CELLULAR LOCALIZATION OF PHLDA1 CONTRIBUTES TO APOPTOSIS

THE FUNCTIONAL DOMAINS OF PHLDA1: MODULATION OF INTRACELLULAR LOCALIZATION IMPACTS APOPTOTIC CELL DEATH

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A Thesis

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ABSTRACT

Pleckstrin homology like domain family A, member 1 (PHLDA1) is a member of the PHLDA family of homologous proteins recognized for their role in apoptotic cell death. PHLDA1 was first reported as a proapoptotic factor involved in Fas-mediated T-cell apoptosis. The role of this protein with regards to apoptosis remains poorly understood, with literature demonstrating both proapoptotic and antiapoptotic functions in a cell and/or pathway specific manner. Intracellular localization may account for the apoptotic potential of this protein, with nuclear accumulation of PHLDA1 increasing its apoptotic potential. We hypothesize that the functional regions of PHLDA1 including its localization signals (pNLS/pNES), pleckstrin homology like domain (PHLD), and PQ region direct cellular localization of PHLDA1, thereby regulating its apoptotic potential.

In this thesis, well-established molecular and cellular approaches were utilized to better define the functional regions within PHLDA1 and to gain further understanding of the role of its localization on apoptosis. Using an EGFP fusion construct and leptomycin B, we confirmed that PHLDA1 contains a weak, CRM1-responsive NES. Using an EGFP- β -galactosidase fusion protein we examined the putative NLS of PHLDA1 and determined that it was not sufficient to direct nuclear localization. However, the PHLD was found to direct cellular localization, mirroring the distribution and punctate patterning of full length PHLDA1. Evidence of association of the PHLD with the membrane was confirmed using fluorescence and electron microscopy, and changes in cell

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morphology indicative of EMT were apparent following overexpression of the PHLD.

Although previous reports have suggested that the PQ region of PHLDA1 is responsible for its proapoptotic function, its cellular localization was not clearly defined. Nuclear accumulation of the PQ region was found to be highly cytotoxic, indicating that it is sufficient to induce apoptosis and that its proapoptotic activity occurs within the nucleus. The findings of this thesis provide fresh insight into the functional regions of PHLDA1 and their respective contributions to the protein's intracellular localization and apoptotic function, demonstrating that localization dictates the apoptotic potential of PHLDA1. This data provides a solid foundation for identifying the cellular mechanisms by which PHLDA1 influences the progression of chronic human diseases including diabetes, cancer and obesity.

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LIST OF ABBREVIATIONS

aa	Amino acid
AICD	Activation induced cell death
АроЕ	Apolipoprotein E
Bg	Background
DAPI	4',6-diamidino-2-phenylindole
DTT	1,4-dithiothreitol
EGFP	Enhanced green fluorescent protein
EM	Electron microscopy
EMT	Epithelial-to-mesenchymal transition
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
GRP78	Glucose-regulated protein, 78 kDa
HK-2	Human proximal tubule kidney cells
HMM	Hidden Markov model
HRP	Horseradish Peroxidase
HUVEC	Human umbilical vein endothelial cells
IGF	Insulin growth factor
LMB	Leptomycin B
MHC	Major histocompatability complex
MEF	Mouse embryonic fibroblasts
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

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PH	Proline-histidine repeat region
PHLD	Pleckstrin homology like domain
PHLDA	Pleckstrin homology like domain family A
PHLDA1	Pleckstrin homology like domain family A, member
PHLDA2	Pleckstrin homology like domain family A, member
PHLDA3	Pleckstrin homology like domain family A, member
PI3 Kinase	Phosphoinositide-3 kinase
PIP	Phosphatidylinositol phosphate
PIKE	Phosphoinositide-3 kinase enhancer
РКС	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
pNES	Putative nuclear export signal
pNLS	Putative nuclear localization signal
cNLS	Classic nuclear localization signal
PKI	Protein kinase A inhibitor
PolyQ (pQ)	Polyglutamine rich linker region
PQ	Proline-glutamine repeat region
Qdot	Quantum dot
rpm	Revolutions per minute
SEM	Standard Error of the mean
SV 40	Simian Virus 40 Large T antigen
TCR	T cell receptor

TDAG51	T-cell death associated gene 51
TEM	Transmission electron microscope
Tg	Thapsigargin
TUNEL	Terminal deoxynucleotidyl transferase mediated dUTP nick end labelling
UPR	Unfolded protein response
-/-	Knockout

DECLARATION OF ACADEMIC ACHIEVEMENT

All research and experimentation in this thesis (Figures 1-25 and Appendices 1-8) was completed by Celeste AF Collins. Electron microscopy fixation, embedding, staining and imaging for immunogold sections was completed with the assistance of Dr. Sărka Lohták (Figures 19-21).

<u>1. INTRODUCTION</u>

1.1 PHLDA1 (TDAG51) an Introduction

T-cell death associated gene 51 (TDAG51) was first identified in a 1996 article by Park *et al.* Initially named based on its detection in mouse T-cell hybridomas and its proapoptotic role, homologues have since been identified in humans and rats (Park *et al.*, 1996; Gomes *et al.*, 1999; Hinz *et al.*, 2001). The human homologue of TDAG51 is now commonly referred to as PHLDA1 (Pleckstrin homology like domain family A, member 1) due to its membership in a unique subclass of pleckstrin homology related proteins (Frank *et al.*, 1999; Neef *et al.*, 2002). The role of TDAG51 and PHLDA1 remains unclear as both proapoptotic and antiapoptotic roles for the protein have emerged.

1.2 Profile of TDAG51 and PHLDA1

1.2.1 Tissue Expression and Cellular Localization

Human and mouse homologues of PHLDA1 share 89.4% amino acid sequence homology but differ significantly in terms of their tissue expression profiles. In adult mice, TDAG51 mRNA expression is highest in the lung and liver with lower levels of expression in the brain, heart, spleen, thymus and white adipose tissue (Frank *et al.*, 1999; Basseri *et al.*, 2013). PHLDA1 mRNA and protein expression is less extensively documented but endogenous expression has been observed in neurons and T-cells. Northern blot analysis of human tissue samples revealed high levels of PHLDA1 in the lung and pancreas with lower levels in the brain, heart, placenta, liver and kidney (Hossain *et al.*, 2003). The

Human Protein Atlas has profiled PHLDA1 protein expression in a variety of human tissues using PHLDA1 monoclonal and polyclonal antibodies (Uhlen *et al.*, 2005; Uhlen *et al.*, 2010). This database suggests that the highest levels of PHLDA1 tissue expression are found in neuronal cells, breast, and tonsil with moderate expression in the kidney, gastrointestinal tract and thyroid (Uhlen *et al.*, 2010).

Within human cell lines, cellular localization of PHLDA1 varies. PHLDA1 localized to the cytoplasm and nucleoli of transfected human T cells (Hinz *et al.*, 2001). However in metastatic melanoma, strong cytoplasmic staining of PHLDA1 was observed (Neef *et al.*, 2002). In human umbilical vein endothelial cells (HUVECs), PHLDA1 localized to the perinuclear area and cell periphery with an increase in perinuclear localization upon treatment with homocysteine (Hossain *et al.*, 2003). The contributions of cellular localization to PHLDA1's pro- and antiapoptotic phenotype have not been extensively documented.

1.2.2 PHLDA1, TDAG51 and Apoptosis

Apoptosis is the healthy, regulatory process of programmed cell death associated with tissue homeostasis. Apoptosis is triggered by both extrinsic and intrinsic signalling pathways, occurring in response to numerous cellular stressors (Guadamillas *et al.*, 2011; Park *et al.*, 2013). A myriad of proteins and second messengers are involved in activation of apoptosis and these components are referred to as proapoptotic factors. Aberrations in apoptotic signalling are

associated with the development of human diseases ranging from inflammation and Alzheimer's to atherosclerosis and cancer (Favaloro *et al.,* 2012).

TDAG51 and PHLDA1 are most frequently characterized as proapoptotic genes, however, conflicting evidence exists throughout the literature, suggesting that PHLDA1 and TDAG51 may play a pro- or antiapoptotic role in a context dependent manner (Wang *et al.*, 1998; Toyoshima *et al.*, 2004; Hossain *et al.*, 2003). PHLDA1 and TDAG51 have been implicated with both intrinsic and extrinsic mechanisms of apoptosis. Overexpression of PHLDA1 was found to be proapoptotic in T-cells, neuronal cells and fibroblasts (Park *et al.*, 1996; Gomes *et al.*, 1998; Wang *et al.*, 1998; Hossain *et al.*, 2003) and in response to various cellular stressors including heat shock and endoplasmic reticulum (ER) stress (Hayashida *et al.*, 2006; Hossain *et al.*, 2003). However, in a 2006 article by Toyoshima *et al.*, TDAG51 was found to play an important role in the anti-apoptotic effects of insulin-like growth factor 1, protecting fibroblasts from apoptosis in response to serum starvation. Recently, TDAG51 has been shown to be protective against reactive oxygen stress in mouse embryonic fibroblasts (Park *et al.*, 2013).

A role for TDAG51 and PHLDA1 in programmed cell death exists, however the apoptotic nature of this protein remains subject to dispute.

TDAG51 and Activation Induced Cell Death

Activation induced cell death (AICD) is a normal self-regulating process for T-cell lymphocytes, modulating population expansion throughout development and following an immune response. AICD is an extrinsic mechanism of apoptosis, with activation of T-cells occuring upon binding of a major histocompatibility complex (MHC) containing peptide to the extracellular region of the T-cell receptor (TCR) (Brownlie and Zamoyska, 2013). Although the TCR complex is enzymatically inert, downstream signalling is initiated by interaction with SRC kinase family members LCK and FYN (Akimzhanov *et al.*, 2010). TCR activation promotes proliferation and differentiation of naïve T-cells into mature T-cells, however, under specific conditions activation of the TCR will initiate AICD (Akimzhanov *et al.*, 2010).

TCR activation is the initiating event for AICD in T-cells, with repeated activation of the complex increasing the cell's susceptibility to Fas-mediated apoptosis (Krammer, 2000; Inaba *et al.*, 1999). Prolonged stimulation of the TCR triggers the AICD pathway within 24 to 48 hours (Akimzhanov *et al.*, 2010).

Upregulation of TDAG51 is associated with AICD, as initially demonstrated by Park *et al.* (1996) showing a T-cell hybridoma population resistant to TCR initiated AICD. This T-cell population was found to be deficient in TDAG51 expression, implicating the protein in the TCR cell death pathway and first exploring the relationship between TDAG51 and the Fas pathway (Park

et al., 1996). Stimulation of the Fas-mediated apoptotic pathway in T-cells has been achieved through direct activation of the TCR using antibodies or through stimulation of protein kinase C (PKC) using ionomycin and phorbol myristate acetate (PMA) (Park *et al.,* 1996; Wang *et al.,* 1998).

Although upregulation of TDAG51 occurs in conjunction with TCR activation, its contribution to this apoptotic signalling pathway remains obscure. It was first postulated that TDAG51 was involved in transcriptional regulation of Fas mRNA expression and that TDAG51 played a pivotal role in linking TCR response to Fas-FasL apoptosis (Park *et al.*, 1996). However TDAG51 knockout murine T-cells showed no defects in Fas signalling, ruling out TDAG51 as an essential component of AICD (Rho *et al.*, 2001). Ultimately, TDAG51 was found to be neither necessary nor sufficient for TCR AICD in murine T-cells, with activated phospholipase C γ 1 (PLC- γ 1) claiming the role of translator between TCR activation to Fas signalling (Rho *et al.*, 2001; Oberg *et al.*, 2004; Akimzhanov *et al.*, 2010).

TDAG51 and ER Stress

The role of TDAG51 and ER stress was first postulated by Hossain *et al.* (2003) through study of homocysteine-induced programmed cell death. Homocysteine is known to disrupt protein folding in the ER of cells, causing ER stress and activating the unfolded protein response (UPR) (Kokame *et al.*, 1996; Outinen *et al.*, 1999; Kokame *et al.*, 2000, Szegezdi *et al.*, 2006). Induction of

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TDAG51 during homocysteine-induced ER stress was observed in mouse embryonic fibroblasts (MEFs), with similar induction of PHLDA1 apparent in homocysteine treated HUVECs (Hossain *et al.*, 2003). This induction occurred in tandem with the known ER chaperone glucose-regulated protein 78 (GRP78) in a time- and concentration-dependent manner (Hossain *et al.*, 2003). Treatment with tunicamycin, an established ER stress inducing agent, also lead to the upregulation of PHLDA1 mRNA and protein in MEFs, reaffirming the association of TDAG51 and PHLDA1 with the ER stress response pathway (Hossain *et al.*, 2003).

ER stress and apoptosis are closely linked, with cells initiating programmed cell death under conditions of prolonged ER stress and UPR activation (Szegezdi *et al.*, 2006). TDAG51 and PHLDA1 have gained recognition as markers of UPR activation and ER stress in the literature, however, their role in ER stress and subsequent cell fate determination remains unknown (Zhou *et al.*, 2005; Carlisle *et al.*, 2012). Upregulation of PHLDA1 may play a key role in cellular commitment to apoptosis following prolonged UPR activation.

1.2.3 Role in Disease

Dysregulation of PHLDA1 and TDAG51 expression has been associated with numerous disease states including atherosclerosis, obesity, diabetes and various cancers (Hossain *et al.*, 2003; Basseri *et al.*, 2013; Chiu *et al.*, 2005; Marchiori *et al.*, 2008; Nagai *et al.*, 2007).

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TDAG51 and Atherosclerosis

Elevated levels of homocysteine can lead to a host of pathologies in humans including pregnancy complications, cognitive impairments and cardiovascular disease (Kim *et al.*, 2012; Bergen *et al.*, 2012; Garcia and Zanibbi, 2004; Refsum *et al.*, 1998). In humans, homocysteine is not obtained from diet but is synthesized from methionine (Refsum *et al.*, 1998). Key vitamins B6, B12 and folic acid catalyze reactions within the homocysteine metabolism pathways, and deficiency leads to an accumulation of homocysteine in the plasma (Refsum *et al.*, 1998). Atherosclerosis, the hardening of the arteries, is often an initiating event for cardiovascular disease and high levels of plasma homocysteine (hyperhomocysteinemia) have been linked to accelerate atherosclerotic progression in the carotid, coronary and peripheral arteries (Zhou *et al.*, 2004; Refsum *et al.*, 1998).

Hyperhomocysteinemia may act through the ER stress pathway, as upregulation of ER stress markers GRP78, GRP94 and PERK has been observed in the lesions of apolipoprotein E knockout (ApoE^{-/-}) mice (Zhou and Tabas, 2013; Zhou *et al.*, 2004). Upregulation of TDAG51 has also been linked to homocysteine-induced atherosclerosis in ApoE^{-/-} mice, conforming with its suspected role as an ER stress response gene (Zhou and Tabas, 2013; Hossain *et al.*, 2003). Intense TDAG51 protein staining was evident in the lesions of the aortic root of ApoE^{-/-} mice on a 4-week high-homocysteine diet, with prominent co-localization to necrotic areas within these atherosclerotic lesions (Hossain *et al.*, 2003).

al., 2003). Furthermore, Hossain *et al.* (2013) have now shown a significant reduction in lesion size and necrotic areas in TDAG51/ApoE double knockout mice relative to ApoE^{-/-} mice alone.

Altogether, this data suggests that TDAG51 contributes to lesion development during atherosclerosis. Atherosclerosis stems from a chronic inflammatory response, which triggers ER stress in lesion-resident cells involved in the arterial microenvironment, including smooth muscle cells, endothelial cells and macrophages (Zhou and Tabas, 2013). Activation of TDAG51 through UPR activation may exacerbate the disease by increasing the rate of cell death within lesions and by contributing to the formation and expansion of necrotic cores (Hossain *et al.*, 2013).

TDAG51 and Obesity

Obesity results from pathological expansion of white adipose tissue through adipocyte hyperplasia and hypertrophy (Basseri *et al.*, 2013). Not only is ER stress activated under conditions of obesity, but studies have shown that TDA51 may play an important role in the development of obesity through activity in both white adipose tissue and the liver.

A recent study using TDAG51 knockout mice suggests a regulatory role for TDAG51 in adipocytes, with significant adipocyte lipid accumulation occurring in its absence (Basseri *et al.*, 2013). TDAG51 is expressed in white

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adipose tissue in healthy wild type mice, but its function in these cells remains poorly understood (Basseri *et al.*, 2013). TDAG51 knockout mice are obese relative to their wildtype controls despite similar food intake levels, likely resultant from their observed decreased metabolic rate and insulin dysregulation (Basseri *et al.*, 2013). These mice also exhibit an age-dependent increase in insulin resistance and development of an overall metabolic syndrome profile on a standard chow diet (Basseri *et al.*, 2013).

In mice, TDAG51 expression is highest in the liver and TDAG51 knockouts develop hepatic steatosis by 28-weeks of age (Basseri *et al.*, 2013). Although TDAG51 has been linked to the PERK/eIF2 α pathway, suppression of the eIF2 α pathway leads to an inverse of this profile in the liver (Scheuner *et al.*, 2001). Transgenic mice overexpressing GADD34, an eIF2 α -specific phosphatase, showed significant impairment in the eIF2 α pathway which lead to reduced body mass and a reduction in hepatic steatosis in mice on a high fat diet (Oyadomari *et al.*, 2008). These mice had dramatically reduced hepatic glucose production and were susceptible to hypoglycemia during a fast (Oyadomari *et al.*, 2008). In another set of studies, mice with a homozygous eIF2 α mutation, which prevents its phosphorylation, lacked hepatic glucose production, but mice heterozygous for the mutation were obese and insulin resistant, a profile more closely resembling TDAG51 knockout mice (Scheuner *et al.*, 2001; Scheuner *et al.*, 2005).

