OxPhos CII (Invitrogen), or rabbit monoclonal anti-COX IV (cell signaling). To reduce photobleaching effects, all staining steps were executed in the dark. High-resolution digital fluorescent images were captured from the stained cells using an LSM 700 confocal laser scanning microscope equipped with a 40x and 63x immersion oil objective (Zeiss, New York, NY). Image acquisition was performed in the multitrack scanning mode with the excitation wavelength at 405, 488 and 555 nm, corresponding to the blue, green and red fluorescence signals, respectively. Consistently, the confocal images were acquired with the same
settings and analyzed using the ZEN 2009 software (Zeiss). Collected images were exported in a 12-bit TIFF RGB format. The LSM 700 confocal microscope system is located in the Cytometry, Screening and Imaging Core Facility, a component of the Border Biomedical Research Center (BBRC-UTEP).

4.2.6 siRNA-mediated silencing of PHB1 and PHB2

PHB1 (SMARTpool Cat. # M-010530-00), PHB2 (SMARTpool Cat. # M-018703-00) and control non-targeting (siControl pool Cat. #D-001206-13) siRNA were purchased from Dharmacon. Transfection of Kit225 cells was carried out by electroporation using the Nucleofection system (Amaxa). Briefly, Kit225 cells (5 × 10⁶) were suspended in 100 μl of transfection solution V and transfected with 1.5 μg of PHB1, PHB2 or control siRNA using the X-001 program. Transfected cells were immediately diluted with pre-warmed complete RPMI containing IL-2 (10 IU/ml) and cultured for the time indicated.

4.3 RESULTS

4.3.1 PHB1 and PHB2 are overexpressed in human lymphoid and myeloid tumor cell lines.

PHB1 and PHB2 protein levels have been reported to be higher in several transformed cells as compared to their non-transformed counterparts. To test this notion within hematologic malignancies, the expression levels of PHB1 and PHB2 were investigated in a panel of lymphoid and myeloid-derived tumor cell lines. As shown (Figure 4.1 A and B), normal naïve (lane a and b) and PHA-activated (lane c) human PBMCs were compared to the chronic lymphocytic leukemia T-cell line Kit225 (lane d), acute lymphoblastic leukemia T-cell line Jurkat (lane e), HTLV-1 transformed T-cell lines MT-2 and Hut102 (lane f and g), cutaneous T-cell lymphoma cell lines HH and H9 (lane h and i), NK-like acute lymphoblastic lymphoma and thymoma cell line YT
(lane j), chronic myelogenous leukemia cell line KCL-22 (lane k), Burkitt’s lymphoma cell lines Raji, Ramos and BJAB (lane l, m and n), pre-B acute lymphoblastic leukemia cell line NALM-6 (lane o), and acute lymphocytic leukemia cell line CCRF-CEM (lane p) by Western blot analysis of total cell lysate (Figure 4.1 A). The membrane was stripped and reprobed for GAPDH to confirm equal loading. Consistent with our previous findings, densitometric analysis indicated PHB1 and PHB2 protein levels were upregulated upon activation of primary human PBMCs (5.34 and 5.44 average fold increase for PHB1 and PHB2 respectively) (Figure 4.1 B) [67]. Compared to naive primary human PBMCs, PHB1 and PHB2 protein levels were 4.3 to 18.4 and 3.6 to 18.4 fold higher (p<0.05) in the tumor cell lines, respectively. Taken together, PHB1 and PHB2 proteins are overexpressed in lymphoid and myeloid tumor cell lines compared to normal naïve and activated primary human PBMCs.

4.3.2 PHB1 and PHB2 co-localize to the mitochondria of Kit225 cells

Identifying the subcellular localization of PHB1 and PHB2 in hematologic tumor cells is important to understanding their function in hematologic cell transformation, as well as the general mechanism of action in normal human lymphocytes. To determine their localization, dual labeled immunofluorescent confocal microscopy was utilized on the model cell line Kit225. The interleukin-2 dependent Kit225 cell line was established from a patient diagnosed with T-cell chronic lymphocytic leukemia [109].

