Identifying Substrates, Interacting Partners And Cofactors Of Pirh2: Characterizing The Pirh2 - PKCδ Interaction

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ABSTRACT

p53 is a central player in the cellular response to stress, allowing cells to cope in the presence of diverse stress signals including DNA damage and oncogene activation. In response to stress, p53 acts as a transcription factor to regulate the expression of protein-coding and non-coding RNA genes that collectively result in cell cycle arrest, senescence or apoptosis. One such p53-regulated gene encodes the Pirh2 protein, an E3 ubiquitin ligase known to ubiquitinate many substrates including p53, p27/Kip1 cell cycle inhibitor and DNA polymerase η. The objective of this project was to validate a putative interaction between Pirh2 and Protein Kinase Cδ and determine if the latter was a substrate for Pirh2-mediated ubiquitination. While data suggest that Pirh2 protein expression negatively correlates with PKCδ protein levels, it could not be confirmed that Pirh2 mediates ubiquitin-dependent degradation of PKCδ protein.

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LIST OF ABBREVIATIONS

A/Ala - Alanine

aPKC - atypical PKC

Asp - Aspartate

ATP - Adenosine Tri-Phosphate

BSA - Bovine Serum Albumin

C/Cys - Cysteine

Ca^{2+} - Calcium

CaMK II - Calmodulin-dependent kinase II

Cdk9 - Cyclin-Dependent Kinase 9

Chk2 - Checkpoint kinase 2

CO_2 - Carbon dioxide

cPKC - conventional PKC

DAG - diacylglycerol

DNA-PK - Deoxyribonucleic Acid (DNA)-dependent Protein Kinase

DTT - Dithiothreitol

E6-AP - E6 - Associated Protein

ECL - Enhanced ChemiLuminescence

ER - Endoplasmic Reticulum

FBS - Fetal Bovine Serum

FCS - Fetal Calf Serum

Gly - Glycine

GPCR - G-Protein Coupled Receptor

GST - Glutathione - S - Transferase

h - Hour(s)

H/His - Histidine

H1299-Pirh2-KD - H1299 cells in which Pirh2 protein expression can be inducibly knocked down

HECT - Homologous to E6- Associated Protein (E6-AP) C-terminus

HIPK2 - Homeodomain-Interacting Protein Kinase 2

HRP - Horseradish Peroxidase

IP_3 - Inositol triphosphate

kDa - kilo-Daltons

Lys - Lysine

MALDI - MS/MS - Matrix-Assisted Laser Desorption Ionization Mass Spectrometry (MS)/MS

Mdm2 - Murine Double Mutant 2

MEM - minimum essential medium

Mg^{2+} - Magnesium

min - Minute(s)

mTOR - mammalian target of rapamycin

NaCl - Sodium Chloride

Nedd8 - Neural precursor cell Expressed, Developmentally Down-regulated protein 8