PHLDA1 and Cancer

The one body of literature where PHLDA1 has been predominantly studied is cancer. PHLDA1 has been implicated with breast cancer, lung carcinoma, colon cancer, basal cell carcinoma and renal cell carcinoma (Chiu *et al.*, 2005; Nagai *et al.*, 2007; Joo *et al.*, 2007; Oberst *et al.*, 2008; Tavares *et al.*, 2008). The role of PHLDA1 in cancer is not yet fully understood but often relates to its proapoptotic properties. In later stage cancers, particularly metastatic cancers, there is a trend towards the downregulation of PHLDA1. This however is not an absolute. As seen in a study on colorectal cancer, upregulation of PHLDA1 has been observed in some human colon cancer samples and was linked to increased anaemia in these patients (Chiu *et al.*, 2005).

The relationship between PHLDA1 and cancer metastasis was reported by Neef *et al* (2002). Using mRNA differential display between primary melanoma and lymph node metastasis from the same patients, they identified 9 differentially regulated genes, of which PHLDA1 was one (Neef *et al.*, 2002). PHLDA1 was found to be preferentially downregulated in metastatic melanoma relative to primary nodular controls. Experiments using a cultured melanoma cell line (Mel Rif) linked PHLDA1 overexpression to reduced cell growth and increased cell death (Neef *et al.*, 2002). It was thereby proposed that downregulation of PHLDA1 may be an important step in metastasis of malignant melanoma cancer. Although downregulation of PHLDA1 correlates positively with cancer metastasis, it has not yet been determined whether PHLDA1

downregulation promotes metastasis or if PHLDA1 downregulation occurs as a consequence of metastatic changes in the cell.

In breast cancer, downregulation of PHLDA1 has been identified as a strong predictor of poor prognosis (Nagai *et al.*, 2007). Notably, the discovery that PHLDA1 is regulated by the serine/threonine kinase Aurora A seemed a promising link to the role of PHLDA1 in breast cancer progression (Johnson *et al.*, 2011). The negative relationship between Aurora A kinase and breast cancer has been well characterized, and has been used to predict the likelihood of breast cancer recurrence in patients (Ferchichi et al., 2013; Johnson et al., 2011; Cronin et al., 2007). Overexpression of Aurora A kinase, linked to increased invasiveness and decreased survival outcomes in breast cancer patients, was found to regulate PHLDA1 protein expression, promoting its degradation through phosphorylation at serine 98 (Johnson *et al.*, 2011; Nadler *et al.*, 2008). However, challenges with nomenclature of this gene have since obscured these findings as PHLDA1 does not contain a serine 98 or serine 78. The serines analyzed in the paper were tracked to the upstream, non-coding region of TDAG51, which authors referred to as (murine) PHLDA1 (Johnson *et al.*, 2011).

Lastly, PHLDA1 is now used as follicular stem cell marker, unique in its ability to distinguish between basal cell carcinoma and trichoepithelioma (Yeh *et al.,* 2012; Sellheyer and Nelson, 2011; Sellheyer *et al.,* 2013). Although imperfect, when used in addition to conventional histomorphological inspection PHLDA1

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staining has proven to be a valuable immunohistological tool with 88% of trichoepitheliomal tumours staining positive for PHLDA1 relative to 12% of basal cell carcinomas (Yeh *et al.*, 2012). Despite interest in the contributions of PHLDA1 to a range of physiological cancers, a limited understanding of the molecular interactions within the cell remains. Correlation between PHLDA1 and cancer has been documented but the implications of these observation remain undetermined.

1.3 PHLDA1 and TDAG51: A Comparison

Although both proteins are homologous in terms of primary structure, sharing 89% sequence homology, it is premature to conclude that TDAG51 and PHLDA1 have a shared intracellular function. Characterization of PHLDA1 has lagged behind TDAG51 for over a decade with most biochemical and protein interaction data studies conducted in mice. Cancer is the one field where study of PHLDA1 has excelled, with particular attention to breast cancer and as a follicular stem cell marker for basal cell carcinoma. Despite critical analysis of TDAG51 and PHLDA1's upstream activation pathways, identification of downstream effector molecules of their activation is notably absent. A limited understanding of TDAG51 and PHLDA1's binding partners and molecular interactions currently exists and a crystal structure of either protein has not yet been resolved. This study focuses exclusively on PHLDA1 and study of its functional role *in vitro* in human cell lines.

1.4 PHLDA1 Protein

The human PHLDA1 protein consists of 259 amino acids and contains: a pleckstrin homology-like domain (PHLD) separated by a glutamine rich linker (Poly Q), a proline-glutamine (PQ) rich repeat region and a C-terminal prolinehistidine (PH) rich repeat (Figure 1 A). TDAG51 mRNA comprises 1955 nucleotides containing two plausible start sequences, one at 297 the other at 439 (Park *et al.*, 1996; Gomes *et al.*, 1999). The start sequence at 439 was found to correspond with the full length 44 kDa PHLDA1 protein (Gomes *et al.*, 1999; Meier-Noorden *et al.*, 2004). The promoter region of PHLDA1 lies immediately upstream of the translated sequence and to date has only been loosely defined (Frank *et al.*, 1999).

1.5 PHLDA Protein Family

In 1999, Frank *et al.* (1999) classified TDAG51 as a member of the pleckstrin homology-like domain family A (PHLDA). TDAG51 is the first protein member of this family and was named accordingly – pleckstrin homology-like domain family A, member 1 or PHLDA1. In current literature, PHLDA1 is used exclusively to describe the human homologue of TDAG51 while conventional naming (TDAG51) is still used when referencing mouse or rat homologues.

Figure 1. PHLDA1 and the PHLDA family

(A) Protein schematic of human PHLDA1 (Pleckstrin Homology Like Domain family A, member 1). PHLDA1 consists of 259 amino acids with a singular functional pleckstrin homology-like domain (PHLD) (orange) spanning 133 amino acids. PHLDA1 contains three additional regions of interest: glutamine rich linker (Poly Q) (grey), which divides the PHLD in two, a proline-glutamine (PQ) rich repeat (yellow) and a C-terminal proline-histidine (PH) rich repeat (blue). (B) The amino acid coding sequence of PHLDA1 with corresponding regions underlined. (C) Protein schematic of all members of the PHLDA family as adapted from Frank *et al.*, 1999. The PHLDA family consists of three members: PHLDA1 (TDAG51), PHLDA2 (Ipl/Tssc3) and PHLDA3 (Tih1) which share a conserved PHLD. PHLDA3 is the smallest family member, consisting almost exclusively of a PHLD domain.



The PHLDA family consists of three members: PHLDA1 (TDAG51), PHLDA2 (Ipl/Tssc3), and PHLDA3 (Tih1) that were grouped based on a high consensus sequence homology within their PHLDs (Figure 1 C, 2). With exclusion of the poly Q region, the PHLD region of PHLDA1 shares 52% and 60% sequence homology with PHLDA2 and PHLDA3 respectively. This is a unique feature of this family, as sequence conservation is not expected within pleckstrin homology domains. Both PHLDA2 and PHLDA3 bind phosphoinositol phosphates (PIP) with moderate affinity through their PHLDs (Saxena *et al.*, 2002). Although several of the key PIP binding residues are conserved across the PHLDA family, PIP binding has not been confirmed *in vitro* with the PHLD of PHLDA1 (Saxena *et al.*, 2002). PHLDA family members also share two conserved tryptophan residues in their PHLD (Frank *et al.*, 1999).

1.5.1 PHLDA2

PHLDA2 (Ipl/Tssc3) is a maternal imprinting gene that was first identified in humans by Qian *et al.* (1997) while searching for novel imprinting genes on chromosome 11 (Qian *et al.*, 1997). PHLDA2 was first named the Imprinted in placenta and liver (Ipl) gene based on its tissue specific expression in the placenta and liver (Qian *et al.*, 1997). *PHLDA2* lies in a chromosomal region rich in imprinting and tumor suppressing genes, and lies between two known imprinting genes $p57^{kip2}$ and hNAP2 (Hu *et al.*, 1997; Lee and Feinberg, 1998).

Figure 2. PHLDA family amino acid sequence analysis

(A) The amino acid coding sequences of all three members of the PHLDA family. With exclusion of the poly-Q region, the PHLD region of PHLDA1 shares 52% and 60% sequence homology with PHLDA2 and PHLDA3 respectively. (B) PHLDA Family Sequence Logo. The amino acid sequences of PHLDA1, PHLDA2 and PHLDA3 were aligned using Clustal Omega multiple sequence alignment prior to generation of the sequence logo using WebLogo 3.3. The degree of conservation is indicated in bits and is represented by the height and width of each amino acid. Key residues have been colour coded : tryptophan (W) (red), cysteine (C) (yellow), leucine (L) (blue), glutamic acid (E) (green) and lysine (K) (purple).

PHLDA Family Amino Acid Sequence PHLDA1 (259 aa)

MLESSGCKAL KEGVLEKRSD GLLQLWKKKC CILTEEGLLL IPPKQLQHQQ QQQQQQQQ QQPGQGPAEP SQPSGPAVAS LEPPVKLKEL HFSNMKTVDC VERKGKYMYF TVVMAEGKEI DFRCPQDQGW NAEITLQMVQ YKNRQAILAV KSTRQKQQHL VQQQPPSQPQ PQPQLQPQPQ PQPQPQPQPQ SQPQPQPQPK PQPQQLHPYP HPHPHPHSHP HSHPHPHPHP HPHQIPHPHP QPHSQPHGHR LLRSTSNSA

PHLDA 2 (152 aa)

MKSPDEVLRE GELEKRSDSL FQLWKKKRGV LTSDRLSLFP ASPRARPKEL RFHSILKVDC VERTGKYVYF TIVTTDHKEI DFRCAGESCW NAAIALALID FQNRRALQDF RSRQERTAPA APAEDAVAAA AAAPSEPSEP SRPSPQPKPR TP

PHLDA 3 (127 aa)

MTAAATATVL KEGVLEKRSG GLLQLWKRKR CVLTERGLQL FEAKGTGGRP KELSFARIKA VECVESTGRH IYFTLVTEGG GEIDFRCPLE DPGWNAQITL GLVKFKNQQA IQTVRARQSL GTGTLVS



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PHLDA2 is also referred to as Tssc3 (tumor-suppressing STF cDNA3) in literature, based on its classification as a tumor suppressor gene. Studies have shown that loss of PHLDA2 in mice results in placental overgrowth, and the protein is markedly absent from Wilms' tumors, which arise from developmental defects in the embryonic kidney (Frank *et al.*, 2002; Takao *et al.*, 2012; Lee and Feinberg, 1998).

PHLDA2 exhibits similar intracellular properties to its PHLDA1 family member (Figure 1 C, 2). Both PHLDA1 and PHLDA2 are cytoplasmic proteins implicated in cellular apoptosis (Takao *et al.*, 2012; Hossain *et al.*, 2003). PHLDA2 has been implicated in Fas mediated-apoptosis, with co-expression of PHLDA2 with Fas reported in fetal kidneys (Lee and Feinberg, 1998). The PHLD of PHLDA2 is known to recruit the protein to the cell membrane through interaction with phosphoinositol phosphate lipids (Takao *et al.*, 2012).

PHLDA2 has a conflicting relationship with Akt (protein kinase B). Both PHLDA2 and Akt compete for binding of PIPs at the membrane, yet upregulation of PHLDA2 leads to increased Akt phosphorylation. Recently, Takao *et al.* (2012) have demonstrated that PHLDA2 induces embryonic differentiation through the PI3 Kinase-Akt pathway and nuclear translocation of the transcription factor specificity protein 1.
1.5.2 PHLDA3

PHLDA3 or Tih1 (TDAG51 Ipl homologous protein 1) is a p53-inducible stress response gene and repressor of Akt. The *PHLDA3* gene is located on chromosome 1 in humans and its promoter contains two consensus p53 response elements – RRRCWWGYYY (Frank *et al.*, 1999). PHLDA3 is the smallest of the PHLDA family at 13.7 kDa consisting almost exclusively of a PH domain (Figure 1 C) (Frank *et al.*, 1999).

PHLDA3 expression is dependent upon p53 activation, and is correspondingly induced by activation of the DNA-damage stress response pathway through exposure to ultraviolet light and gamma radiation (Kawase *et al.*, 2009). PHLDA3 and PHLDA2 are structurally and functionally similar proteins with 53% sequence conservation in their 100 aa PH domains. While PHLDA2 expression is limited to select tissues, PHLDA3 is expressed almost ubiquitously in adult and fetal tissues with high concentrations found in both skeletal tissue and lungs (Frank *et al.*, 1999).

Like PHLDA1, PHLDA3 is proapoptotic in nature, with *in vitro* overexpression of the protein linked to an increase in cell death (Kawase *et al.*, 2009). Much like its sister protein PHLDA2, PHLDA3 acts through the Akt pathway, competing with Akt for binding to PIPs (Kawase *et al.*, 2009; Liao and Hung, 2010). PHLDA3 expression is strongly correlated with a decrease in Akt phosphorylation and inhibition of the PI3K/Akt pathway (Kawase *et al.*, 2009).

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1.6 The Pleckstrin Homology-Like Domain of PHLDA1

Pleckstrin homology domains are distinct from most other domains in that they are identified by conserved secondary structural motifs rather than by amino acid sequence (Lemmon and Ferguson, 2000). A classic pleckstrin homology domain consists of a seven beta sheet, single alpha helix motif with a conserved tertiary conformation (Lemmon and Ferguson, 2000; Lemmon, 2005) (Figure 12 A,B) . Although the disordered loops of classic pleckstrin homology domains are hypervariable in nature, the PHLD of PHLDA1 is unique in that it is interrupted between the third and fourth beta sheet by an 11-amino acid poly Q repeat (Frank *et al.*, 1999; Rebecchi *et al.*, 1998).

In 1999, PHLDA was included in a novel pleckstrin homology-related family with PHLDA2 and PHLDA3 based on their > 50% shared sequence identity, non-canonical start and polyadenylation signal sequences (Frank *et al.*, 1999). Since $TDAG51^{-/-}$ mice appear phenotypically normal, some researchers postulate that other proteins, perhaps members of this family, are also capable of executing PHLDA1's task in the cell (Rho *et al.*, 2001).

The functional role of the PHLD of PHLDA1 has not yet been studied however the classic pleckstrin homology domain is known for its ability to target proteins to the membrane and facilitate cytoskeletal interactions (Lemmon and Ferguson, 2000; Lemmon, 2005). Such a role has been postulated for the PHLD of PHLDA1, and holds promise since all other members of the PHLDA family have

demonstrated the ability to bind to PIPs at the plasma membrane (Saxena *et al.,* 2002). Furthermore, binding interactions between PHLDA1 and heat shock proteins have implicated the PHLD as a viable binding site for protein interaction (Hayashida *et al.,* 2006). PHLDA1's cytoplasmic localization, association with focal adhesions and presence during anoikis all favour involvement of the PHLD (Hossain *et al.,* 2003; unpublished data).

1.7 Amino Acid Repeat Tracts of PHLDA1 (PH, PQ and poly Q)

The long stretches of amino acid repeats are one of the distinctive features of PHLDA1. The C-terminal portion of PHLDA1 is disordered and consists primarily of single and tandem amino acid repeats. Repeat regions of a protein often arise as a result of polymerase slipping during DNA duplication events, however their conserved presence in proteins suggests important functional roles (Katti *et al.*, 2000).

The poly Q repeat linker of TDAG51 effectively divides the pleckstrin homology-like domain in two. Poly Q repeats are often associated with transcription regulatory proteins, one of the proposed functions for PHLDA1 upon its nuclear localization (Katti *et al.*, 2000; Labaj *et al.*, 2010; Al-Bayati *et al.*, unpublished). Poly Q repeats are one of the hallmark features of poly-glutamine neurodegenerative diseases including Huntington's disease and spinal muscular atrophy (Truant *et al.*, 2008; Sambataro and Pennuto, 2012). However, unlike the poly-glutamine tracts of neurodegenerative diseases, the poly Q tract of PHLDA1 M.Sc. Thesis

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is not prone to expansion (Butland *et al.*, 2007). Due to redundancies in the genetic code both CAA and CAG codons code for glutamine. The CAG form of glutamine, present in PHLDA1, is associated with the disease phenotype however no polymorphisms in PHLDA1 have been identified in human populations (Butland *et al.*, 2007). A 2007 study by Butland *et al.*, looked at 212 alleles in the human population and found no variance in poly Q tract length in PHLDA1 excluding it as a candidate for expansion disorders (Butland *et al.*, 2007).

The proline-glutamine (PQ) and proline-histidine (PH) repeat C-terminal regions of PHLDA1 consist of 16 and 15 repeats respectively (Park *et al.*, 1996). Identification of PQ region briefly resulted in PHLDA1 being classified as a proline-glutamine rich or PQR protein (Gomes *et al.*, 1999). The repeat rich C-terminal region of PHLDA1 has been described as the death domain and is believed responsible for the proapoptotic phenotype of the protein. A truncated fragment of PHLDA1 containing the PH and PQ C-terminal regions resulted in strong inhibition of cellular translation in reticulocyte extracts (Hinz *et al.*, 2001). Subsequent truncation experiments distinguishing between the PQ and PH rich regions demonstrated a two-fold decrease in cell survival of MEFs following transfection of the PQ repeat region relative to the full length protein and PH repeat peptide (Hinz *et al.*, 2007)

Associations between PQ repeat regions and transcriptional regulation have been made previously, and PQ domains are believed to mediate apoptosis in neurodegenerative diseases (Toyoshima *et al.*, 2004). Previous research in the Austin laboratory has confirmed the importance of the PQ region towards the apoptotic phenotype of TDAG51, as proteins lacking the PQ region were unable to trigger apoptosis in HeLa cells (Al-Bayati *et al.*, unpublished). Ultimately, the interplay between the proapoptotic PQ C-terminal region of TDAG51 and the anti-apoptotic N-terminal PHLD may account for the perceived inconsistency in TDAG51's function between different cell types and under different conditions.