The nucleus was identified with the DNA binding fluorescent stain DAPI (Figure 4.2 A, B and B1). PHB1 and PHB2 co-localize primarily to peri-nuclear regions with a specific punctuate staining pattern in Kit225 cells. There were no detectable levels of PHB1 or PHB2 at the plasma membrane or in the nucleus. To determine whether the prohibitin complex can be localized to
mitochondria in Kit225 cells, the inner mitochondrial membrane marker protein OxPhos CII was utilized (Figure 4.2 C, D and D1). Dual immunofluorescent confocal microscopy detecting PHB2 and OxPhos CII in tandem with DAPI showed co-localization of PHB2 with OxPhos CII. Taken together, PHB1 and PHB2 were identified to be primarily confined to the mitochondria in lymphoid tumor cell lines, including Kit225 cells.
Figure 4.1: PHB1 and PHB2 protein expression in human lymphoid and myeloid derived tumor cell lines. (A) Naïve (lane a and b) or PHA activated primary human PBMCs (lane c), CLL T cell line Kit225 (lane d), ALL T cell line Jurkat (lane e), HTLV-1 transformed T cell lines MT-2 and Hut102 (lane f and g), CTCL cell lines HH and H9 (lane h and i), NK-like lymphoma cell line YT (lane j), CML cell line KCL-22 (lane k), Burkitt’s lymphoma cell lines Raji, Ramos and BJAB (lane l, m and n), pre-B-ALL cell line NALM-6 (lane o), and ALL cell line CCRF-CEM (lane p) cell lysates (10 µg) were separated by 10% SDS-PAGE and subjected to Western blot analysis with antibodies directed to PHB1, PHB2 or GAPDH. (B) PHB1 and PHB2 band intensities were normalized to GAPDH using densitometric analysis and the fold increase plotted for each cell type. Values represent the mean ± S.D. of three independent experiments.
Figure 4.2: PHB1 and PHB2 co-localize primarily to mitochondria of Kit225 cells. Kit225 cells were cytocentrifuged onto glass slides and subjected to analysis by immunofluorescent confocal microscopy. (A and B) PHB subcellular localization was determined by staining the nucleus with DAPI; PHB1-Cy2 and PHB2-Cy3 and overlay. (C and D) Nuclear staining with DAPI, OxPhos CII-Cy2, PHB2-Cy3 and overlay were detected in Kit225 cells using immunofluorescent confocal microscopy. B1 and D1 represent zoomed views of their corresponding image. The 20 µm and 5 µm size marker is indicated.
4.3.3 PHB1 and PHB2 knock-down alone was not sufficient to induce apoptosis of Kit225 cells.

siRNA mediated knockdown and cell viability assay were performed in order to investigate if downregulation alone of prohibitins could cause cell death. Cells were electroporated with non-targeting control siRNA (Figure 4.3 A, lane a), PHB1 specific siRNA (lane b), or PHB2 specific siRNA (lane c) and cultured for 48 hr before cell lysis, SDS-PAGE and Western blot detection of PHB1 and PHB2 to confirm knockdown. Then, cell viability assays were performed (B). Also, Western blot was performed to evaluate the expression levels of caspase-8, caspase-9, and caspase-3 (C). Interestingly, the levels of cleaved caspases remained unchanged among controls and experimental samples, as well as cell viability. In conclusion, PHB1 and PHB2 knock-down alone was not sufficient to induce apoptosis of Kit225 cells.

4.3.4 PHB1 and PHB2 are upregulated during ROS-mediated apoptosis of Kit225 cells, while reduced PHB1/2 expression results in increased cell death during oxidative stress

Accumulating evidence suggests that prohibitins play a role in preventing oxidative stress in an array of cell types [71, 72]. Previously, we showed PHB1 and PHB2 protein levels were upregulated during cytokine deprivation-mediated apoptosis in Kit225 cells, suggesting the complex is induced in response to oxidative stress [67]. To test this hypothesis and explore the functional role of PHB1 and PHB2 during ROS-mediated apoptosis, Kit225 cells were treated with 500 µM H$_2$O$_2$ for 0, 1, 3, 6, 12 and 24 hr. Hydrogen peroxide (H$_2$O$_2$) has been widely used as a ROS in various cell models as an inducer of apoptosis [127]. H$_2$O$_2$ is endogenously generated in the mitochondria and cytosol and is one of the major contributors to oxidative damage [74].
Western blot analysis of protein lysate from treated Kit225 cells revealed PHB1 and PHB2 protein levels increase in a time-dependent manner (Figure 4.4 A). Detection of GAPDH protein levels was performed to confirm equal loading, whereas oxidative stress mediated caspase activation readily was verified by detection of caspase-cleaved PARP which reached maximum levels at approximately three hours.