1.8 Localization Signals and PHLDA1

1.8.1 Nuclear Export Signals

Movement of particles across the nuclear envelope can occur through both passive diffusion and active transport through nuclear pore complexes called nucleoporins (Allen *et al.*, 2000). Nuclear export receptors are responsible for the nuclear export of NES-containing proteins and RNA cargo (Allen *et al.*, 2000). Although consensus patterns for nuclear export signal identification have been attempted, they remain a crude measure for prediction of a NES. The most definitive commonality between positively identified NESs remains the increased presence of hydrophobic residues in the signal. Most signals are found in or near flexible regions of the protein for maximal access and tend to be negatively charged (la Cour *et al.*, 2004). PKI (protein kinase A inhibitor) and HIV Rev are the strongest NESs found in nature, and are rapidly transported out of the nucleus (Gerace, 1995).

Consensus sequence from la Cour *et al.,* 2004:



Where accepted hydrophobic residues include leucine (L), isoleucin (I), valine (V), phenylalanine (F) and methionine (M). X indicates a non-specific residue.

1.8.2 Nuclear Localization Signals

Nuclear import receptors are responsible for the transport of proteins and other cargo into the nuclear envelope. The best characterized nuclear import receptors remain importin α and importin β , although the import family is believed to contain at least seven members (Mattaj and Englmeier, 1998; Allen *et al.*, 2000). The first nuclear localization signal was identified in the simian virus 40 large T-antigen (SV 40) and is the strongest classical NLS (cNLS) currently identified in nature. (Allen *et al.*, 2000; Kosugi *et al.*, 2009; Marfori *et al.*, 2011). NLSs can be either monopartite or bipartite in nature with 4 classes identified for monopartite NLSs and 6 for bipartite NLSs (Kosugi *et al.*, 2009). Classical monopartite NLSs are much larger, composed of two NLS regions interrupted by a linker of 10-12 nucleotides (Kosugi *et al.*, 2009). Many strong and weak

monopartite and bipartite NLSs have been characterized and structural studies have revealed a high degree of conservation for cNLSs (Allen *et al.*, 2000; Kosugi *et al.*, 2009).

Consensus sequences from Kosugi *et al.*, 2009:

Class I Monopartite NLS:

 $[\frac{S}{P}]$ -P-x-K- $[\frac{K}{R}]$ -x- $[\frac{K}{R}]$

Class I Bipartite NLS:

SV 40 T-antigen NLS from Gerace, 1995:

SV 40 T antigen NLS:

P-K-K-R-K-V

Amino acid residues include serine (S), threonine (T), proline (P), valine (V) and basic residues lysine (K) and arginine (R). X indicates a non-specific residue.

1.8.3 Putative Localization Signals of PHLDA1

PHLDA1 is a 44 kDa soluble protein that localized to both the nucleus and cytoplasm in mammalian cells. Previous research in the Austin laboratory has identified a potential nuclear localization signal (pNLS) and nuclear export signal (pNES) in PHLDA1 that play a role in its nuclear-cytoplasmic shuttling capabilities. (Al-Bayati *et al.,* unpublished). Both the pNES and pNLS are located within the PHLD region of PHLDA1, with the pNLS preceding the poly Q region and the pNES located 15 amino acids downstream (Figure 1). Point mutation of residues within these regions lead to changes in PHLDA1 distribution in HK-2 cells, altering its apoptotic potential (Al-Bayati et al., unpublished). Alanine substitution of two lysines within the pNLS region lead to nuclear exclusion of PHLDA1 while alanine substitution of two hydrophobic residues in the pNES lead to increased nuclear localization (Al-Bayati *et al.*, unpublished). Furthermore, exclusion of PHLDA1 from the nucleus of HeLa cells through mutation of the pNLS resulted in less apoptosis when compared to overexpression of wildtype PHLDA1, which suggests that localization may influence PHLDA1's proapoptotic activity (Al-Bayati et al., unpublished).

1.9 The future of PHLDA1

Characterization of PHLDA1 has lagged behind TDAG51 for over a decade in terms of functional analysis of the protein. Cancer is the one field where study of PHLDA1 has excelled, with particular attention to breast cancer and as a follicular stem cell marker for basal cell carcinoma. However, despite an

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established correlation between the downregulation of PHLDA1 and cancer, the contributions of PHLDA1 to development of the disease remains poorly understood. More specifically, the mechanism of PHLDA1 activation, the signalling pathways it contributes to and the mechanisms of its behaviour in the cell are all areas of limited understanding. Identifying differential expression levels of PHLDA1 across different disease states provides little insight into the role of the protein in healthy cells and its contributions to homeostasis.

The most definitive mechanistic study for TDAG51 remains the knockout mouse model, which indicates that TDAG51 is not critical to normal growth and development in mice. However knockout mouse models, although a valuable technique in the study of signalling pathways, are not without limitation. Residual genetic information in the regions flanking the ablated gene and compensatory gene activation may conceal the impact of *TDAG51* gene knockout (Eisener-Dorman *et al.*, 2009). Such findings do not exist for PHLDA1, although the common consensus remains that both proteins behave in an analogous fashion. A limited understanding of TDAG51 and PHLDA1's binding partners and molecular interactions currently exists, and a crystal structure of either protein has not yet been resolved. Molecular analysis of this protein including its cellular localization, activation, binding partners and functional domains could provide valuable insight into the mechanism of action of PHLDA1 and its contributions to cellular homeostasis.

The relationship between PHLDA1 localization in the cell and its apoptotic potential has not been extensively studied. Evidence for a correlation between nuclear localization of PHLDA1 and apoptosis exists in the literature but has received little attention. TGDA51's inhibition of general protein synthesis in reticulocyte extracts and identification of ribosomal protein L14 by yeast-two hybrid screen suggest that PHLDA1 may play an important role in the nucleus (Hinz *et al.*, 2001). Furthermore, research in the Austin laboratory suggests that PHLDA1-induced apoptosis may occur through nuclear-cytoplasmic shuttling of the protein with increased nuclear accumulation of PHLDA1 associated with increased cell death (Al-Bayati *et al.*, unpublished).

1.10 Hypothesis and Objectives

Based on the observation that nuclear accumulation of PHLDA1 promotes apoptosis, we hypothesize that the functional regions of PHLDA1 including its localization signals and pleckstrin homology like domain direct cellular localization of PHLDA1 and through localization, regulate its proapoptotic effects. Furthermore we propose that nuclear localization contributes to the cytotoxicity of the PQ region of PHLDA1.

First, through analysis of the putative NES and NLS identified in PHLDA1 we assess the nuclear-cytoplasmic shuttling capabilities of the protein under both normal and stress conditions. Then, through overexpression of the PHLD we assess the contributions of the PHLD to cellular localization of PHLDA1 using

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both light and electron microscopy. Lastly, to determine the influence of localization on protein function, we assess the cytotoxicity of nuclear accumulation of the PQ region.

Through molecular analysis of the functional regions of PHLDA1, we hope to gain a further understanding of the contributions of localization to the apoptotic role of this enigmatic protein.

2. MATERIALS AND METHODS

2.1 Reagents

All materials and reagents purchased were of the highest possible grade. The following chemicals were purchased from Sigma-Aldrich (St. Louis, USA): phorbol 12-myristate 13-acetate (PMA), ionomycin, thapsigargin, 1,4dithiothreitol (DTT), leptomycin B (LMB), 4',6-diamidino-2-phenylindole (DAPI), acridine orange solution, sodium cacodylate buffer (pH 6.5) and glutaraldehyde solution. Ampicillin, kanamycin, LB broth, LB agar, bovine serum albumin (BSA), fetal bovine serum (FBS), β -mercaptoethanol, agarose and human insulin solution were also purchased from Sigma-Aldrich (St. Louis, USA). Reagents for electron microscopy immunostaining were purchased from EM Sciences (Hatfield, USA): Blocking solution for goat gold conjugates, Aurion acetylated bovine serum albumin (BSA-c) and Aurion R-Gent SE-EM silver enhancement reagents. Restriction endonucleases, one kb DNA ladder, T4 DNA ligase and 10x DNA ligase buffer were purchased from Fermentas (Thermo Fisher Scientific, Waltham, USA). DH5 α competent bacteria and PCR SuperMix were purchased form Invitrogen (Life Technologies, Carlsbad, USA). All other reagents and materials were purchased as specified.

2.2 Plasmids

2.2.1 Synthesis of EGFP PHLDA1 fusion proteins-

All enhanced green fluorescent protein (EGFP) fusion proteins were generated using the EGFPC1 plasmid (Clontech, Mountain View, USA). pNES and pNLS DNA sequences were synthesized by Integrated DNA Technologies (IDT, Coralville, USA). Forward and reverse oligonucleotides were annealed prior to sticky-end ligation into the <u>BglII/KpnI</u> sites of EGFPC1.

pNES

Fwd:	5' <u>GATCT</u> GTCGCCAGCCTCGAGCCGCCGGTCAAGCTC
	<u>GGTAC</u>
Rev:	5' <u>C</u> GAGCTTGACCGGCGGCTCGAGGCTGGCGAC <u>A</u>

pNLS

 Fwd:
 5' <u>GATCT</u>AAGCGCAGCGACGGGTTGTTGCAGCTCTGG

 AAGAAAAG<u>GGTAC</u>

 Rev:
 5' <u>C</u>CTTTTTCTTCCAGAGCTGCAACAACCCGTCACT

GCGCTT<u>A</u>

The EGFP-PHLD fusion protein was generated using PCR and cloned directly into the intermediate PCR vector pGEM-T (Promega, Madison, USA). PHLDA1 cDNA encoding the PHLD of PHLDA1 was amplified by polymerase chain reaction (PCR) prior to ligation into the T-overhang transition vector.

PHLD

Fwd:	5' <u>AGATCT</u> CTGAAGGAGGGCGTG
Rev:	5' <u>GGTACC</u> GTACTGCACCATCTGCAGC

The pGEM-T transition vector containing the PHLD was digested with <u>BglII</u> and <u>KpnI</u>, and cloned into the <u>BglII/KpnI</u> sites of pEGFP-C1. All constructs were verified by fluorescence-based double-stranded DNA sequencing (Institute for Molecular Biology (MOBIX), McMaster University, Canada) (Appendix 1,2).

2.2.2 Synthesis of pEF PQ

A pEF myc/nuc vector (pShooterTM, Life Technologies, Carlsbad, USA) was used for creation of a nuclear-targeted PQ fusion protein. cDNA encoding the PQ region of PHLDA1 was amplified by PCR from a full-length human PHLDA1 template. <u>Ncol</u> and <u>Xhol</u> restriction sites were incorporated into the primer sequences and the resultant PCR product (PQ) was subcloned into the pGEM-T intermediate vector (Promega, Madison, USA).

PQ

Fwd:	5' <u>CCATGG</u> TCCTGGCGGTCAAATCC
Rev:	5' <u>CTCGAG</u> ATACGGGTGGAGCTGCTG

The pGEM-T transition vector containing the PQ region was digested with restriction enzymes *NcoI* and *XhoI* prior to cloning into the *NcoI/XhoI* sites of pEF myc/nuc. Authenticity of the construct was confirmed by fluorescence-based double-stranded DNA sequencing (Institute for Molecular Biology (MOBIX), McMaster University, Canada) (Appendix 3).

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2.2.3 Synthesis of pHM830-pNLS2

The NLS site was extended from 13 to 17 amino acids (pNLS2 - VLEKRSDGLLQLWKKKC) to maximize signal recognition. The pHM830 vector expresses a large protein complex consisting of an EGFP fused to β -galactosidase with a multiple cloning site fusing the two proteins together (Gift from Dr. Ray Truant). New oligonucleotides were designed containing overhangs for the restriction sites <u>SacII</u> and <u>XbaI</u> and synthesized by IDT.

pNLS2

Fwd: 5'

GGGTGCTGGAGAAGCGCAGCGACGGGTTGTTGCAGCTCTGGAA GAAAAAGTGT<u>T</u> Rev: 5' <u>CTAGA</u>ACACTTTTTCTTCCAGAGCTGCAACAACCCGTCGCTGCG CTTCTCCAGCAC<u>CCGC</u>

Forward and reverse oligonucleotides were annealed prior to ligation into the *SacII/XbaI* sites of pHM830. Construct authenticity was confirmed by fluorescence-based double-stranded DNA sequencing (Institute for Molecular Biology (MOBIX), McMaster University, Canada)(Appendix 4).

2.3 Bacterial Transformation and Plasmid Purification

Competent DH5 α bacteria were transformed with the desired plasmid using standard heat shock conditions (45 seconds at 42°C). Transformed bacterial colonies were selected by antibiotic resistance (100 µg/mL ampicillin or 50 µg/mL kanamycin) and verified by sequencing. Logarithmic phase bacteria were obtained by 8-hour incubation in antibiotic-containing LB broth with shaking at 250 rpm. Plasmids were purified from DH5 α transformed bacteria using endotoxin-free Qiagen plasmid Mini, Midi and Maxi Kits following manufacturers instructions (Qiagen, Venlo, Netherlands). Purified plasmids were resuspended in DNase- and RNase-free distilled water and stored at -20°C (UltraPureTM, Life Technologies, Carlsbad, USA).

2.4 Cell Culture

HK-2 cells, derived from a human proximal tubule cell line were obtained from American Type Cell Collection (ATCC, Rockville, USA) (Ryan, 1994). HK-2 cells were cultured in Dulbecco's Modified Eagle Media (DMEM) and Ham's F-12 nutrient media containing non-essential amino acids along with penicillin (100 units/mL) and streptomycin (100 μ g/mL) (Gibco, Life Technologies, Carlsbad, USA). Media was supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, USA). Cells were cultured at 37°C under 5% CO₂ conditions.

HeLa cells, derived from a well-established cervical cancer cell line were obtained from American Type Cell Collection (ATCC, Rockville, USA). Cells

were cultured in high glucose Dulbecco's Modified Eagle Media (DMEM) containing penicillin (100 units/mL) and streptomycin (100ug/mL) (Gibco, Life Technologies, Carlsbad, USA). Cells were cultured at 37°C under 5% CO₂ conditions.

2.5 Immunofluorescence Staining

Cells were grown on gelatin-coated glass coverslips prior to treatment (Attachment Factor - Gibco, Life Technologies, Carlsbad, USA). Following treatment, cells were then fixed using 4% paraformaldehyde, unless otherwise specified, and incubated for 20 minutes at room temperature or overnight at 4°C. Following permeabilization with 0.1% Triton-X 100, cells were stained with DAPI and immunostained according to protocols described previously (Hossain et al., 2003). Briefly, cells were washed with phosphate buffered saline (PBS) after permeabilization and blocked for 1 hour in PBS containing 1% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, USA). Cells were then incubated for 1 hour in primary antibody, washed three times in PBS and then incubated for 30 minutes in secondary antibody. All antibody dilutions were prepared in PBS containing 1% BSA. Following immunostaining, incubation with 100 ng/mL of the nuclear dye DAPI for 30 minutes was used for visualization of the cell nucleus. Coverslips were mounted on glass slides using PermaFluor[™] aqueous mounting medium (Thermo Fisher Scientific, Waltham, USA). All steps were conducted at room temperature unless otherwise specified. Immunofluorescence images were taken using Olympus IX81Nipkow spinning disk confocal

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microscope (Center Valley, USA) and a Carl Zeiss 510 laser-scanning confocal microscope (Jena, Germany).

2.6 Antibodies and Conjugates

Primary Antibodies:

c-Myc mouse monoclonal 9E10 Santa Cruz Biotechnology (1:200 IF)

GFP mouse monoclonal 4B10 Cell Signaling (1:1000 WB)

GFP rabbit polyclonal Ab290 AbCam (1:1000 WB)

EGFP chicken polyclonal GFP-1020 Aves Lab Inc. (1:1000 IF, 1:5000 WB, 1:500 EM)

TDAG mouse monoclonal sc-23866 Santa Cruz Biotechnology (1:200 IF)

Anti β -catenin mouse monoclonal L54E2 Cell Signaling (1:200 IF)

Secondary Antibodies:

Alexa Fluor 488 Goat anti-mouse IgG A-21121 Molecular Probes (1:200 IF)

Biotinylated Goat anti-chicken IgY B-1005 Aves Lab Inc. (1:200 IF, 1:5000 WB)

Goat anti-mouse IgG HRP Conjugate K4000 Dako EnVision+ (1:1000 WB)

Goat anti-mouse IgG (H+L) HRP Conjugate 172-1011 Bio-Rad (1:5000 WB)

Alexa Fluor 488 Donkey anti-mouse IgG A-21202 Life Technologies (1:200 IF)

Biotin-XX Goat anti-mouse IgG B-2763 Life Technologies (1:200 IF)

Goat anti-rabbit IgG HRP Conjugate K4002 Dako EnVision+ (1:3000 WB)

Quantum dot (Qdot) Conjugates:

Qdot 605 Streptavidin Conjugate Q10101 Molecular Probes (1:50 IF)

Qdot 625 Streptavidin Conjugate Q22063 Molecular Probes (1:2000 WB)

Colloidal Gold Conjugate:

Goat-anti chicken IgG 6 nm EM grade 25587 EM Sciences

IF = immunofluorescence WB = Western blot

 $EM = electron\ microscopy$

2.7 Western Blotting

Transiently transfected HK-2 cells were grown in 6-well plates and cell lysates were generated in 100 μ L of 4x SDS lysis buffer with protease inhibitor (complete Mini, Roche, Laval, Canada). Cells were then homogenized by cell scraping and repeated passage through a 25-gauge needle. Protein sample concentrations were determined using the Bio-Rad DC Protein Assay to ensure even sample loading (Bio-Rad, Hercules, USA). Lysates were separated under reducing conditions by SDS-PAGE using 10% acrylamide gels (Bio-Rad, Hercules, USA). Proteins were transferred to an appropriate membrane for Immunoblotting. Ponceau S solution (0.1% (w/v) Ponceau S in 5% acetic acid) was used to resolve total protein and verify protein transfer prior to immunoblotting (Bio-Rad, Hercules, USA).