The differential regulation of PHB1 and PHB2 during ROS-mediated cell death suggested the complex plays a functional role in the oxidative stress response of Kit225 cells. To determine the cellular consequences of PHB1 and PHB2 loss during ROS-mediated cell death, siRNA-mediated knockdown was performed and cell viability monitored following an H2O2 treatment time course for 24 hr. Kit225 cells were electroporated with non-targeting control siRNA (lane a), PHB1 specific siRNA (lane b), or PHB2 specific siRNA (lane c) and cultured for 48 hr before cell lysis, SDS-PAGE and Western blot detection of PHB1 and PHB2 to confirm knockdown (Figure 4.4 B). The membrane was stripped and reprobed for GAPDH to confirm equal loading. An H2O2 time course was then performed after treatment with siRNA for 12 hr and cell viability determined by MTS assay at 0, 3, 6 and 12 hr after addition of H2O2. The percent viability of Kit225 cells following PHB1 and PHB2 depletion was 78%, 58% and 45% after 3, 6 and 12 hr H2O2 treatment, respectively, compared to control siRNA treated cells (Figure 4.4 C). Only when treated with H2O2, did Kit225 cells with diminished PHB1 and PHB2 expression display greater levels of cell death.

4.3.5 PHB1 and PHB2 co-localize primarily to mitochondria of Kit225 during oxidative stress.
Identifying the subcellular localization of prohibitins during oxidative stress is significant, as these proteins have been reported to elicit multiple functions that may be defined by their cellular localization. To explore the subcellular localization of PHB1 and PHB2 during ROS-mediated apoptosis, Kit225 cells were treated with 500 µM H₂O₂ for 0, 1, 3, 6, 12 and 24 hr. Immunofluorescent confocal microscopy was utilized to evaluate PHB1 or PHB2 co-localization and prohibitin subcellular localization. In all time points, PHB1 and PHB2 co-localized primarily to peri-nuclear regions in Kit225 cells. There were no detectable levels of PHB1 or PHB2 at the plasma membrane or in the nucleus (Figure 4.5 A and A1). To confirm mitochondrial localization of the prohibitin complex in Kit225 cells, co-staining with the inner mitochondrial membrane marker protein COX IV was performed. Dual immunofluorescent confocal microscopy detecting PHB2 and COX IV in tandem with DAPI showed co-localization of PHB2 primarily with COX IV (B and B1). Oxidative stress mediated caspase activation was verified via caspase-cleaved PARP (C). Overall, the results displayed prohibitins be localized mainly in the mitochondria throughout ROS-mediated cell death.
Figure 4.3: PHB1 and PHB2 knock-down alone was not sufficient to induce apoptosis of Kit225 cells. (A) Kit225 cells (5x10^6) were electroporated with either non-targeting control siRNA (100 nM) (lane a), PHB1 specific siRNA (100 nM) (lane b), or PHB2 specific siRNA (100 nM) (lane c) and harvested at 48 hrs post-transfection. Cell lysates (10 µg) were subjected to 12% SDS-PAGE and Western blot analysis with antibodies directed toward PHB1, PHB2 and GAPDH as indicated. (B) Cell viability was measured at 48 hr post-transfection. (C) Western blot was done with antibodies against caspase-8, caspase-9 and caspase-3.
Figure 4.4: Kit225 cells unable to upregulate PHB1 and PHB2 are more sensitive to ROS-mediated cell death. (A) Kit225 cells (5x10^6) were treated with 500 µM H_2O_2 for 0, 1, 3, 6, 12 or 24 hr. Cell lysate (10 µg) was separated by 10% SDS-PAGE, transferred to PVDF membrane, and PHB1, PHB2, GAPDH and PARP protein levels detected by Western blot analysis as indicated. (B) Kit225 cells (5x10^6) were electroporated with either non-targeting control siRNA (100 nM) (lane a), PHB1 specific siRNA (100 nM) (lane b), or PHB2 specific siRNA (100 nM) (lane c) and harvested at 48 hr post-transfection. Cell lysates (10 µg) were subjected to 12% SDS-PAGE and Western blot analysis with antibodies directed toward PHB1, PHB2 and GAPDH as indicated. (C) Kit225 (5x10^6) cells were electroporated with control non-targeting siRNA (100 nM), PHB1 specific siRNA (100 nM), or PHB2 specific siRNA (100 nM), and treated with H_2O_2 (500 µM) for the indicated time points after 48 hr post-transfection with siRNA. Cell viability was determined by MTS assay. Values are the mean ± standard deviation of PHB1 and PHB2 siRNA treated percent viability normalized to control siRNA treated percent viability from three independent experiments.
Figure 4.5: PHB1 and PHB2 co-localize primarily to mitochondria of Kit225 during oxidative stress. Kit225 cells were treated with 500µM H₂O₂ for 0, 1, 3, 6, 12 and 24 hr. Cells were cytocentrifuged onto glass slides and subjected to analysis by immunofluorescent confocal microscopy. (A and A1) PHB subcellular localization was determined by staining the nucleus with DAPI; PHB1-Alexa488 and PHB2-Cy3 and merge. (B and B1) Nuclear staining with DAPI, PHB1-Alexa488, COX IV-Cy3 and overlay. (C) Nuclear staining with DAPI, cleaved PARP-Alexa488 and overlay were detected in Kit225 cells using immunofluorescent confocal microscopy. A1 and B1 represent zoomed views of their corresponding image.
4.3.6 PHB1 and PHB2 are overexpressed in tumor cells from patients diagnosed with lymphoid and myeloid malignancies