2.7.1 Western Blot using Chemiluminescence

Nitrocellulose membranes were blocked in TBST (20 mM Tris-base, 150 mM NaCl, 0.05% Tween-20) containing 5% milk prior to 1-hour incubation with primary antibodies for GFP (1020, Ab290) or c-myc (9E10). The membranes were then incubated for 30 minutes with either anti-mouse or anti-rabbit horseradish peroxidase (HRP) tagged IgG secondary antibodies (172-1011, K4002). Both primary and secondary antibodies were diluted in TBST containing 1% milk and blots were washed in TBST.

Chemiluminescent Western blots were visualized using horseradish HRP labelled secondary antibodies and Amersham or West-Zol Plus ECL (GE Healthcare, Buckinghamshire, UK; Froggabio, Toronto, Canada). Blots were exposed on Amersham ECL autoradiography film (GE Healthcare, Buckinghamshire, UK) or visualized using the Bio-Rad ChemiDoc XRS+ System and Image Lab Software (Bio-Rad, Hercules, USA).

2.7.2 Western Blot using Quantum Dots

Western blotting using quantum dots (Qdots) was done on Immobilon-FL low fluorescence polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were blocked in WesternDot blocking buffer (Invitrogen, Life Technologies, Carlsbad, USA) prior to 1-hour incubation with a GFP primary antibody (4B10). Membranes were subsequently incubated for 30 minutes with a goat anti-mouse biotinylated secondary antibody (B-2763) and then incubated for 30-60 minutes in Qdot 625 conjugate solution (Q22063). Both primary and secondary antibodies were diluted in TBST containing 1% milk; Qdot beads were diluted in TBST alone. Blots were washed three times in TBST and once with ultrapure water prior to imaging on Bio-Rad ChemiDoc XRS+ System using Image Lab Software (Bio-Rad, Hercules, USA).

2.8 Intracellular Localization

2.8.1 EGFP Fusion Proteins

Intracellular localization of EGFP fusion proteins was examined using an Olympus IX81Nipkow spinning disk confocal microscope (Center Valley, USA) and a Carl Zeiss 510 laser-scanning confocal microscope (Jena, Germany), following protocols previously established in the Austin laboratory. To quantify nuclear and cytoplasmic ratios the following method was established. Nuclear regions were defined using DAPI staining. Images were analyzed using Metamorph for Olympus software and ImageJ. Optical sectioning was performed to define the centre of the nucleus and localization analysis was conducted in this plane. Nuclear and cytoplasmic fluorescence were quantified using 10 μ m² regions in Metamorph. Nuclear and cytoplasmic fluorescence values were normalized to a corresponding background (Bg) region. Nuclear:cytoplasmic ratios were determined using the following equation:

Nuclear: cytoplasmic ratio =
$$\frac{(Nuc^r - Bg^r)}{(Cyt^r - Bg^r)}$$

2.8.2 pHM800 Suite and pEF PQ

Localization of the pHM800 suite (830, 840, 830pNLS2) and pEF PQ was determined using the Olympus spinning disk confocal microscope following protocols previously established in the Austin laboratory. Nuclear regions were defined using DAPI staining and images were analyzed using Metamorph for

Olympus software and ImageJ. Values represent an average of 20 fields per slide with a minimum of three independent experiments.

2.9 Live Cell Imaging

HK-2 cells were grown and visualized in Ibidi Slide $IV^{0.4}$ live cell chambers. Cells were cultured at 37°C under 5% CO₂ conditions. Cells were warmed on a heated stage to 37°C during imaging sessions at room CO₂ levels. An infusion system involving sterile syringe, clamps and tubing were used to minimize evaporation of media when imaging for longer than 1 hour. The infusion system was also used for addition of acridine orange in real time imaging. Under the microscope, a single channel was slowly infused by syringe with 1 mL of 10 μ M acridine orange in standard growth media. For live cell imaging of the actin cytoskeleton, HK-2 cells were transduced with 4 μ L/well of Cellular LightsTM Actin-RFP (Invitrogen, Life Technologies, Carlsbad, USA) 24 hours prior to imaging.

2.10 TUNEL Assay with Immunofluorescence

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) was used to detect apoptotic cells. Cells were grown on coverslips and fixed using 4% paraformaldehyde 24 to 48 hours after transfection with pEF EGFP or pEF PQ. pEF PQ cells were first permeabilized with 0.1% Triton-X 100 and immunostained as described previously to tag the c-myc epitope of pEF PQ (9E10 Santa Cruz, Dallas, USA). TUNEL staining was performed using the In Situ Cell

Death Detection Kit TMR Red (HL Roche, Mississauga, Canada), which detects single and double stranded breaks in DNA. Staining was conducted according to manufacturers instructions. Briefly, cells were incubated for 1 hour at 37°C with a TUNEL reagent mixture consisting of 5 μ L enzyme solution and 45 μ L label solution. Cells were washed three times with PBS prior to staining with 200 μ L of 100 ng/mL DAPI. Cells undergoing DNA fragmentation were positively identified by fluorescence microscope at 20x magnification. TUNEL staining was quantified by counting the number of transfected cells and determining the ratio of TUNEL positive transfected cells to the total number of transfected cells per field.

2.11 Annexin V with Immunostaining

Annexin V staining was also used to detect apoptosis in HK-2 cells. The Vybrant Apoptosis Assay Kit (Kit #2, #6, Molecular Probes, Life Technologies, Carlsbad, USA) was used for detection of phosphatidylserine on the outer surface of the cell membrane, an early marker of cellular commitment to apoptosis. Assays were conducted on live cells plated on coverslips and methods were adapted from those specified for flow cytometry. Cells were plated on coverslips in 6-well plates and grown in low glucose (LG) DMEM (10% FBS, 1% P/S, 0.5% NEAA). Once 70-90% confluent, HK-2 cells were transfected for 24 to 48 hours with pEF EGFP or pEF PQ using Extreme Gene HP (HL Roche, Mississauga, Canada) prior to Annexin V staining.

Media was collected for LDH release assay and adherent HK-2 cells were washed twice with PBS. Cells were subsequently washed with 1x Annexin binding buffer (ABB) and incubated for 15 minutes at room temperature in 1x ABB containing 4% (v/v) Annexin V conjugated to biotin. Following two washes with 1x ABB, cells were incubated in 1x ABB containing 3% (v/v) streptavidin conjugated Alexa Fluor 350 (Molecular Probes, Life Technologies, Carlsbad, USA) for 30 minutes on ice. Plated cells were rinsed with 1x ABB prior to fixation in 250 μ L of 4% paraformaldehyde and overnight incubation at 4°C. Following annexin V staining, cells were lysed with 0.1% Triton-X 100 for immunostaining. pEF PQ transfected cells were subsequently immunostained for detection of PQ peptide expression using the C terminal c-myc epitope tag (9E10). All cells were immunostained with rhodamine phallodin for resolution of the actin cytoskeleton.

Positive and background staining controls were used. In the positive control, 1 μ L of Triton-X 100 was added per mL of media and cells were incubated on ice for 15- 20 minutes prior to live cell Annexin V staining. As a background control, Annexin V incubation was omitted and cells were incubated with streptavidin conjugated Alexa Fluor 350. The number of annexin-positive transfected cells was determined by manual cell counting. The percentage of annexin-positive transfected cells was calculated using the following equation:

% Annexin V positive transfected cells = <u># of Annexin V positive transfected cells</u> x 100 Total # of transfected cells

2.12 Lactate Dehydrogenase (LDH) Release Assay

LDH release assays were conducted according to protocol using the Roche *Cytotoxicity Detection Kit (LDH)* (HL Roche, Mississauga, Canada). Cells were plated on coverslips in 6-well plates and transfected with either pEF PQ or pEF EGFP vectors for 24 to 48 hours. Cells were transferred to media containing 1% FBS 24 hours prior to media collection. A high control (100% cytotoxicity) value was determined using media collected from untransfected HK-2 cells exposed to 0.1% Triton-X 100 in PBS for 15 minutes on ice. Media collected from healthy, untransfected HK-2 cells was used to establish a low control. Percent cytotoxicity was determined using the following equation:

 $C_{L} = low control$

 $C_{H} = high control$

$$Cytotoxicity(\%) = \frac{n - C_L}{C_H - C_L} \times 100$$

n = experimental value

Assays were conducted in a 96-well plate. 50 μ L of sample media and 50 μ L of reaction mixture were loaded per well. Samples were run in triplicate and incubated in the dark for 20 minutes at room temperature prior to reading. Plates were read using Molecular Devices SpectraMax Plus384 Absorbance Microplate Reader (Sunnyvale, USA) and absorbance was recorded at 490 nm.

2.13 Qdot Staining and Fixation

2.13.1 For Immunofluorescence

HK-2 cells were grown in 6-well plates on coverslips and transfected with EGFP-PHLD or EGFP plasmid using Extreme Gene HP. Cells were fixed, permeabilized and immunostained according to protocol (see Section 2.4). A primary chicken anti-EGFP antibody was used along with a secondary goat antichicken biotinylated antibody (GFP-1020, B-1005). Following secondary antibody incubation, cells were washed three times with PBS prior to a 30 minute incubation with streptavidin conjugated Qdot 605 beads (Q10101) diluted in PBS containing 1% BSA. Excess Qdots were washed off with PBS prior to mounting in PermaFluor[™] aqueous mounting medium (Thermo Fisher Scientific, Waltham, USA) and imaging using an Olympus IX81Nipkow spinning disk confocal microscope (Center Valley, USA).

2.13.2 For Transmission Electron Microscopy

HK-2 cells were grown up in 6-well plates and transfected with EGFP-PHLD or EGFP plasmid using Extreme Gene HP. Cells were fixed with 4% paraformaldehyde, permeabilized and immunostained according to protocol (see Section 2.3). For EGFP constructs, a primary chicken anti-EGFP antibody was used along with a secondary goat anti-chicken biotinylated antibody (GFP-1020, B-1005). For β -catenin labelling, a primary anti- β -catenin mouse monoclonal antibody was used along with a secondary biotinylated goat anti-mouse IgG (L54E2, B-2763). Following secondary antibody incubation, cells were washed

three times with PBS prior to a 30 minute incubation with streptavidin conjugated Qdot 605 beads (Q10101) diluted in PBS containing 1% BSA. Excess Qdots were washed off with PBS prior to fixation in a sodium cacodylate solution containing 2% gluteraldehyde. Cells were fixed overnight at 4°C prior to processing at McMaster University's Electron Microscopy Facility. Images were taken on JEOL 1200EX TEMSCAN electron transmission microscope (McMaster University, Hamilton, Canada).

2.14 Immunogold Transmission Electron Microscopy

2.14.1 Cell Processing

Cells grown in 6-well plates were rinsed with PBS, fixed with 4% formaldehyde in PBS, dehydrated in 50, 75, 95 and 100% ethanol, all in the 6-well plates. The final dehydration step was performed with propylene oxide which was squirted from a glass Pasteur pipette under the layer of cells to release them. Propylene oxide's ability to dissolves polystyrene was used to lift the intact cells off the plate. The cells were transferred with a glass Pasteur pipette into an Eppendorf microtube (Eppendorf, Hamburg, Germany), pelleted, resuspended in fresh propylene oxide and pelleted again. The tip of the microtube containing the pellet was cut off with the razor blade and the pellet was then infiltrated with the LR White resin mixture: LR White resin + 0.5% benzoin ethyl ether (full LR White): propylene oxide 1:1, and with the full LR White, for 1 hour each, while still inside and being protected from disintegration by the microtube tip. The

pellet was then scraped out of the tip, placed in a gelatin capsule and polymerized at -20° C by UV light.

2.14.2 Immunogold Staining

Sections were cut using an ultramicrotome (Leica Microsystems, Wetzlar, Germany) at 80 nm thickness and picked up on formvar-coated nickel grids. The grids were then floated on drops of the following reagents: Blocking on 1% glycine + 1% normal goat serum in PBS for 10 minutes, further blocking on the Blocking solution for goat gold conjugates (Cat #25596) for 15 minutes, incubation on the Incubation solution (20 mM PBS + 0.2% Aurion BSAc Cat #25557) for 2 x 5 min, incubation on drops of anti-EGFP chicken antibody (EGFP-1020, 1:500) in the Incubation solution for 1 hour and washing on large drops of the Incubation solution for 6 x 5 minutes. Samples were further incubated on the secondary antibody-colloidal gold conjugate (25587) for 1 hour and washed on large drops of the Incubation solution for 6 x 5 minutes, followed by large drops of distilled water for 4 x 5 minutes. The colloidal gold particles were then silver enhanced using the R-Gent SE-EM (Cat #25521). Images were taken on JEOL 1200EX TEMSCAN electron transmission microscope (McMaster University, Hamilton, Canada).

2.15 Statistical Analysis

Statistical analyses were performed using GraphPad Prism software and Student's *t*-test assuming two-tailed distribution (GraphPad, La Jolla, USA). Statistical significance was defined as p < 0.05. All results are shown as mean \pm standard error of the mean (SEM). Averages represent a minimum of three independent experiments.

3. RESULTS

3.1 Identification of the putative NLS and NES of PHLDA1

A putative NLS, KRSDGLLQLWKKK, in PHLDA1 was confirmed using the cNLS Server which identifies importin α -dependent nuclear localization signals in submitted protein queries (Kosugi *et al.*, 2009; Kosugi *et al.*, 2009) (Figure 3). The full amino acid sequence of PHLDA1 was submitted to the server and parameters were selected to maximize NLS identification. Zero monopartite NLSs and 13 bipartite NLSs were identified using this server. Four results contained the region previously identified in this laboratory. These four bipartite sequences were expanded versions of our NLS containing 10 upstream amino acids and a variable number downstream. These signals received an activity score ranging from 2 – 3.9 making them weak NLSs. Proteins containing NLS with scores in this range were found to distribute evenly to both nucleus and cytoplasm (Kosugi *et al.*, 2009; Appendix 5).

PHLDA1's pNES, VASLEPPVKL, was identified based on visual inspection of the hydrophobic resides present in the protein (Figure 4). Although consensus patterns for nuclear export signal identification have been attempted, they remain a crude measure for prediction of a NES (Fornerod and Ohno, 2002). The identified pNES fit the broad consensus sequence defined by La Cour *et al.*, 2004, to which 72% of the verified NESs in their study conformed (la Cour *et al.*, 2004) (Figure 4 A).

Figure 3. Putative nuclear localization signal (pNLS) of PHLDA1.

(A) Consensus sequence for importin α -dependent NLSs as reported in Kosugi *et al.*, 2009. (B) The putative nuclear localization site of PHLDA1 as identified using the cNLS server (Kosugi, 2009). (C) Identification of the position of the pNLS within the full amino acid sequence of PHLDA1. The pNLS site of PHLDA1 is highlighted within a red box; it is located within the PHLD of PHLDA1 close to the N-terminal of the protein. (D) A basic vector map of the EGFP-pNLS plasmid with the promoter region and direction indicated by an arrow. This plasmid was designed by PCR amplification of the pNLS region of PHLDA1 from complementary DNA (cDNA) and ligation into the EGFP-C1 vector. The resultant fusion protein consists of an N-terminal EGFP with the pNLS at the C-terminal.

A Consensus Sequence

KR-xxxxxxxxx(xx)-KKK

B PHLDA1 pNLS Sequence

KR-sdgllqlw-KKK

C PHLDA1 pNLS Site

MLESSGCKAL KEGVLEKRSD GLLQLWKKKC CILTEEGLLL IPPKQLQHQQ QQQQQQQQQ QQPGQGPQEP SQPSGPAVAS LEPPVKLKEL HFSNMKTVDC VERKGKYMYF TVVMAEGKEI DFRCPQDQGW NAEITLQMVQ YKNRQAILAV KSTRQKQQHL VQQQPPSQPQ PQPQLQPQPQ PQPQPQPQPQ SQPQPQPQPK PQPQQLHPYP HPHPHPHSHP HSHPHPHPH HPHQIPHPHP QPHSQPHGHR LLRSTSNSA



Figure 4. Putative nuclear export signal (pNES) of PHLDA1.

(A) Consensus sequence for NESs as defined by La Cour *et al.*, 2004. (B) The putative nuclear export signal site of PHLDA1 as identified by manual inspection of the hydrophobic regions of the protein. (C) Identification of the position of the pNES within the full amino acid sequence of PHLDA1. The pNES site of PHLDA1 is highlighted within a red box; it is located within the PHLD of PHLDA1 after the poly Q tract. (D) A basic vector map of the EGFP-pNES plasmid with the promoter region and direction indicated by an arrow. This plasmid was designed by PCR amplification of the pNES region of PHLDA1 from cDNA and ligation into the EGFP-C1 vector. The resultant fusion protein consists of an N-terminal EGFP with the pNES at the C-terminal.



3.2 The putative NES and NLS sites of PHLDA1 are not sufficient to direct localization of EGFP

Others have previously reported on the nuclear to cytoplasmic shuttling abilities of PHLDA1 (Al-Bayati *et al.*, unpublished). To examine if this behaviour is directed by nuclear localization and export signals within PHLDA1, fusion constructs containing the pNLS and pNES linked to EGFP were generated. Cellular localization of EGFP-pNES and EGFP-pNLS proteins over expressed in HK-2 cells was monitored using fluorescence microscopy (Figure 5). HK-2 cells over expressing wildtype EGFP were used as a control. Initial transfection of EGFP-pNES and EGFP-pNLS constructs did not result in robust nuclear or cytosolic accumulation (Figure 5). EGFP-pNES and EGFP-pNLS were abundant throughout the cell with only a slight perceived increased nuclear or cytosolic presence. Due to their low molecular weight, EGFP, EGFP-pNES and EGFPpNLS proteins readily diffuse across the nuclear membrane and are present in both the nucleus and cytoplasm of HK-2 cells, rendering quantification of active transport difficult.

3.3 The putative NES of PHLDA1 responds to LMB treatment

To determine whether active transport contributed to the cellular distribution of EGFP-pNES observed in HK-2 cells, leptomycin B (LMB) treatment was used. Leptomycin B is a cytotoxic agent that blocks active nuclear export through covalent binding to the CRM1 transporter (Fukuda *et al.*, 1997; Fornerod and Ohno, 2002; Mutka *et al.*, 2009). Cells were transiently transfected

Figure 5. Overexpression of EGFP, EGFP-pNLS and EGFP-pNES in HK-2 cells.

EGFP, EGFP-pNLS and EGFP-pNES were overexpressed by transient transfection in HK-2 cells grown on coverslips. Cells were fixed 48 hours post-transfection and cell nuclei were stained with DAPI (blue) prior to imaging. Approximately 60-70% transfection efficiency was achieved using Extreme Gene HP with no significant difference between plasmids (data not shown). EGFP (green) overexpression can be seen in EGFP, EGFP-NLS and EGFP-NES transfected cells. Distribution of EGFP-pNLS and EGFP-pNES did not differ substantially from EGFP controls, although a slight increase in nuclear accumulation was apparent for EGFP-pNLS relative to both EGFP and EGFP pNES. All images were taken using spinning disk confocal fluorescence microscopy and 100x objective with oil immersion.


with EGFP-pNES for 24 hours prior to a 24-hour treatment with 1 or 5 ng/mL LMB. The NES of protein kinase A inhibitor (PKI), a well documented CRM1 binding protein, was tagged to EGFP and used as a positive control and EGFP was used as a negative control (Figure 6 A). Relative fluorescence counts from both the cytosol and nucleus of fixed HK-2 cells were used to quantify localization and DAPI staining was used to define the nuclear region of the cell (Figure 6). A significant increase in nuclear localization was observed for both EGFP-pNES and EGFP tagged PKI NES upon 1 ng/mL LMB treatment, indicating that the pNES of PHLDA1 is a functional, CRM1-dependent nuclear export signal. An increase in nuclear localization was observed at 5 ng/mL LMB for both EGFP-pNES and PKI NES; however this increase was paralleled by an increased nuclear signal for the control EGFP and was not significant.

3.4 Expansion of the putative NLS does not alter its localization

The NLS site was extended from 13 to 17 amino acids (VLEKRSDGLLQLWKKKC) to include an upstream valine and downstream cysteine (Figure 7). The valine to cysteine range included in our extended NLS2 was present in all of the top five queries returned using the cNLS mapper, however, further extension was limited by purchased oligonucleotide synthesis length (Kosugi *et al.*, 2009; data not shown). An EGFP-β-galactosidase plasmid (pHM830) was used to generate a pNLS2 fusion protein unable to passively diffuse across the nuclear membrane (pHM830-pNLS2). Empty pHM830 vector was used as a negative control and an EGFP- β -galactosidase plasmid containing

Figure 6. Effect of leptomycin B treatment on localization of EGFP-pNES

(A) HK-2 cells were transfected with EGFP, EGFP-pNES or EGFP PKI NES (green) for 24 hours prior to leptomycin B (LMB) treatment. EGFP PKI NES was used as a positive control. Cells were treated with 1 or 5 ng/mL LMB for 24 hours with PBS used as a control (NT). Cells were fixed 24 hours after LMB treatment and nuclei were stained with DAPI (blue). Cells were imaged by spinning disk confocal fluorescence microscopy using a 100x objective with oil immersion. (B) Nuclear to cytoplasmic ratios were calculated using Metamorph for Olympus software with ratios quantified based on fluorescence in a single optical section. Experiments were conducted in triplicate and values represent an average of 20 images per slide. Error bars indicate the mean ± SEM of three independent experiments. A significant increase in nuclear localization was observed at 1 ng/mL LMB for both EGFP PKI NES and EGFP-pNES constructs.

* p < 0.05



Figure 7. Expanded putative nuclear localization signal (pNLS2) of PHLDA1.

(A) Consensus sequence for importin α -dependent NLSs as reported in Kosugi *et al.*, 2009. (B) The putative nuclear localization site of PHLDA1 as identified using the cNLS server expanded to include an upstream valine (V) and downstream cysteine (C)(Kosugi, 2009). (C) Identification of the position of the pNLS2 within the full amino acid sequence of PHLDA1. The pNLS2 site of PHLDA1 is highlighted within a red box; it is located within the PHLD of PHLDA1 close to the N-terminal of the protein. (D) A basic vector map of the pHM830-NLS2 plasmid with the promoter region and direction indicated by an arrow. This plasmid was designed by PCR amplification of the pNLS2 region of PHLDA1 from complementary DNA (cDNA) and ligation into the pHM830 vector. The resultant fusion protein consists of an N-terminal EGFP, central pNLS2 site and C-terminal β -galactosidase.

A Consensus Sequence

KR-xxxxxxxxxx(xx)-KKK

B PHLDA1 pNLS2 Sequence

VLE-KR-SDGLLQLW-KKK-C

C PHLDA1 pNLS2 Site

MLESSGCKAL KEQVLEKRSD GLLQLWKKKC CILTEEGLLL IPPKQLQHQQ QQQQQQQQQ QQPGQGPQEP SQPSGPAVAS LEPPVKLKEL HFSNMKTVDC VERKGKYMYF TVVMAEGKEI DFRCPQDQGW NAEITLQMVQ YKNRQAILAV KSTRQKQQHL VQQQPPSQPQ PQPQLQPQPQ PQPQPQPQPQ SQPQPQPQPK PQPQQLHPYP HPHPHPHSHP HSHPHPHPH HPHQIPHPHP QPHSQPHGHR LLRSTSNSA



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the strong monopartite NLS from the SV40 Large T-antigen (pHM840) was used as a positive control. Protein expression was confirmed by Western blotting using an EGFP primary antibody (Appendix 6 B). Overexpression of pHM830, pHM840 and pHM830-pNLS2 fusion constructs was evident in both transiently transfected HK-2 and HeLa cells (Figure 8). pHM830-pNLS2 failed to gain access to the nucleus in both HK-2 and HeLa cell lines. The negative control pHM830 was exclusively cytosolic, while the positive control pHM840 showed a robust nuclear signal in both human cell lines.

3.5 Stress conditions failed to induce nuclear accumulation of PHLDA1's pNLS

It was postulated that the hydrophobic residues within the pNLS2 of PHLDA1 might promote interaction of the NLS with lipids at the cell membrane. The NLS of PHLDA1 contains 6 hydrophobic residues including leucines, valine and a tryptophan. To verify that PHLDA1's NLS was not being restricted by capture at the membrane, transiently transfected HK-2 cells were subjected to numerous cellular stressors known to disrupt membrane integrity and stress the cells.

HK-2 cells plated on coverslips were transfected for 24 hours with pHM830-pNLS2, pHM830 or pHM840 (green) prior to treatment. Cells were subjected to cold shock for 20 minutes, 24-hour 200 nM thapsigargin treatment or 14-hour 1 mM dithiothreitol (DTT) exposure prior to fixation (Figure 9). The actin cytoskeleton was stained using rhodamine phallodin (red) and cell nuclei were

Figure 8. Overexpression of pHM830-pNLS2 in HK-2 and HeLa cells.

(A) HK-2 cells were transfected with pHM830-pNLS2, pHM830 or pHM840 (green) to test the ability of PHLDA1's pNLS2 sequence to direct nuclear localization. The pHM800 suite contains an N-terminal EGFP and C-terminal β galactosidase protein, resulting in expression of a bulky fusion construct incapable of diffusing into the nucleus. Transfection of the pHM830 vector, lacking an NLS, was used as a negative control and pHM840, containing the strong monopartite NLS of SV 40 large T-antigen, was used as a positive control. Cells were fixed 24 hours after transfection and cell nuclei were stained with DAPI (blue). Overexpression of pHM830-pNLS2 in HK-2 cells resulted in a strong cytosolic accumulation. The negative control, pHM830, remained in the cytosol while the positive control pHM840 exhibited robust nuclear accumulation. (B) Transfection experiments were repeated in the HeLa cell line. At 48 hours post-transfection, pHM830-NLS2 was again excluded from the nucleus and accumulated in the cytosol. As seen in HK-2 cells, controls pHM830 and pHM840 localized to the cytosol and nucleus respectively of HeLa cells. Cells were imaged by fluorescence microscopy using a 40x objective.



Figure 9. Effect of cellular stress on pHM830-NLS2 localization in HK-2 cells.

HK-2 cells transfected for 24 hours with pHM830, pHM840 or pHM830-NLS2 (green) were subjected to the following stress conditions: 24 hour treatment with 200nM thapsigargin, 20 minute cold shock on ice or 14 hour treatment with 1 mM dithiothreitol prior to fixation. Cells were stained with rhodamine phalloidin to visualize the actin cytoskeleton (red) and DAPI for cell nuclei (blue). pHM830-NLS2 remained cytosolic under all stress conditions, suggesting that its cytosolic localization is not solely due to capture of the NLS at the membrane. The negative control pHM830 remained cytosolic under all conditions, while the positive control pHM840 maintained its nuclear phenotype, confirming that the nuclear membrane and active transport machinery were not impaired by treatment conditions. Cells were imaged by fluorescence microscopy using a 40x objective.



visualized using DAPI (blue). pHM830-pNLS2 remained cytosolic despite cold shock, incubation with thapsigargin or exposure to DTT. Both pHM830 and pHM840 maintained their respective cytosolic or nuclear localization under all conditions, confirming that the nuclear membrane and active transport machinery were unaffected during treatment.

3.6 Stimulation with various agents failed to induce nuclear accumulation of PHLDA1's pNLS

Known stimulants of PHLDA1 upregulation were used to investigate whether the NLS of PHLDA1 requires activation by an associated protein or interaction with a co-chaperone to gain access to the nucleus. HK-2 cells were plated on coverslips and transfected for 24 hours with pHM830-pNLS2, pHM830 or pHM840 (green) prior to treatment. Cells were treated under the following conditions: 24-hour serum starvation, 24-hour 5 μ g/mL insulin treatment or 14hour induction using 10 ng/mL PMA and 500 ng/mL ionomycin (Figure 10).

pHM830-pNLS2 remained cytosolic and did not accumulate in the nucleus despite serum starvation, insulin treatment or stimulation with PMA and ionomycin. Stimulation with PMA and ionomycin, an activator of protein kinase C, resulted in complete cell death at 24 hours (data not shown) and substantial cell death at 14 hours, independent of transfection. Although a relationship between nuclear accumulation of PHLDA1 and cell death has been established, no change in localization was apparent for pHM830-pNLS2 upon PMA and

Figure 10. Effect of various stimuli on pHM830-NLS2 localization in HK-2 cells.

HK-2 cells transfected for 24 hours with pHM830 (negative control), pHM840 (positive control) or pHM830NLS2 (green) were subjected to the following stress conditions: 24 hour serum starvation, 24 hour 5ug/mL insulin treatment or 14 hour induction using 10 ng/mL PMA and 500 ng/mL ionomycin prior to fixation. Cells were stained with rhodamine phalloidin to visualize the actin cytoskeleton (red) and DAPI for cell nuclei (blue). pHM830-NLS2 remained cytosolic under all conditions, suggesting that the NLS of PHLDA1 does not respond to PHLDA1 activation stimuli. pHM830 remained cytosolic under all conditions, while pHM840 maintained it nuclear phenotype, confirming that the nuclear membrane and active transport machinery were not impaired by the treatment conditions. Cells were imaged by fluorescence microscopy using a 40x objective.



ionomycin treatment despite evidence of cell death. Both pHM830 and pHM840 maintained their respective cytosolic or nuclear localization under all conditions, confirming that the nuclear membrane and active transport machinery were unaffected during treatment.

3.7 Endogenous PHLDA1 localizes to the cytoplasm and plasma membrane of HK-2 cells

Previous PHLDA1 localization studies in the Austin laboratory were conducted in untreated and homocysteine treated HUVECs (Hossain *et al.*, 2003). Distribution within untreated cells was concentrated to vesicles, categorized by the author as being either perinuclear or associated with the cell periphery (Hossain *et al.*, 2003). Upon 30-minute treatment with homocysteine, vesicles at the cell periphery were reduced, with typical distribution restored 24 hours post-treatment.

HK-2 cells, an immortalized human proximal tubule kidney cell line, also express PHLDA1 endogenously. In order to gain a full understanding of the localization of PHLDA1, the localization of endogenous protein was examined in the HK-2 cell line. Low passage number HK-2 cells were grown on coverslips until 80% confluent. Cells were then fixed and immunostained using a mouse monoclonal anti-PHLDA1 primary antibody (sc23866) and fluorescently tagged anti-mouse goat secondary Alexa 594 (Figure 11 A, B). At lower magnification, association of endogenous PHLDA1 with the plasma membrane is evident with

Figure 11. Endogenous PHLDA1 localizes to the plasma membrane, perinuclear region and cytoplasm in HK-2 cells.

(A-B) HK-2 cells were plated on gelatin-coated glass coverslips and endogenous PHLDA1 (red) was stained by immunofluorescence using an anti-PHLDA1 primary antibody and Alexa594 tagged secondary antibody. Cell nuclei were stained with DAPI (blue). Concentration of PHLDA1 at the plasma membrane and perinuclear region was evident at low magnification, whereas a less specific cytoplasmic distribution was apparent at high magnification. (C) HK-2 cells were plated on gelatin-coated glass coverslips and grown to 80% confluency prior to 24-hour treatment with 10 ng/mL PMA and 500 ng/mL ionomycin. Upregulation of endogenous PHLDA1 (green) using PMA and ionomycin lead to increased PHLDA1 protein expression and uniform cytoplasmic distribution. (D) HK-2 cells were plated on gelatin-coated glass coverslips and grown to 80% confluency prior to 24-hour treatment with thapsigargin, an ER stress agent. Upregulation of endogenous PHLDA1 (green) was observed in these cells, with a strong cytoplasmic presence. (E) Specificity of the PHLDA1 antibody was confirmed using HK-2 cells overexpressing EGFP-PHLDA1. Montage shows EGFP-PHLDA1 (green) transiently transfected cells, anti-PHLDA1 antibody (red) sc23866 positive cells, DAPI stained nuclei (blue), and resultant merged image. Strong colocalization between EGFP-PHLDA1 and anti-PHLDA1 antibody confirms specificity of anti-PHLDA1 antibody for PHLDA1 protein in paraformaldehyde fixed HK-2 cells. All images were obtained by fluorescence microscopy using a 100x objective with oil immersion.



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cell boundaries illuminated by the immunofluorescence signal. Endogenous PHLDA1 was found distributed throughout the cytoplasm with a slight increase in fluorescence within the perinuclear region. At higher magnification, these features were less apparent relative to the strong cytoplasmic distribution. PHLDA1 was present in the nucleus however at a lower concentration relative to the cytoplasm.

To observe native distribution of PHLDA1 upon upregulation, HK-2 cells were treated with chemicals known to upregulate PHLDA1 expression. HK-2 cells were treated for 14 hours with 10 ng/mL PMA and 500 ng/mL ionomycin, agents known to induce protein kinase C (PKC) activation and induce upregulation of PHLDA1 (Hinz *et al.*, 2001). Cells were fixed 14 hours post treatment and immunostained for endogenous PHLDA1 using the anti-PHLDA1 primary monoclonal antibody (sc23866) and an Alexa 488 (green) secondary antibody (Figure 11 C). Cell nuclei were stained with DAPI. Upregulation of PHLDA1 was readily apparent in these cells after 14-hour treatment. Protein localized non-specifically to the cytoplasm with an increased nuclear presence.

HK-2 cells were also treated for 24 hours with thapsigargin (Tg) a wellcharacterized ER stress agent known to upregulate PHLDA1 expression (Carlisle *et al.*, 2012)(Figure 11 D). Once fixed, these cells were immunostained with anti-PHLDA1 and Alexa 488 (green) secondary antibody prior to DAPI staining. Upregulation of PHLDA1 was evident in these cells after 24-hour Tg treatment with a similar cytoplasmic and nuclear distribution to PMA/ionomycin treatment.

Specificity of the anti-PHLDA1 antibody sc23866 was verified by immunofluorescence using the EGFP-PHLDA1 vector. HK-2 cells overexpressing EGFP-PHLDA1 were fixed 24 hours after transfection and then immunostained as described previously using an Alexa 594 (red) secondary antibody (Figure 11 E). Colocalization between fluorescently tagged protein and fluorescently tagged antibody confirmed specificity of the PHLDA1 antibody sc23866 for PHLDA1 *in situ*. Two other polyclonal anti-PHLDA1 antibodies 6142 and 6143 were excluded using this method based on poor colocalization and non-specific binding (data not shown).

3.8 Protein modelling of PHLDA1 reveals strong consensus to PI3 Kinase Enhancer (PIKE)

To confirm the identity of the PHLD of PHLDA1 as a genuine pleckstrinlike domain, the sequence was analyzed using the Phyre2 protein-threading server (Kelley and Sternberg, 2009). The 133 amino acid sequence of PHLDA1 was entered as a query and returned a high sequence consensus with a variety of pleckstrin homology-like domain structures (Appendix 8). The strongest alignment, with 96.6 % confidence, was associated with the split pleckstrin homology domain of PI3-kinase enhancer (PIKE) (Figure 12). PIKE is a signalling molecule consisting of three isoforms, only two of which (PIKE-A and PIKE-L)

Figure 12. Secondary structure prediction of PHLDA1 using the protein fold recognition server Phyre2.