To further evaluate the expression of PHB1 and PHB2 in hematologic malignancies, Western blot analyses were performed on total cell lysates of 115 leukemia/lymphoma patient samples from El Paso Texas regional hospitals and compared with PBMCs purified from healthy donors. PHB1 and PHB2 band intensities were normalized to GAPDH using densitometric analysis and fold increase relative to the average expression in the normal PBMC donors was plotted for each sample (Figure 4.6 A and B). For this sample set, densitometry analysis indicated that 76% and 83% of tumor samples have a fold increase of one or more (>1 fold) in PHB1 and PHB2 expression respectively (C).

In other to better categorize our results, we select only new-onset hematopoietic malignancies and we evaluate the expression of PHB1 and PHB2 in these samples. Western blot analyses were performed on total cell lysates from PBMCs purified from healthy donors (n=5) and tumor cells obtained from individuals diagnosed with B-ALL (n=15), B-NHL (n=18), CML (n=10), T-ALL (n=5) and T-NHL (n=8). The membranes were stripped and reprobed for GAPDH levels to confirm equal loading. Densitometric analysis indicated that PHB1 and PHB2 were overexpressed (>1 fold) in 61% and 79% of tumor samples, respectively compared to normal donor PBMCs (Figure 4.7 A). The range of normalized prohibitin expression relative to normal PBMCs was 0.1 to 37.6 fold for PHB1 and 0.2 to 55.9 fold for PHB2 (Figure 4.7 B). The median fold increase in PHB1/PHB2 protein expression in the B-ALL, B-NHL, CML, T-ALL, T-NHL patient samples were 2.9/4.6, 3/5.7, 1.9/4.3, 2.3/2.8, and 6.4/4.1 respectively (Figure 4.7 B). Thus, concordant with prohibitin expression observed in tumor cell lines (Figure 4.1), PHB1 and PHB2
are overexpressed in lymphoid (T- and B-cell leukemia/lymphoma) and myeloid (CML) primary patient tumor cells.

4.3.7 PHB1 and PHB2 co-localize primarily to the mitochondria in primary hematologic tumor cells

To determine PHB1 and PHB2 localization in primary CML and T-ALL cells, dual labeled immunofluorescent confocal microscopy was utilized (Figure 4.8). Similar to prohibitin localization in normal PBMCs [67] and Kit225 cells (Figure 4.2), PHB1 and PHB2 co-localize primarily to peri-nuclear regions in CML (A) and T-ALL (B and B1). There were no detectable levels of PHB1 or PHB2 observed at the plasma membrane or in the nucleus. Subcellular localization was evaluated in a CML (Figure 4.8 C and C1) and T-ALL (D and D1) tumor samples using the inner mitochondrial membrane marker COX IV. Dual immunofluorescent confocal microscopy detecting PHB1 and COX IV in tandem with DAPI showed co-localization of PHB1 with COX IV (C, C1, D, and D1). These findings further support the role of the prohibitin complex in promoting hematologic tumor cell survival through mitochondrial stabilization.