Top: (A-B) Ribbon diagram of the classic pleckstrin homology domain of dynamin consisting of a 7 beta sheets and a single C-terminal alpha helix. Adapted from Ferguson *et al.*, 1994. **(C)** Ribbon diagram of the split pleckstrin homology domain of PIKE. This pleckstrin homology domain consists of 6 beta sheets split by a short alpha helical region, flanked by a C-terminal beta sheet. **Bottom: (D-E)** Ribbon diagram of the PHLD of PHLDA1 modelled after the split PH domain of PIKE. Using the Phyre2 protein fold recognition server, the PHLD of PHLDA1 was modelled to PIKE with 96.6% confidence. **(F)** Theoretical modelling of the tertiary structure of the full length PHLDA1 protein with its PHLD and large disordered C terminal region. The full length query returned matches to theoretical proteins with significantly lower confidence.



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have a distinctive split pleckstrin homology domain (Yan *et al.,* 2008). PIKE-L is found exclusively in neural tissue whereas PIKE-A expression occurs in various tissues (Yan *et al.,* 2008).

The pleckstrin-homology domain of PIKE contains a short alpha helical interruption between the third and fourth beta sheets and a larger interrupter region between the fifth and sixth beta sheets which spans ~120 amino acids, depending on isoform. The results from the query do not specify which PIKE isoform was used. Based on the Phyre2 alignment model, the poly-Q linker of PHLDA1 corresponds to the helical linker between the third and fourth beta sheet, while the flexible linker which divides the PHLD in two corresponds to a 28 amino acid run from L73 to Y101 (Figure 13). This is the first evidence suggesting a disordered region in the PHLD flanking the poly-Q region. The putative nuclear export signal of PHLDA1 also lies within this disordered region. Phyre2 provides compelling evidence to support the existence of a genuine PHLD in PHLDA1.

3.9 The PHLD of PHLDA1 localizes to the cytoplasm and plasma membrane in HK-2 cells

To gain further understanding of the functional role of the PHLD of PHLDA1 and its contributions towards cellular localization, an EGFP-PHLD fusion protein was constructed. The EGFP-PHLD fusion was obtained by PCR amplification of the PHLD from full-length PHLDA1 cDNA and cloning into the

Figure 13. Secondary structure and disorder prediction for the PHLD of PHLDA1

Results from the Phyre2 protein structure prediction server generated from a query containing the full PHLD (aa 9-142) of PHLDA1. Amino acid sequence is indicated at the top, followed by secondary structure predictions (helix in blue, beta sheet in green) and corresponding confidence scale (SS confidence). Regions of disorder are indicated by a question mark (?), and the corresponding confidence scale for this value is located at the bottom (Disorder confidence). Regions predicted with the highest confidence are indicated in red with regions of most uncertainty in blue – See confidence key legend in figure for full details (Kelley and Sternberg, 2009).



pEGFP-C1 plasmid. Protein expression of EGFP-PHLD was confirmed by Q-dot Western blotting using a GFP primary antibody (Appendix 6 A). The EGFP-PHLD fusion ran significantly higher than both pNES and pNLS at ~48 kDa. A lower level of overexpression was observed for the EGFP-PHLD fusion relative to both EGFP-pNES and EGFP-pNLS fusions. Overexpression of EGFP-PHLD was also confirmed by HRP Western blotting using a primary chicken antibody (GFP-1020) (Appendix 6 B). A strong band was observed at ~48kDa, below full length EGFP-PHLDA1 but well above the EGFP control.

Cellular localization of EGFP-PHLD protein in transiently transfected HK-2 cells was first assessed by spinning disk confocal fluorescence microscopy. HK-2 cells were fixed 48 hours after transfection and nuclei were stained using DAPI (Figure 14 A). EGFP-PHLD distributed to both the nucleus and cytoplasm with unique punctate structures forming in the cytoplasm and at the cell periphery. Cytoskeletal organization was further monitored by immunofluorescence for β catenin (Figure 14 B). EGFP-PHLD protein again distributed throughout the cell with bright punctate structures evident in the cytosol. A change in cell morphology from an adherent phenotype to an extended fibroblast-like state was observed in approximately 10% of transfected cells.

Using laser scanning confocal fluorescence microscopy a series of optical sections of EGFP-PHLD transfected cells was obtained (Figure 14 C). Consecutive optical sections were captured from the basal to apical region of the cells.

Figure 14. Localization of the PHLD of PHLDA1 in HK-2 cells.

(A) HK-2 cells were plated on gelatin-coated glass coverslips and transiently transfected with EGFP-PHLD (green) for 24 hours prior to fixation. Cell nuclei were stained with DAPI (blue). Punctate structures are indicated by white arrows. (B) EGFP-PHLD transfected HK-2 cells were immunostained for β -catenin (red) using an alexa 594 fluorescent secondary antibody. Approximately 10% of EGFP-PHLD transfected cells exhibited changes in morphology at 24 hours. These adherent cells adopted a more extended, fibroblast-like shape. (C) Consecutive confocal images of EGFP-PHLD transiently transfected cells moving from the basal to apical membrane. Punctate structures can be seen at the basal membrane and throughout the cytosol. Cells were imaged using a Zeiss LSM 510 laser scanning confocal microscope and 63x objective.



Punctate structures were evident at the basal membrane and throughout the cytosol. B-catenin disruptions and fibroblast-like phenotype were again observed in a small percentage of EGFP-PHLD cells.

3.10 PHLD exhibits similar behaviour to full length PHLDA1 in HK-2 cells

As the major domain of PHLDA1, many of the functional roles of the protein including localization have been attributed to the PHLD. Both putative NES and NLS regions of PHLDA1 lie within the PHLD regions and the domain is a strong candidate for interaction with PIPs at the plasma membrane. Overexpression of EGFP-PHLD differs significantly from control EGFP transfected HK-2 cells (Figure 15 A, B). Although both EGFP and EGFP-PHLD are present in both cytoplasm and nucleus, they have distinct patterns of distribution. EGFP diffuses evenly throughout the cytoplasm and accumulates strongly in nucleus. EGFP-PHLD diffuses throughout the cytoplasm but also forms distinct punctate aggregates. Nuclear localization of the EGFP-PHLD was reduced relative to overexpression of EGFP alone in HK-2 cells.

PHLD and full-length PHLDA1 shared similar distribution patterns in transiently transfected HK-2 cells (Figure 15 B, C). Both distribute throughout the cytoplasm and form punctate aggregates. Notably, cell death is reduced in PHLD transfectants relative to PHLDA1 transfected cells (data not shown). Substantial condensing and detachment is observed in PHLDA1 transfected cells at 48 hours while PHLD transfected cells remain attached to the gelatin-coated coverslips.

Figure 15. The PHLD of PHLDA1 localizes similarly to full length PHLDA1 in HK-2 cells.

(A) HK-2 cells were transiently transfected with EGFP (green) as a control. Cells were fixed 24 hours post transfection and cell nuclei were stained with DAPI (blue). (B) HK-2 cells were transiently transfected with EGFP-PHLD and distribution was observed 24 hours post-transfection. (C) Corresponding image of HK-2 cells transiently transfected with full length EGFP- PHLDA1 for 24 hours prior to fixation and staining. Punctate structures (white arrows) are evident in both PHLD and full length PHLDA1 positive cells. All images were obtained by spinning disk confocal fluorescence microscopy using a 100x objective with oil immersion.







These findings imply that the PHLD of PHLDA1 directs localization of the fulllength protein and support previous findings that the C-terminal region of PHLDA1 is responsible for apoptosis.

3.11 Characterization of the punctate structures in EGFP-PHLD transfected HK-2 cells

Live cell imaging techniques were used to further assess the localization and activity of the PHLD of PHLDA1 in HK-2 cells. HK-2 cells were grown, transfected and visualized within Ibidi cell culture slide chambers. Cells transfected with EGFP-PHLD were visualized at 100x using confocal fluorescence microscopy (Figure 16 A, B). Punctate structures were evident throughout the cell with larger stationary blebs evident at the periphery of the cell and associated with the basal membrane. Throughout the cytoplasm an abundance of smaller, rapidly moving bright dots were apparent. Free particles moved in a random manner with no evidence of lysosomal trafficking whereas fixed particles maintained their position for an extended period (> 2 hours) (Supplemental video).

Acridine orange, a cell permeable dye that stains DNA/RNA green and lysosomes orange, was used to increase contrast in the cell during live imaging (Figure 16 C). Cells were bathed in media containing 10 μ M acridine orange immediately prior to imaging. Nuclei, lysosomes and cell membranes were

Figure 16. Live cell imaging of EGFP-PHLD transfected HK-2 cells.

Live cell imaging was used to track the movement of EGFP-PHLD protein aggregates in transiently transfected HK-2 cells. All images were obtained using fluorescence microscopy and 100x objective with oil immersion. (A) Image captured during a 15-minute time laps of the cell floor of a living EGFP-PHLD transfected HK-2 cell. Large, static punctate structures (white arrow) are evident along the basal cell membrane. **(B)** Image captured during a 15-minute time lapse of the same transiently transfected cell in the mid-plane. Static punctate structures were evident at the cell periphery while smaller aggregates moved rapidly within the cytoplasm. (C) Image captured during live cell imaging of an EGFP-PHLD positive cell bathed in 10 µM acridine orange. (D) HK-2 cell transiently transfected with EGFP-PHLD and fixed 24 hours after infection with Cellular LightsTM Actin-RFP. (E) Montage of image D showing the DAPI stained nucleus (blue), actin-RFP expression (red) and EGFP-PHLD (green). Colocalization between actin and EGFP-PHLD can be seen in the larger aggregates.



clearly defined using acridine orange, however the intense green fluorescence from the stain significantly limited resolution of our particles of interest.

Transduction of HK-2 cells with red fluorescent protein (RFP)-tagged actin (Cellular Lights[™] Actin-RFP, Invitrogen) was used to study the relationship between the PHLD and the actin cytoskeleton in living cells (Figure 16 D,E). This technique was limited by the low frequency of cells overexpressing both actin-RFP and EGFP-PHLD. The larger fixed EGFP-PHLD punctate structures on the basal cell membrane localized well with actin clusters in paraformaldehyde fixed cells. Actin staining remained faint in co-transfected cells despite increased incubation time and increases in transfection/transduction efficiency, thereby limiting analysis.

3.12 Transmission Electron Microscopy of EGFP-PHLD and EGFP-PHLDA1

Cellular localization of EGFP-PHLDA and EGFP-PHLDA1 was assessed by electron microscopy. An EGFP-chicken antibody was used for both preembedding and post-embedding immunostaining for transmission electron microscopy (TEM). Specificity of this antibody for EGFP was first confirmed by Western blotting (Appendix 6 B) and subsequently by light microscopy (Figure 17). HK-2 cells were transiently transfected with EGFP-PHLD for 24 hours prior to being fixed and stained for EGFP using a streptavidin-coated secondary and biotinylated Qdots 605 (red). Cell nuclei were stained using DAPI (blue). Strong

Figure 17. Colocalization of EGFP-PHLD and EGFP antibody immunostaining. HK-2 cells were grown to 80% confluency and transfected with EGFP-PHLD (green) for 24 hours prior to fixation. Cells were then stained for EGFP (red) using a primary chicken antibody, biotinylated secondary and streptavidinconjugated Qdot 605, cell nuclei were stained using DAPI (blue). **(A)** Fixed and stained cells grown directly on 6-well plate and imaged immediately prior to processing for EM. Image obtained using 20x objective. **(B)** Merged image of a single HK-2 EGFP-PHLD positive cell obtained using a 100x objective with oil immersion and **(C)** a detail montage showing the same image with EGFP-PHLD alone, colocalization between EGFP-PHLD and the EGFP antibody, and EGFP antibody alone. The EGFP chicken antibody showed high specificity and low background for immunostaining. Colocalization of EGFP and EGFP-PHLD fluorescence is apparent, with the exception of cell nuclei.


colocalization was evident between EGFP-PHLD (green) and EGFP immunostaining (red), however the antibody was occluded from the nucleus.

Using TEM, Qdot size was determined to be between 5-10 nm (Figure 18 A, B). Qdots were not highly electron dense so TEM thin section staining with uranyl acetate and lead citrate was omitted. HK-2 cells were transfected with EGFP or EGFP-PHLD and immunostained prior to embedding and processing for TEM. Qdots were evident in both EGFP (Figure 18 B) and EGFP-PHLD (Figure 18 C, D) transfected cells however no significant difference in localization was apparent.

Post-embedding staining using immunogold staining with silver enhancement was used for improved visualization (Figure 19). EGFP was seen diffused throughout the cytoplasm and nucleus of transfected cells (Figure 19 A). EGFP-PHLDA1 cells also exhibited a diffuse distribution, present throughout the cytoplasm and nucleus of transfected cells (Figure 19 B). Particles were found in proximity to the plasma membrane and in association with actin filaments, though not exclusively. Evidence of punctate structures was apparent for EGFP-PHLD transfected cells with large clusters of protein found within the cytosol (Figure 19 C, D). EGFP-PHLD staining was apparent within the nucleus and cytoplasm of positively transfected cells.

Figure 18. Transmission electron microscopy of Qdots and thin sections of Qdot immunostained EGFP and EGFP-PHLD HK-2 cells.

(A-B) TEM of Qdot 605 streptavidin-conjugated beads on a formvar grid. Qdot beads average 5-10 nm in size. (C-D) Thin section of EGFP transfected HK-2 cells at both 25,000x and 50,000x magnification, immunostained for EGFP using Qdot 605 prior to embedding and processing for TEM. Qdots are indicated by arrows and can be seen diffused throughout the cells. (E-F) Thin section of EGFP-PHLD transfected HK-2 cells at both 30,000x and 40,000x magnification, immunostained for EGFP using Qdot 605 prior to embedding and processing for TEM. Qdots are indicated by arrows and can be seen throughout the cells. (E-F) Thin section of EGFP-PHLD transfected HK-2 cells at both 30,000x and 40,000x magnification, immunostained for EGFP using Qdot 605 prior to embedding and processing for TEM. Qdots are indicated by arrows are indicated by arrows and can be seen throughout the cytosol.



Figure 19. Transmission electron microscopy of EGFP, EGFP-PHLDA1 and EGFP-PHLD thin sections with immunogold labelling and silver enhancement.

Transfected HK-2 cells were processed for TEM and 80 nm sections were immunostained with colloidal gold and silver enhancement prior to imaging. (A) EGFP transfected cell and two adjacent untransfected HK-2 cells. Gold particles (arrows) can be seen diffused throughout the cytoplasm. (B) EGFP-PHLDA1 transfected cell with heavy metal staining. Features of the cell ultrastructure including ribosomes and actin filaments can be seen. EGFP-PHLDA1 is seen throughout the cytoplasm, at the membrane and associated with actin filaments. (C-D) EGFP-PHLD transfected HK-2 cells. Large clusters of EGFP-PHLD can be seen within the cytoplasm with more diffuse staining in the nucleus



TEM imaging was repeated for PHLDA1 with increased silver enhancement and additional staining with heavy metals (Figure 20, 21). The larger, more electron dense particles remained visible after uranyl acetate and lead citrate staining, allowing for higher resolution of cellular ultrastructure. The association of PHLDA1 with the cell membrane was evident, especially in narrow cell extensions. Again, EGFP-PHLDA1 was found in both the cytosol and nucleus of positively transfected cells. Concentration of EGFP-PHLDA1 at the membrane supports PHLDA1's proposed interaction with focal adhesion complexes.

3.13 Nuclear accumulation of the PQ region of PHLDA1 induces apoptosis in HK-2 cells

Previous research in this lab has indicated that the PQ domain of PHLDA1 may be responsible for the proapoptotic behaviour of the protein. To examine if this behaviour is directed by nuclear accumulation of the PQ region, a nuclear PQ fusion protein was generated. This plasmid (pEF PQ) was constructed by ligation of the PCR-amplified PQ region of PHLDA1 into the pEF/myc/nuc plasmid (pShooter[™], Life Technologies) which contains an N-terminal triple NLS and C-terminal c-myc epitope. Overexpression of pEF-PQ was confirmed by Western blotting using a c-myc antibody and by immunofluorescence. The previously constructed vector pEF EGFP was used as a control. HK-2 cells were plated on gelatin-coated glass coverslips and transiently transfected for 24 hours.

Figure 20. Transmission electron microscopy of EGFP-PHLDA1 transfected cell with heavy metal staining.

EGFP-PHLDA1 transfected HK-2 cells were processed for TEM and 80 nm sections were immunostained with colloidal gold and silver enhanced. Sections were stained with uranyl acetate and lead citrate prior to imaging. Full EGFP-PHLDA1 cell can be seen with gold particles present in both the cytoplasm and nucleus. Gold particles (arrows) were found associated with the cell membrane and concentrated in narrow, pseudopodial-like, cell extensions (see inset).



Figure 21. Transmission electron microscopy of a second EGFP-PHLDA1 transfected cell with heavy metal staining.

EGFP-PHLDA1 transfected HK-2 cells were processed for TEM and 80 nm sections were immunostained with colloidal gold and silver enhanced. Sections were stained with uranyl acetate and lead citrate prior to imaging. Full EGFP-PHLDA1 cell can be seen with gold particles (arrows) present in both the cytoplasm and nucleus (top inset). At higher magnification, association of EGFP-PHLDA1 with the membrane is evident, again concentrated within cell extensions (bottom inset).



Overexpression of pEF PQ and pEF EGFP resulted in strong nuclear accumulation in HK-2 cells (Figure 22). Substantial cell detachment was evident at 48 hours post-transfection for pEF PQ relative to pEF EGFP.