4.3.8 Proteomic Identification of PHB1 and PHB2 interacting proteins in Kit225 cells

PHB1 and PHB2 have been reported to interact with a vast number of proteins, however these studies were primarily performed in adherent cell lines [2, 6, 55]. To gain insight into the prohibitins mechanism of action in T cells and during IL-2 induction, differentially expressed co-associating proteins were identified using co-immunoprecipitation and LC-MS/MS analysis. PHB2 was immunoprecipitated from IL-2 activated Kit225 cell lysate. SDS-PAGE separation of the prohibitin complex of proteins was subjected to Coomassie blue staining and the detected
proteins of interest were named for their corresponding relative molecular weights (kDa) (Figure 4.9). In total, ten proteins of interest were detected, including p175, p150, p130, p100, p70, p40, p35, p33, p30, and p28. The successful identification of all proteins was achieved by searching the NCBI database with the peptide sequences obtained from LC-MS/MS analysis using the SEQUEST algorithm (Table 4.1). The theoretical molecular weight (MW) of each identified protein was compared to the observed MW from the SDS-PAGE. Remarkably, Stress-70 protein (HSPA9) and transaldolase 1 (TALDO1) are mitochondrial proteins that function as negative regulator of apoptosis and as a transferase in the pentose-phosphate pathway respectively.
Figure 4.6: PHB1/PHB2 are overexpressed in tumor cells obtained from individuals diagnosed with hematopoietic malignancies. PBMCs were isolated from normal donors (N) and patients diagnosed hematopoietic malignancies (P). Cell lysates (10 µg) were separated by 12% SDS-PAGE and subjected to Western blot analysis with antibodies directed to PHB1, PHB2 and GAPDH (A). PHB1 and PHB2 band intensities were normalized to GAPDH using densitometric analysis and fold increase relative to the average expression in the normal PBMC donors plotted for each sample (B). Heat map depicting the relative fold expression of PHB1 and PHB2 across the different tumor cell types of 115 Leukemia/Lymphoma patient samples from El Paso Texas regional hospitals.
Figure 4.7: PHB1/PHB2 are overexpressed in tumor cells obtained from individuals diagnosed with lymphoid or myeloid malignancies. PBMCs were isolated from normal donors (n=5) and patients diagnosed with B-ALL (n=15), B-NHL (n=18), CML (n=10), T-ALL (n=5) and T-NHL (n=8). Cell lysates (10 µg) were separated by 12% SDS-PAGE and subjected to Western blot analysis with antibodies directed to PHB1, PHB2 and GAPDH. (A) PHB1 and PHB2 band intensities were normalized to GAPDH using densitometric analysis and fold increase relative to the average expression in the normal PBMC donors plotted for each sample as a heat map. (B) Box plot depicting the relative fold expression of PHB1 and PHB2 across the different tumor cell types with the median fold increase denoted in the table (dots represent outlier values).
Figure 4.8: PHB1/PHB2 co-localize to peri-nuclear regions and mitochondria in tumor cells obtained from patients diagnosed with hematologic malignancies. Samples were cytocentrifuged onto glass slides and subjected to analysis by immunofluorescent confocal microscopy. (A and B) PHB subcellular localization was determined by staining the nucleus with DAPI; PHB1-Cy2 and PHB2-Cy3 and overlay in a CML and ALL patient samples respectively. (C and D) Nuclear staining with DAPI, PHB2-Cy2, COX IV-Cy3, and overlay were detected in CML and ALL patient samples using immunofluorescent confocal microscopy. The 10 µm and 5 µm size marker is indicated. B1, C1 and D1 represent zoomed views of their corresponding image.
Figure 4.9: Immunoprecipitation of PHB2 co-associating proteins in IL-2 stimulated Kit225 cells. PHB2 immunoprecipitate from IL-2 stimulated Kit225 cells was separated by SDS-PAGE and subjected to Coomassie blue stain. The detected proteins of interest were excised, trypsin digested and resulting peptides sequenced by LC-MS/MS analysis. Arrows denote proteins of interest and Immunoglobulin G (IgG) heavy chain and light chain (LC).
Table 4.1: LC-MS/MS Identification of PHB2 co-immunoprecipitating proteins.

| Protein | Identification                  | Gene   | Theoretical MW (kDa) |
|---------|---------------------------------|--------|----------------------|
| P175    | Filamin                         | FLNA   | 281                  |
| P150    | Myosin-9                        | MYH9   | 226.5                |
| P130    | RAS protein activator like-3    | RASAL-3| 111.86               |
| P100    | α-Actinin-4                     | ACTN4  | 105.02               |
| P70     | Stress-70 protein               | HSPA9  | 75                   |
| P40     | γ-Actin                         | ACTG1  | 41.8                 |
| P35     | Transaldolase 1                 | TALDO1 | 37.4                 |
| P33     | Prohibitin 2                    | PHB2   | 33.3                 |
| P30     | Swiprosin-1                     | EFHD2  | 26.8                 |
| P28     | Prohibitin 1                    | PHB1   | 29.8                 |
4.4 DISCUSSION

Prohibitins have been proposed to play key roles in a variety of disease states, however their function in lymphocytes has not been clearly defined [57]. Evidence is provided herein that PHB1 and PHB2 are overexpressed in a panel of leukemia and lymphoma cell lines compared to normal naïve PBMCs (Figure 4.1). Likewise, primary tumor cells obtained from leukemia and lymphoma patients displayed similar levels of overexpressed prohibitins (Figure 4.6 and 4.7). Although prohibitins have been reported to localize to a variety of subcellular locations in tumor cells [2, 6], PHB1 and PHB2 were shown to co-localize mainly to mitochondria in Kit225 and leukemia/lymphoma patient tumor cells (Figure 4.2 and 4.8) [67]. Furthermore, PHB1 and PHB2 were upregulated upon oxidative stress-induced cell death, suggesting these proteins play a protective or anti-apoptotic function in lymphoid tumor cells (Figure 4.4). PHB1 and PHB2 knockdown significantly enhanced the susceptibility of Kit225 cells to ROS-induced cell death (Figure 4.4) but PHB1 and PHB2 knockout alone was not sufficient to induce apoptosis (Figure 4.3). Remarkably, prohibitins localize in mitochondria during oxidative stress (Figure 4.5). The mitochondrial chaperone action of PHB1 and PHB2 supports the hypothesis that prohibitins can play a protective function for cells undergoing a transformation.

Mitochondrial dysfunction is a hallmark of cellular transformation and increasing evidence supports its importance in human pathology. It is well established that cancer cells have an enhanced energy demands including an increase in glycolysis, glucose transport, gluconeogenesis and pentose phosphate pathway activity [128-130]. Considering the critical role of chaperones in the maintenance of mitochondrial integrity, it is reasonable to expect prohibitin overexpression is functionally linked to oncogenesis. Indeed, PHB1 and PHB2 protein levels were overexpressed in
tumor cells isolated from patients with leukemia and lymphoma compared to normal naïve PBMCs (Figure 4.6 and 4.7). Previous reports suggest PHB1 mRNA levels are inversely proportional to cellular proliferation in a number of cell types [46, 131, 132], however the results presented herein indicate PHB1 and PHB2 are upregulated during the oxidative stress response in hematologic tumor cells.

The regulation of prohibitins in response to various stimuli has been reported in a number of tumor cell types. Luan et al. established that PHB1 knockdown reverses the “epithelial-to-mesenchymal transition” phenotype in pancreatic cancer cell lines. It was also concluded that disrupting the Ras/Raf/MEK/ERK pathway by blocking prohibitin-cRaf interaction diminished the viability of pancreatic cancer cells in vitro, and inhibits their migration in vitro and in vivo [80]. Moreover, phosphorylation of PHB1 at T258 on the plasma membrane activates PI3K/AKT and the Ras/Raf/MEK/ERK pathways promoting proliferation and metastasis of cancer cells in lung and cervical adenocarcinoma cell lines [133]. Additionally, the oncoprotein c-Myc, whose activation and deregulation by chromosomal translocations is a major feature of certain leukemias and lymphomas [134], was shown to target the PHB1 gene by binding and inducing its transcription on a specific consensus sequence [135]. This interplay of oncogenic signaling molecules suggests that one mechanism of cellular transformation occurs through the upregulation of prohibitins to protect mitochondria during the increased bioenergetic requirements demanded by tumor cells. Similarly, interleukin 6 signaling through STAT3 was shown to control PHB1 expression in intestinal epithelial cells where it was shown to protect against oxidative stress [76]. The pathophysiological role of the JAK/STAT signaling pathway in hematologic malignancies is well established [135-137]. In conclusion, these observations indicate that PHB1 and PHB2 activity contribute to tumor cell survival in the context of mitochondrial protection and therefore
strengthen the potential value of these proteins as therapeutic targets in the treatment hematologic cancers.
CHAPTER 5