Apoptosis was first quantified by detection of DNA fragmentation using a TUNEL assay kit (Roche) (Figure 23). pEF EGFP was used as a negative control. TUNEL positive cells were quantified as a percentage of TUNEL positive transfected cells, from the total number of transfected cells per field. A significant increase in TUNEL positive cells was observed in cells expressing pEF PQ at both 24 and 48 hours post-transfection. Over 50% of pEF PQ cells were TUNEL positive at both 24 and 48 hours, many had irregular nuclei and aberrations in cell shape, indicative of cellular distress.

3.14 Nuclear accumulation of the PQ region initiates apoptosis within 24 hours in HK-2 cells

The increase in apoptosis observed upon nuclear accumulation of the PQ region of PHLDA1 was confirmed using two alternative methods of apoptosis detection. Annexin V staining was selected as the secondary method for apoptosis detection since it is an intermediate stage marker for apoptosis, detecting phosphatidylserine (PS) translocation to the outer face of the plasma membrane. pEF EGFP was again used as a negative control, and cells were transfected for either 24 or 48 hours prior to live cell staining with biotinylated annexin V (Figure 24 A). A 15-minute incubation on ice with 0.1% Triton X-100

Figure 22. pEF PQ localizes exclusively to the nucleus of HK-2 cells.

HK-2 cells were grown on gelatin-coated glass coverslips prior to transfection with pEF PQ for 24 hours. HK-2 cells transfected with pEF EGFP were used as a control. pEF PQ cells were immunostained with a c-myc antibody (green) and cell nuclei were stained using DAPI (blue). Both pEF EGFP and pEF PQ localized exclusively to the nucleus of HK-2 cells, as indicated by strong colocalization with the fluorescent DAPI signal. Cells were imaged using fluorescent microscopy and 100x objective with oil immersion.



Figure 23. TUNEL quantification of pEF PQ induced DNA fragmentation in HK-2 cells.

HK-2 cells were transfected with pEF PQ or pEF EGFP for 24 or 48 hours prior to fixation, TUNEL staining (red) and immunostaining for c-myc (green, pEF PQ only). Cell nuclei were visualized using DAPI (blue). **(A)** Percentage of TUNEL positive transfected cells as calculated by averaging 20 fields per slide at 20x magnification for three independent experiments. TUNEL positive transfected cells were determined based on colocalization of the TUNEL (red) and c-myc or EGFP (green) fluorescence. A significant increase in pEF PQ TUNEL-positive cells was apparent at both 24 and 48 hours post-transfection, relative to pEF EGFP controls. Error bars indicate the mean \pm SEM of three independent experiments. **(B)** TUNEL stained pEF EGFP transfected cells at 24 hours. **(C)** TUNEL stained pEF PQ transfected cells at 24 hours and **(D)** a montage showing pEF PQ transfected cells with the merged image and corresponding DAPI (blue – nuclei), pEF PQ (green – c-myc) and TUNEL (red) channels. * p < 0.05



was used as a positive control for annexin V staining and omission of the annexin V antibody during the staining protocol was used as a negative control (Figure 24 B).

Although transfected control cells (pEF EGFP) could readily be distinguished by fluorescence microscopy, immunostaining was required for visualization of pEF PQ. Preliminary studies were conducted to determine the impact of fixation and immunostaining on the annexin V signal and adaptations were made to the manufacturer's recommended protocol (data not shown). Leaching of propidium iodine into fixed and immunostained cells lead to its omission from the protocol. Both paraformaldehyde and gluteraldehyde fixation agents were tested. Paraformaldehyde fixation was preferred, since high levels of autofluorescence were observed in gluteraldehyde fixed HK-2 cells, obscuring the annexin V signal. As anticipated, a decrease in annexin V staining was observed with fixed and immunostained cells relative to cells mounted on coverslips immediately following live cell staining. All controls and pEF PQ cells were subjected to fixation, lysis and similar staining protocols to limit differences in annexin V signal due to post-annexin V treatment conditions.

Rhodamine phallodin staining was added to the protocol to facilitate quantification of the annexin V signal. Addition of the F-actin probe allowed for cytoskeletal resolution, enabling more accurate positive cell counting. Apoptosis was determined as a percentage of annexin V positive transfected cells from the

Figure 24. Annexin V staining of pEF PQ induced apoptosis in HK-2 cells.

(A) HK-2 cells were transfected with pEF PQ and pEF EGFP (control) for 24 or 48 hours prior to fixation and staining with annexin V conjugated to Alexa 350 (blue), immunostaining for c-myc (green - pEF PQ only), rhodamine phallodin (red), and DAPI staining (blue). (B) Annexin V staining was confirmed using a negative control, omission of the annexin V antibody incubation, to test Alexa 350 background. Rupture of the cell membrane by incubation with 0.1% Triton X-100 was used as a positive control. All cells were transfected when 70-90% confluent, however by 48 hours post-transfection substantial cell loss was evident in pEF PQ transfected wells at low magnification. Large gaps between cell clusters can be seen across the coverslips.



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total number of transfected cells per field. A significant increase in apoptosis was observed in cells expressing pEF PQ at 24 hours post-transfection relative to the pEF EGFP control. However, by 48 hours, annexin V staining for pEF EGFP had increased and differences between pEF EGFP and pEF PQ were no longer statistically significant. Cells were transfected at 70-90% confluency, however by 48 hours post-transfection substantial cell loss was evident in pEF PQ transfected wells at low magnification. Large, cell-free gaps formed on the glass coverslips with an abundance of cells floating in the media.

Lactate dehydrogenase (LDH) release assay was also used to determine the apoptotic potential of nuclear accumulation of PQ (Figure 25). During both TUNEL and annexin V assays, it was apparent that a large amount of cell loss occurred for the pEF PQ transfected cells by 48 hours. Media collected from the annexin V experiments was used for the LDH release assays. A significant increase in cell death was evident for pEF PQ at both 24 and 48 hours posttransfection relative to EGFP controls. Only a slight increase in cell death occurred in pEF EGFP between 24 and 48-hour samples. Altogether these results indicate that nuclear accumulation of the PQ region of PHLDA1 is sufficient to direct apoptosis in HK-2 cells.

Figure 25. Quantification of annexin V staining and LDH release in pEF PQ and pEF EGFP transfected HK-2 cells.

(A) Percentage of annexin V positive transfected pEF PQ and pEF EGFP (control) HK-2 cells as calculated by averaging 20 fields per slide at 20x magnification for three independent experiments. The number of annexin V positive transfected cells were determined by manual cell counting of transfection-positive, annexinpositive cells. A significant increase in pEF PQ annexin V positive cells relative to pEF EGFP controls occurred at 24 hours, but this increase was no longer significant at the 48-hour time point. Error bars indicate the mean \pm SEM of three independent experiments. * p < 0.05 (B) LDH release assays were performed using media collected from HK-2 cells 24 and 48 hours after transient overexpression of pEF PQ or pEF EGFP (control). Percent cytotoxicity was determined using both high (media collected from cells ruptured by Triton-X 100) and low controls (healthy non-transfected HK-2 cells). A significant increase in LDH release was observed for pEF PQ transfected cells at both 24 and 48-hour time points relative to their corresponding pEF EGFP controls. Samples were run in triplicate and error bars indicate the mean \pm SEM of three independent experiments. * p < 0.05 .



4. DISCUSSION

4.1 Study of the pNES of PHLDA1

Although EGFP-pNES was present in both the nucleus and cytoplasm of HK-2 cells, nuclear accumulation was observed upon treatment with 1 ng/mL of the fungicide LMB. This suggests that the pNES is recognized by the CRM1 transporter and can be actively exported from the nucleus. Previous studies in the Austin laboratory confirmed the nuclear accumulation of EGFP-PHLDA1 with 1 ng/mL LMB treatment, supporting current findings that PHLDA1 contains a NES.

The increase in nuclear accumulation of EGFP-pNES was not significant at 5 ng/mL LMB relative to the EGFP control. This may be due to the cytotoxic effects of LMB at 5 ng/mL. LMB is a known cytotoxic agent due to its covalent binding and inhibition of the CRM1 transporter, and is lethal at nanomolar doses in cancer cell lines (Mutka *et al.*, 2009). LMB is also known to be strongly cytotoxic in human papillomavirus (HPV 16) cell lines. The HK-2 cell line used in this study was immortalized by transduction with HPV 16 E6/E7 genes, making it susceptible to the cytotoxic effects of LMB even at low doses (Ryan *et al.*, 1994; Gray *et al.*, 2007). Five ng/mL LMB was found to be cytotoxic to HK-2 cells overexpressing EGFP-PHLDA1 (unpublished data).

PHLDA1's pNES could not be confirmed using NetNES 1.1 Server, which predicts leucine rich nuclear export signals in eukaryotes (la Cour *et al.,* 2004;

Appendix 7). Query submission of the full length PHLDA1 did not result in positive identification of a potential NES region; only a single leucine (L25) crossed the threshold for NES significance (Appendix 7 A, B). Submission of a subsection of PHLDA1, residues 74 – 109, proved more promising (Appendix 7 C). Six residues (LKELHF) met the threshold for NES identification and 11 residues (VKLKELHFSNM) satisfied the hidden Markov model threshold (HMM). However, of the six residues positively identified using the NetNES server, only the leading leucine was present in the pNES tested. Of the 11 residues that satisfied the HMM only the first three were present in the pNES selected. This suggests that the pNES studied may have excluded key hydrophobic residues, limiting its efficacy for nuclear clearance by CRM1.

Although active transport may occur, both the EGFP-pNES and PHLDA1 are small enough to passively diffuse across the nuclear membrane. The cytosolic and nuclear distribution observed by both PHLDA1 and EGFP-pNES may result from passive diffusion, with CRM1 active transport occurring at a level too low for complete clearance of either protein from the nucleus.

4.2 Study of the pNLS of PHLDA1

PHLDA1 has been observed to accumulate in the nucleus upon ER stress, thereby promoting apoptosis (Al-Bayati *et al.*, unpublished). It was therefore proposed that PHLDA1 contains a nuclear localization signal, which promotes its nuclear accumulation under stress conditions. Overexpression of the EGFP-pNLS

in HK-2 cells resulted in a peptide that localized to both the nucleus and cytoplasm, with a similar distribution pattern to EGFP overexpression alone. However, the EGFP-pNLS fusion protein is small enough to passively diffuse into the nucleus and does not require active transport.

The pNLS2 is an expansion of the pNLS, containing an additional four residues including a hydrophobic cysteine, valine and leucine. The pNLS2 was incorporated into the pHM830 vector, which contains an N-terminal EGFP and C terminal lacZ gene. This fusion construct was ideal for nuclear localization studies since it was easily tracked by fluorescence microscopy and was too large for passive diffusion into the nucleus. pHM830-pNLS2 was occluded from the nucleus of both healthy HK-2 and HeLa mammalian cell lines.

Hydrophobic NLSs can behave like switches, remaining bound to the plasma membrane though their hydrophobic interactions until a conformational change leads to its release from the membrane and activation of the NLS. This mechanism of NLS "switch" activation has been proposed for the protein PIKE (Yan *et al.*, 2008). When PIKE's split pleckstrin homology domain interacts with PIPs at the membrane, its NLS interacts favourably with the membrane shielding it from activation (Yan *et al.*, 2008). As the PHLD of PHLDA1 modelled closely to PIKE, a similar "switch" mechanism for PHLDA1's NLS was proposed.

Perturbation of the cell membrane by cold shock or DTT failed to induce nuclear accumulation of the PHLDA1 pNLS2 fusion construct, confirming that activity of the NLS2 is not limited by hydrophobic trapping of the NLS at the cell membrane. Thapsigargin treatment, which raises intracellular calcium levels and induces endogenous PHLDA1 in HK-2 cells, failed to promote nuclear accumulation of the construct. This suggests that activation of the pNLS2 is not dependent upon induction of components involved in the cellular response to increased cytosolic calcium and ER stress (Jan *et al.*, 1999).

Both insulin treatment and serum starvation failed to promote nuclear accumulation of the construct suggesting that activation of PHLDA1's pNLS does not occur through a mechanism analogous to PIKE (Yan *et al.,* 2008). Furthermore, induction of endogenous PHLDA1 by PMA/ionomycin treatment in transfected cells failed to induce nuclear accumulation of pHM830-pNLS2 despite causing substantial cell death.

Altogether, this data confirms that the pNLS sequence identified for PHLDA1 is not a classical nuclear localization signal. Although EGFP-pNLS localized to the nucleus and cytoplasm, the bulkier EGFP-pNLS- β -galactosidase protein, pHM830 pNLS2, failed to gain access to the nucleus under optimal culturing conditions as well as during conditions of stress and PHLDA1 induction.

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The candidate bipartite NLSs identified using the cNLS Server were all longer than the putative sequences we analysed (Appendix 5). An additional 10 amino acid residues upstream of the pNLS start site were present in the top four NLSs identified by the server. It is possible that the region selected for the pNLS and pNLS2 contained only a portion of PHLDA1 nuclear localization signal and a larger peptide containing the 10 upstream residues may show more activity. However, all candidates from the cNLS Server query obtained localization scores below 3. According to la Cour *et al.* (2004), proteins with NLS scores in this range are found distributed in both the nucleus and cytoplasm with a preference for cytoplasmic localization. This distribution accurately reflects localization patterns observed in HK-2 cells overexpressing PHLDA1.

Based on *in vitro* experimentation of both pNLS and pNLS2, VLEKRSDGLLQLWKKKC is not a rapid, classical nuclear localization signal. Predictions from the cNLS Server suggest that the theoretical NLS of PHLDA1 would have very weak activity and would not direct pronounced nuclear accumulation of the protein. It is important to consider that PHLDA1 does not require an NLS to gain entry to the nucleus. As a 44 kDa protein, PHLDA1 falls within the size restriction for entry to the nucleus through passive diffusion (Allen *et al.*, 2000). It is therefore postulated that accumulation of PHLDA1 under stress conditions, as observed by Al-Bayati *et al.* in HeLa cells, occurs in an NLS-independent manner (Al-Bayati *et al.*, unpublished).

4.3 Study of the PHLD of PHLDA1

The PHLD of PHLDA1 plays an important role in protein localization. Previous PHLDA1 localization studies conducted in untreated and homocysteine treated HUVECs showed concentration of PHLDA1 within vesicles in untreated cells (Hossain *et al.*, 2003). These vesicles were categorized by the author as being either perinuclear or associated with the cell periphery (Hossain *et al.*, 2003). Upon 30-minute treatment with homocysteine, vesicles at the cell periphery were reduced, with typical distribution restored 24 hours post-treatment.

Endogenous PHLDA1 staining of quiescent HK-2 cells revealed a similar perinuclear staining and uniform staining at the cell membrane, outlining the cell (Figure 11 A). Membrane staining strongly resembled β -catenin staining supporting association of PHLDA1 with cytoskeletal elements (Figure 11 A, B). Distribution of the PHLD within HK-2 cells was less definitive, more closely resembling EGFP-PHLDA1's observed distribution in the cytosol and clustering into punctate structures (Carlisle *et al.*, 2012). A change in cell morphology from an adherent phenotype to an extended fibroblast-like state was observed in approximately 10% of PHLD transfected cells. Similar changes in cell morphology have been documented in EGFP-PHLDA1 transfected cells (Carlisle *et al.*, 2012). These cells often deviated from the adherent monolayer, extending over neighbouring cells and exhibited disruptions in β -catenin suggestive of EMT.

Overexpression of EGFP-PHLD resulted in similar cytosolic vesicular patterning to endogenous PHLDA1 in HUVEC cells, and using live cell imaging two distinct behaviours were observed for these punctate structures. Large, static punctate structures could be seen associated with the cell membrane, while smaller, rapidly moving particles were found throughout the cytosol and nucleus. No trafficking to lysosomes or accumulation of PHLDA1 within lysosomal vesicles was apparent, which suggests that these PHLD-containing structures are not simply aggregates caused by overexpression. Detailed examination of EGFP-PHLD localization by EM revealed cytoplasmic vesicles of EGFP-PHLD (punctate structures), however association with the cell membrane could not be confirmed.

For the first time, membrane association of EGFP-PHLDA1 is confirmed by EM. Clear association of EGFP-PHLDA1 is evident within cytoplasmic extensions of adherent HK-2 cells in addition to distribution throughout both cytoplasm and nucleus of HK-2 cells. Using optimized immunogold staining with silver enhancement and heavy metal staining we hope to obtain similar results with EGFP-PHLD.

4.4 PHLDA1 and PIKE

Based on results from the Phyre2 protein fold recognition server, the PHLD of PHLDA1 modelled with 96.6% confidence to the split-pleckstrin homology domain of PIKE. This is the first evidence suggesting that the

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disordered region in the PHLD is not the poly Q region but a region immediately adjacent to it containing PHLDA1's NES. Phyre2 provides compelling evidence to support the existence of a genuine PHLD in PHLDA1.

Key similarities between the PIKE isoforms and PHLDA1 also indicate that PIKE is a fair candidate for functional modelling of PHLDA1. PIKE contains a NLS that directs its nuclear accumulation upon PIP depletion at the membrane (Yan *et al.*, 2008). The split PH domain of PIKE-L contains a NLS structural motif and localizes predominantly to the nucleus, while PIKE-A contains a truncated NLS motif and predominantly occupies the cytosol (Yan *et al.*, 2008). PI3 kinase enhancers are recognized proto-oncogenes, acting through promotion of the PI3kinase/Akt signalling pathway (Yan *et al.*, 2008). Although the relationship between PHLDA1 and Akt has not been documented, induction of TDAG51 by Akt has been demonstrated in murine fibroblasts. Truncation experiments involving the split pleckstrin homology domain of PIKE-L showed localization of the protein to the plasma membrane, cytoplasm and nucleus (Yan *et al.*, 2008). Endogenous and exogenous PHLDA1 localizes to both the nucleus and cytoplasm, with the proapoptotic activity of the protein linked to its nuclear accumulation (Al-Bayati *et al.*, unpublished).