Overview and Future Directions
5.1 Overview

Lymphocyte proliferation and differentiation is coordinated with great precision in healthy humans and is vital to maintaining a normal immune system. Imbalance of this regulation can result in the development of autoimmune diseases, immunodeficiency, and hematopoietic malignancies. These pathologies in specific leukemia and lymphoma have a high incidence of relapse and mortality due to their limited treatment options. Therefore, there is a critical need to characterize the signal transduction pathways and molecular hallmarks that mediate T cell activation in order to develop new strategies for diagnosis and treatments of immunologic diseases.

The first objective of this dissertation was to identify IL-2 inducible phosphorylation sites of prohibitins. The strategy of large-scale immunoprecipitation of PHB2 followed by identification of phosphopeptides through LC-MS/MS revealed eight residues that are phosphorylated in PHB2, four of them were constitutively phosphorylated, and for the first time, PHB2 T21, T62, T155, and T169 were identified to become differentially phosphorylated in response to IL-2 induction (Figures 2.1-2.6 and Table 2.1). Moreover, T62 and T169 are phosphorylated residues that never have been reported before. The novel IL-2 inducible threonine 62 and 169 were chosen for further examination. Amino acid sequence alignment of the region surrounding each of the phosphosites from different organisms revealed conservation among different species.

In order to verify if PHB2 is phosphorylated at T62 and to investigate the regulatory role of this phosphorylation sites, phosphospecific polyclonal antibodies were generated. The strategy for characterization of the antibodies included ELISA, dot blots, peptide competition assays, Western blot of immunoprecipitated (PHB2 and anti-pT62) and immunofluorescent confocal microscopy. These results revealed that PHB2 anti-pT62 antisera preferentially recognize their phosphorylated peptide and are specifically blocked by phospho- but not non-phosphopeptide. In
addition, one of our antibodies **PHB2 and anti-pT62 (17632)** displayed specificity towards the **T62 phosphorylated form of PHB2** in Western blot analysis (immunoprecipitated) protein and in immunofluorescent confocal microscopy with variable degrees in reproducibility probably because the low rate of phosphorylation in prohibitins (Figures 3.1-3.6).

The second part of our study was initiated in an effort to gain insight into the prohibitin molecular mechanism of action in lymphoid and myeloid-derived malignancies, **PHB1 and PHB2 were determined to be overexpressed in tumor cell lines** compared to normal primary human PBMCs (Figure 4.1) and **localized primarily to the mitochondria** in Kit225 cells (Figure 4.2). **siRNA-mediated knockdown of PHB1 and PHB2 in Kit225 cells significantly enhanced their sensitivity to ROS-induced cell death**, suggesting a protective function in human hematologic tumor cells (Figure 4.4). These findings were substantiated by evidence demonstrating **PHB1 and PHB2 are upregulated during ROS-mediated apoptosis** (Figure 4.4). Furthermore, **PHB1 and PHB2 protein levels were significantly higher in tumor cells isolated from leukemia and lymphoma patients compared to healthy individual PBMCs** (Figure 4.6 and 4.7) and **localized to primarily to the mitochondria** (Figure 4.8). Taken together, these findings support the role of PHB1 and PHB2 in hematologic tumor cells for maintenance of mitochondrial integrity, which may facilitate the energy requirements of these tumor cells.

In conclusion, we were able to identify novel phosphosites in PHB2, generated and characterized a polyclonal antibody for a specific site. In addition, evidence is provided that PHB1 and PHB2 are upregulated during tumorigenesis to possibly maintain mitochondrial integrity and therefore may serve as novel biomarkers and molecular targets for therapeutic intervention in certain types of hematologic malignancies.
5.2 **Future Directions**

5.2.1 **Prohibitins as a therapeutic target**

The cellular localization and expression of prohibitins is a determinant of their function, especially in oncogenic cells. Hence, these proteins could serve as potential therapeutic targets. Treatments with the recently described small molecule inhibitor fluorizoline which has been shown to interact with prohibitins and cause apoptosis or the natural compound rocaglamide which disrupts their interaction with c-Raf could result in selective toxicity towards leukemia/lymphoma cell lines or patient samples [103, 105, 138]. The cellular pathways altered during treatment with these drugs could be evaluated, as well their synergistic effect with other chemotherapeutic agents or inhibitors. Also, we can assess the hypothesis that knockdown of prohibitins in primary cancer cells leads to increased susceptibility to chemotherapeutic agents.

5.2.2 **Investigate the functional role of T62 phosphorylation on signal transduction pathways.**

Regulation of T62 phosphorylation has yet to be explored. Determine the kinases and phosphatases involved in mediating pT62 phosphorylation/dephosphorylation events with IL-2 induction in order to identify the mechanism behind this phenomenon. Likewise, we could investigate these events with the induction of different cytokines and growth factors such as IL-6, CD3/CD28 or PHA. Other human lymphoid and myeloid tumor cell lines, as well as other tissues like breast or prostate cancer, could be used for this purpose.

5.2.3 **Determine involvement of T62 phosphorylation in disease**

It has been demonstrated that aberrant phosphorylation of proteins serves a crucial role in numerous cancers, including leukemia and lymphoma. Therefore, PHB2 T62 phosphorylation
status could be explored in leukemia and lymphoma patient samples as well as other malignancies. Whole cell lysates from these samples could be evaluated for phosphorylation of T62 by Western blot and immunofluorescence using our phosphospecific antibody.

5.2.4. Evaluate the Relation of Prohibitins co-associated Proteins

Prohibitins have been reported to interact with a considerable number of proteins, however the co-associated proteins during IL-2 stimulation have never been reported. We identified prohibitins co-associating proteins that were differentially expressed during IL-2 induction by co-immunoprecipitation and LC-MS/MS analysis (Figure 4.9 and Table 4.1). Interestingly, Stress-70 protein (HSPA9) and transaldolase 1 (TALDO1) are mitochondrial proteins that function as negative regulator of apoptosis and as a transferase in the pentose-phosphate pathway respectively. Moreover, these two proteins have never been reported to interact with prohibitins. We can investigate the expression of these proteins after prohibitin knockdown and viceversa.
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Vita

Elisa Robles-Escajeda earned her medical degree from the Universidad Autónoma de Ciudad Juárez. After years working in private practice, she decided to join The University of Texas at El Paso (UTEP) and earned her Bachelor of Science degree in Microbiology in 2011. She then continued her education by enrolling in the Pathobiology Doctoral Program within the Department of Biological Sciences at UTEP with an emphasis on cancer research.

While pursuing her degree, Dr. Robles was the recipient of numerous honors and awards. She was part of The RISE (Research Initiatives for Scientific Enhancement) Graduate Scholars Program and received various travel awards to present her research findings at national and international conferences including The American Association of Immunologists annual meeting in Seattle, WA, The American Society for Cell Biology conference in San Francisco, CA, and Denver, CO, and the Research Centers in Minority Institutions International Symposium on Health Disparities, in San Juan, Puerto Rico. She also received The Institute on Teaching and Mentoring Travel Award for five consecutive years allowing her to travel to Atlanta, GA, Tampa, FL, and Washington, DC, to broaden her knowledge about research, academia, and succeed as a member of the professoriate.

Dr. Robles worked as a research assistant in the Department of Biological Sciences and published several peer-reviewed scientific articles. Aside from her academic responsibilities, she enjoys mentoring students in translational medicine and exposing them to the many opportunities offered within the field of biomedical research.

Dr. Robles’s dissertation “The Prohibitin Protein Complex Promotes Mitochondrial Stabilization and Cell Survival in Hematologic Malignancies” was performed under the supervision of Dr. Robert A. Kirken. The findings of her dissertation were submitted for publication in a peer-reviewed scientific journal. Elisa’s current plan is to continue her exciting career in cancer research as a post-doctoral research fellow at UTEP.