In all, PIKE may serve not only as a structural model for the PHLD of PHLDA1 but as a true functional model. As with PHLDA2 and PHLDA3, the

split PH domain of PIKE interacts with PIPs at the plasma membrane, supporting the theory that PHLDA1 is capable of such interactions.

4.5 Study of the PQ Region

As previously reported by Hayashida *et al.* (2006), a 51 aa region of PHLDA1 containing the PQ region was found to be highly cytotoxic relative to both full length PHLDA1 and either N- or C-terminal halves of the protein 36-hours post transient transfection. Our previous findings have demonstrated that nuclear accumulation of PHLDA1 leads to a significant increase in cell death in HeLa cells (Al-Bayati *et al.,* unpublished). This proapoptotic phenotype was then abolished in PHLDA1 mutants lacking the PQ region (Al-Bayati *et al.,* unpublished).

Nuclear accumulation of the PQ region in HK-2 cells induced apoptosis within 24 hours of transfection, with more than 50% of cells positive for TUNEL staining. Cellular disruption was apparent by visual inspection under the light microscope 48 hours after transfection, where sheets of cells could be seen floating in the media, having detached from the plate or gelatin-coated slides. Nuclear accumulation of the PQ region of PHLDA1 is sufficient to direct apoptosis in HK-2 cells, as confirmed using TUNEL, annexin V and LDH release assays. It has yet to be determined whether exclusion from the nucleus limits the apoptotic activity of the PQ region.

The mechanism through which the PQ region of PHLDA1 induces apoptosis remains unknown, however its localization in the nucleus has proven to be highly cytotoxic. Based on its potency when concentrated in the nucleus, the PQ region may act through direct interaction with DNA or through interaction with nuclear proteins. Hinz *et al.* (2001) found that the C-terminal region of PHLDA1 attenuated translation in reticulocyte extracts, and a concurrent yeasttwo hybrid screen of PHLDA1 showed interaction with two mRNA binding proteins (eIF3-p66 and iPABP) and the ribosomal protein L14 (Hinz *et al.*, 2001). The cytotoxicity observed upon nuclear localization of PQ suggests that a correlation exists between localization and apoptotic potential of PHLDA1.

5. CONCLUSION AND FUTURE DIRECTIONS

In this thesis, I explored the functional regions of PHLDA1 in order to further characterize the protein's function and localization within human kidney cells. Through a series of truncation experiments, we assessed the two main functional regions of PHLDA1: the pleckstrin homology-like domain (PHLD) with its putative nuclear localization and nuclear export signals (pNLS/pNES), and the proline-glutamine (PQ) rich region.

Using an EGFP-fusion construct and LMB we confirmed that PHLDA1 contains a weak but CRM1 responsive NES. Using an EGFP- β -galactosidase-fusion we examined the putative NLS of PHLDA1 and determined that it is not sufficient to direct nuclear localization, and thereby not a classical NLS. Nonetheless at 44 kDa, PHLDA1 may not require active transport to shuttle in and out of the nucleus.

The PHLD was found to direct protein localization, mirroring the distribution and punctate patterning of full-length PHLDA1. Using a variety of techniques, we provide some evidence for the association of the PHLD with the cell membrane and actin cytoskeleton, indicative of a possible role for PHLDA1 in focal adhesion complexes. For the first time, we confirm the association of EGFP-PHLDA1 with the cell membrane of HK-2 cells by electron microscopy, and using our optimized protocol, we hope to achieve similar results for EGFP-PHLD. Although the PHLD of PHLDA1 is believed to interact with PIPs at the

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cell membrane, an *in vitro* spot binding assay using PIPs and purified PHLDA1 should be conducted to confirm this interaction. Saxena *et al.* (2002) established PIP binding of both PHLDA2 and PHLDA3 using this technique and were able to determine that both proteins bind non-specifically to most PIPs, PIP₂s and PIP₃. Changes in cell morphology, indicative of EMT, were apparent in a subset of PHLD transfected cells and should be further explored.

Nuclear accumulation of the PQ region was found to be highly cytotoxic, reaffirming its role as the proapoptotic region of PHLDA1. Futhermore, using several cell death assays we demonstrated that the PQ region alone is sufficient to induce apoptosis in HK-2 cells. It has yet to be determined if nuclear localization is a requirement for activity of the PQ region or if it remains cytotoxic when confined to the cytosol. Using the pHM800 suite an EGFP- β -galactosidase fusion containing the PQ region of PHLDA1 could be designed. The β -galactosidase would provide bulk, restricting the PQ to the cytoplasm. Apoptosis could be quantified using TUNEL, annexin V and LDH release with pHM830 used as a control. Design of an EGFP- β -galactosidase fusion containing the full length PHLDA1 would provide further insight into the role of nuclear localization with regard to the apoptotic potential of PHLDA1.

This thesis represents but a first step towards establishing a strong molecular understanding of PHLDA1. Additional work remains in gaining an understanding of this enigmatic protein and its cellular role. Obtaining a

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structural model of the protein using x-ray crystallography would provide us with a first view of PHLDA1 and may reveal keys to its function and activity within the cell. A strong next step for PHLDA1's characterization, with regards to molecular biology, consists of establishing binding partners of PHLDA1 in both quiescent and stressed cell states. Using tandem affinity purification, one could determine novel binding partners of PHLDA1 and further clarify this protein's role in the various apoptotic signalling pathways to which it has been attributed.

Lastly, as downregulation of PHLDA1 is associated with breast cancer and upregulation associated with progression of atherosclerosis, finding novel chemical stimulants of the PHLDA1 promoter could prove valuable towards *in vivo* studies of the protein, allowing for non-invasive modulation of protein expression levels. Through molecular analysis of PHLDA1 we hope to build a strong foundation of knowledge on the protein, on which future studies can be built.

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7. APPENDICES

Appendix 1 – Multiple cloning site and DNA sequencing results for EGFPpNES and EGFP-pNLS plasmids

Multiple Cloning Site pEGFP-C1

EGFP

Stop codons TAC AAG TCC GGA CTC AGA TCT CGA GCT CAA GCT TCG AAT TCT GCA GTC GAC GGT ACC GCG GGC CCG GGA TCC ACC GGA TCT AGA TAA CTG ATC A Bgl II Konl

NLS PHLDA1

Amino Acid Sequence: KRSDGLLQLWKKK Nucleotide Sequence: AAG CGC AGC GAC GGG TTG TTG CAG CTC TGG AAG AAA AAG

DNA Sequencing Results NLS PHLDA1 EGFP Bgl II Stop codons -NNN AG CGCAGCGACGGG GG AAG NNN NN N N NT NN NN

NES PHLDA1

Amino Acid Sequence: V A S L E P P V K L Nucleotide Sequence: GTC GCC AGC CTC GAG CCG CCG GTC AAG CTC

DNA Sequencing Results

Bgl II EGFP **NES PHLDA1** Stop codons N N NNNN N NNGT CGC CG C CT CG N CCGC CG 111111.11.1111 1111 1111111

Appendix 2 – Multiple cloning site and DNA sequencing results for EGFP-PHLD

Multiple Cloning Site pEGFP-C1

EGFP

Stop codons TAC AAG TCC GGA CTC AGA TCT CGA GCT CAA GCT TCG AAT TCT GCA GTC GAC GGT ACC GCG GGC CCG GGA TCC ACC GGA TCT AGA TAA CTG ATC A Bgl II Kpn I

PHLD of PHLDA1

Amino Acid Sequence: LKEGVLEKRSDGLLQLWKKKCCILTEEGLLLIPPKQLQHQQQQQQQQQQQQQQ Q G P A E P S Q P S G P A V A S L E P P V K L K E L H F S N M K T V D C V E R K G K Y M Y F T V V M A E G K E IDFRCPQDQGWNAEITLQMVQY

Nucleotide Sequence:

CTG AAG GAG GGC GTG CTG GAG AAG CGC AGC GAC GGG TTG TTG CAG CTC TGG AAG AAA AAG TGT TGC ATC CTC ACC GAG GAA GGG CTG CTG CTT ATC CCG CCC AAG CAG CTG CAA CAC CAG CAG CAG CAG CAA CAG CAG CAG CAG CAG CAG CAA CAA CAG CCC GGG CAG GGG CCG GCC GAG CCG TCC CAA CCC AGT GGC CCC GCT GTC GCC AGC CTC GAG CCG CCG GTC AAG CTC AAG GAA CTG CAC TTC TCC AAC ATG AAG ACC GTG GAC TGT GTG GAG CGC AAG GGC AAG TAC ATG TAC TTC ACT GTG GTG ATG GCA GAG GGC AAG GAG ATC GAC TTT CGG TGC CCG CAA GAC CAG GGC TGG AAC GCC GAG ATC ACG CTG CAG ATG GTG CAG TAC

DNA Sequencing Results									
EGFP	Bgl II				PH	ILD of PHLDA1			
NNN NNN NN I O	N NNN NNN CTGANG		GCAGCGACGGGT TGT TG	CAGC TCTGG AG ANA	VETETTECATOCTO				
MAR							110 120		
160	170 180	190 200	210	220 230	240 2	50 260	270 280	290	300 310
						Kpn I		Stop codor	IS
GTACATGTACTTOK	TGTOGT GAT GGCAG 4000	AAGGAGATCGACTTTCG	TGCCCCGCAAGACCAGGG	CTGGAACGCCGAG AT	DAC GC TGCAGATOGT				
	340	300 30	370						

Appendix 3 – Multiple cloning site and DNA sequencing results for pEF PQ plasmid

Multiple Cloning Site pEF myc/nuc

Start

CGT GAA CAC GTG GCC A<u>CC ATG G</u>CC CAG GTG CAG CTG CAG GTC GAC <u>CTC GAG</u> ATC AAA CGG GCC GCA - 3x NLS - c-myc - Nco I

PQ Region of PHLDA1

Nucleotide Sequence: TCC TGG CGG TCA AAT CCA CGC GGC AG AAG CAG CAG CAC CTG GTC CAG CAG CAG CCC CCC TCG CAG CCG CAG CCG CAG CCG CAG CTC CAG CCC CAA CCC CAG CCT CAG CCT CAG CCG CAA CCC CAG CCC CAA TCA CAA CCC CAG CCT CAG CCC CAA CCC AAG CCT CAG CCC CAG CAG CTC CAC CCG TAT



Appendix 4 - Multiple cloning site and DNA sequencing results for pHM830pNLS2 plasmid

Multiple Cloning Site pHM830

 BamHI

 ATG GAT CCG TGC AGC TGG CCG ACC ATT ATC AAC AGA ACA CTC CAA TCG GCG ACG GCC CTG TGC TCC CAG ACA ACC ATT ACC TGT CCA CCC

 AG TCT GCC CTG TCT AAA GAT CCC AAC GAA AAG AGA GAC CAC ATG GTC CTG CTG GA GTT TGT GAC CGC TGC TGG GAT CAC ACA TGG CAT GGA

 AG TCT GCC CTG TCT AAA GAT CCC AAC GAA AAG AGA GAC CAC ATG GTC CTG CTG GA GTT TGT GAC CGC TGC GG GAT CAC ACA TGG CAT GGA

 CGA GCT GTA CAA GGC TAT CGA TGC TCT CGA CGC TAG GCC CGG GCC CGC TAG CGG CCT TAA GGG GCC CGC GGG GCC ACG TCG GCT CTA GAG

 GGC CCG GGC CTA GAA TGC CTT CTG AAC AAT GGA AAG GCA TTA TTG CCG TAA GCC GTG GCG GTC TGA TAC CGG TGG AGG

NLS2 of PHLDA1

Amino Acid Sequence: V L E K R S D G L L Q L W K K K C Nucleotide Sequence: GTG CTG GAG AAG CGC AGC GAC GGG TTG TTG CAG CTC TGG AAG AAA AAG TGT



Appendix 5 – NLS prediction results for PHLDA1 using the cNLS Mapper server

cNLS results returned only bipartitie NLSs. Regions identified are indicated in red. Proteins with a NLS that scored 1-2 remain that remain in the cytosol while those with a score of 3-4 were present in both nucleus and cytoplasm (Kosugi *et al.*,2009).





Predicted bipartite NLS							
Pos.	Sequence						
6	GCKALKEGVLEKRSDGLLQLWKKKCCI	3.9					
6	GCKALKEGVLEKRSDGLLQLWKKKCCI	2.9					
6	GCKALKEGVLEKRSDGLLQLWKKKCCILT	2					
6	GCKALKEGVLEKRSDGLLQLWKKKCCILTE	2.2					
8	KALKEGVLEKRSDGLLQLWKKKCCI	2.6					
12	EGVLEKRSDGLLQLWKKKCCILTEEGLLLIPPK	2					
25	LWKKKCCILTEEGLLLIPPKQLQHQQQQ	2.5					
26	WKKKCCILTEEGLLLIPPKQLQHQQQQQ	2.4					
26	WKKKCCILTEEGLLLIPPKQLQHQQQQQQ	2.5					
100	DCVERKGKYMYFTVVMAEGKEIDFRCPQDQGW	2.4					
145	RQAILAVKSTRQKQQHLVQQQPPSQPQPQLQP	2.4					
154	TRQKQQHLVQQQPPSQPQPQLQPQPQPQP	2.4					
198	PQPKPQPQQLHPYPHPHPHPHSHPHSHPHP	2.8					

Appendix 6 – Western blots of newly synthesized plasmids probed for EGFP (**A**) EGFP-pNES, EGFP-pNLS and EGFP-PHLD probed using biotinylated secondary and Qdots. (**B**) pHM800 suite probed using HRP secondary and ECL. EGFP and EGFP-PHLDA1 were used as positive controls.



Appendix 7 – Identification of putative NES sites in both full length and truncated region of PHLDA1 using NetNES

The NES score is calculated using the hidden markov model (HMM) and artificial neural network (ANN) scores indicated (la Cour *et al.,* 2004).



#Seq-Pos-Residue	ANN	HMM	NES	Predicted
	0.127	0.000	0.000	-
PH-2-L	0.187	0.009	0.000	-
PH-3-E	0.114	0.009	0.000	-
PH-4-S	0.137	0.009	0.000	-
PH-5-S	0.100	0.009	0.000	-
PH-6-G	0.111	0.009	0.000	-
PH-7-C	0.134	0.009	0.000	-
PH-8-K	0.088	0.016	0.000	
PH-9-A	0.090	0.016	0.000	_
PH-10-L	0.506	0.259	0.475	-
PH-11-K	0.137	0.266	0.090	
PH-12-E	0.114	0.266	0.090	-
PH-13-G	0.070	0.266	0.077	-
PH-14-V	0.122	0.278	0.096	-
PH-15-L	0.225	0.417	0.262	-
PH-16-E	0.073	0.416	0.164	-
PH-17-K	0.102	0.416	0.158	-
PH-18-R	0.082	0.416	0.151	-
PH-19-S	0.121	0.416	0.161	-
PH-20-D	0.117	0.416	0.160	-
PH-21-G	0.094	0.416	0.134	-
PH-22-L	0.145	0.430	0.162	-
PH-23-L	0.216	0.458	0.214	-
PH-24-0	0.093	0.458	0.216	-
PH-25-L	0.503	0.508	0.752	Yes
PH-26-W	0.085	0.094	0.000	-
PH-27-K	0.208	0.147	0.007	-



Appendix 8 – The top 10 protein models returned for fold-recognition of the PHLD of PHLDA1 using Phyre2

Phyre2 is an online server that predicts protein structure of unknown queries using a library of known protein structures (Kelley and Sternberg, 2009).

	Template	Alignment Coverage	3D Model	Confidence	% i.d.	Template information
1	c2rloA_	Alignment		96.9	21	PDB headensignaling protein Chain: A: PDB Moleculescentaurin-gamma 1; PDBTitle: split ph domain of pi3-kinase enhancer
2	dibaka_	Alignment	\mathbb{N}	96.8	16	FeldiPH domain-like barrel Superfamily:PH domain-like Family:Pieckstrin-homology domain (PH domain)
3	<u>c2d9vA_</u>	Alignment	Ŵ	96.7	18	PDB header:membrane protein Chalin: A: PDB Molecule:pleckstrin homology domain-containing protein PDBTItles solution structure of the ph domain of pleckstrin homology2 domain-containing protein family b member 1 from mouse
4	d2bcja2	Alignment	XX.	96.6	37	FeldiPH domain-like barrel Superfamily:PH domain-like Family:Pieckstrin-homology domain (PH domain)
5	<u>c2dhiA_</u>	Alignment	Ś	96.5	38	PDB headerssignaling protein Chain: A: PDB Moleculespleckstrin homology domain-containing family b PDBTIELes solution structure of the ph domain of evectin-2 from mouse
6	dlpisa_	Alignment	1 and the second	96.3	19	FeldiPH domain-like barrel Superfamily:PH domain-like Family:Pieckstrin-homology domain (PH domain)
7	<u>dlupga</u>	Alignment	EZ.	96.3	20	FeldiPH domain-like barrel Superfamily:PH domain-like Family:Pieckstrin-homology domain (PH domain)
8	<u>c1u5fA</u>	Alignment	1	96.2	36	PDB header:signaling protein Chain: A: PDB Moleculessrc-associated adaptor protein; PDBTIBle crystal structure of the ph domain of skap-hom with 8 vector-derived2 n-terminal residues
9	<u>d2/5fa1</u>	Alignment	A	96.1	18	FeldiPH domain-like barrel Superfamily:PH domain-like Family:Pleckstrin-homology domain (PH domain)
10	<u>c2dkpA</u>	Alignment		96.1	18	PDB headerssignaling protein Chalms A: PDB Moleculespleckstrin homology domain-containing family a PDBTitles solution structure of the ph domain of pleckstrin homology2 domain-containing protein family a member 5 from human