nPKC - novel PKC

PB1 - Phox and Bem1 domain

PBS - Phosphate-Buffered Saline

PDK1 - Phosphoinositide-Dependent Kinase 1

PI - Phosphatidylinositol

PIP_2 - Phosphatidylinositol (4,5) biphosphate

Pirh2 - p53-induced protein with a RING-H2 domain

Pirh2-/- - null for Pirh2 expression

PLC - Phospholipase C

PKC - Protein Kinase C

PMA - Phorbol 12-Myristate 13-Acetate

PS - Psuedosubstrate

PVDF - Polyvinylidene Difluoride

RACK - Receptor for Activated C Kinase

RINCK - RING-finger protein that interacts with C kinase

RING - Really Interesting New Gene

RTK - Receptor Tyrosine Kinase

SCF - Skp-Cullin- F-box protein E3 ligase

SDS - PAGE - Sodium Dodecyl Sulphate (SDS)-Poly-Acrylamide Gel Electrophoresis

Ser - Serine

siRNA - short interfering Ribonucleic Acid

SUMO-1 - Small Ubiquitin-related Modifier 1

TBST - Tris-Buffered Saline - Tween 20

Tet - Tetracycline

Thr - Threonine

Tip60 - Tat-interactive protein of 60 kDa

ts - Temperature-sensitive

Tyr - Tyrosine

Ub - Ubiquitin

UV - Ultraviolet

WT - Wild-Type

Zn^{2+} - Zinc

INTRODUCTION

Normal cellular function relies on both intra- and inter-cellular communication, the integration of such signals and the mounting of appropriate cellular responses such that cells grow, proliferate, develop or die. Central to such cellular functions are thousands of proteins and enzymes that relay intracellular signals and mediate intracellular trafficking, cellular motility and metabolism. Cells must thus regulate protein function such that proteins are only made and active when needed, in response to a specific signal or a cellular event. Different cellular mechanisms are elicited to modulate protein function including regulation at the transcriptional, translational and, more commonly, the post-translational levels. One such mechanism is through control of target protein intracellular levels by covalent conjugation of the ubiquitin (Ub) protein moiety to specific lysine (Lys) residues on substrate proteins, in a process termed ubiquitination, which mostly results in protein degradation by the 26S proteasome (Glickman and Ciechanover, 2002). In addition to targeting proteins for proteolysis, ubiquitination modulates countless cellular processes including cell cycle progression, protein trafficking, transcription factor activity and cellular apoptosis (Deshaies and Joazeiro, 2009). The objective of this thesis was to investigate a putative interaction between the p53-induced protein with a RING-H2 domain (Pirh2) and the protein kinase Cδ (PKCδ) and elucidate its significance. Pirh2 functions as an E3 ubiquitin ligase for many cellular substrates including p53 and p27. The first part of the introduction will focus on the physiological process of ubiquitination.

UBIQUITINATION

Ubiquitin is a 76 amino acid polypeptide that can act as a protein tag through conjugation of its C-terminus to an amino group within a protein substrate (Pickart, 2001). The C-terminal glycine residue (Gly76) of ubiquitin is usually conjugated to the ε-NH_2 group of a substrate lysine or, less frequently, to the free α-NH_2 group of specific proteins such as the cell cycle inhibitor p21 protein (Bloom *et al.*, 2003; Reinstein *et al.*, 2000). The attachment of a polyubiquitin chain, consisting of at least four ubiquitin moieties, to a protein targets it for destruction through the 26S proteasome while conjugation of ubiquitin monomers and chains through specific linkages may modulate protein function by influencing protein-protein interactions, subcellular location or activity (Passmore and Barford, 2004). The attachment of a ubiquitin polypeptide to a protein can take on multiple forms, and it is the topology of the attached ubiquitin tag that mediates protein fate: (a) monoubiquitination, the conjugation of a ubiquitin monomer to a single lysine residue; (b) multiubiquitination, the attachment of multiple ubiquitin monomers to different lysines; and (c) polyubiquitination, the attachment of a ubiquitin polymer to one or more lysine residues within a protein substrate (Henry *et al.*, 2003; Haglund *et al.*, 2003; Bloom *et al.*, 2003). The formation of a ubiquitin polymer relies on conjugation of ubiquitin monomers to Lys residues within an already appended ubiquitin moiety, and indeed, ubiquitin has seven lysines that can participate in polymerization (Lys6, 11, 27, 29, 33, 48 and 63) (Fang and Weissman, 2004; Glickman and Ciechanover, 2002). The Lys^{48}-linked polymer targets tagged proteins to the 26S proteasome for degradation, while the Lys^{63}-linked polymer may signal non-proteolytic functions such as changes in subcellular localization and protein activation (Schnell and Hicke,

2003; Ikeda and Dikic, 2008). Ubiquitin polymers through Lys^{6}, Lys^{11} and Lys^{29} have also been reported, and while the former resembles the Lys^{63}-linked polymer in function, the latter two polymers may target proteins to the proteasome (Passmore and Barford, 2004; Schnell and Hicke, 2003). In addition to ubiquitin, a family of ubiqutin-like proteins are also found including the Small Ubiquitin-related Modifier 1 (SUMO-1) and Neural precursor cell Expressed, Developmentally Down-regulated protein 8 (Nedd8) proteins, and these proteins are thought to modulate protein function both in concert and independently of ubiquitin through conjugation to specific substrates (Ciechanover, 1998).

The conjugation of ubiquitin is thought to proceed through a complex and highly regulated mechanism involving the sequential actions of three classes of enzymes: the E1 ubiquitin-activating enzyme, the E2 ubiquitin-conjugating enzyme and the E3 ubiquitin-ligating enzyme (Pickart, 2001). Mechanistic details of ubiquitin conjugation are discussed next.

The ubiquitination mechanism

The ubiquitination reaction starts with the E1 ubiquitin-activating enzyme, which sequentially binds Magnesium (Mg^{2+})-coordinated ATP followed by ubiquitin (Pickart, 2001). Binding of ATP greatly increases the affinity of the E1 enzyme for ubiquitin (Haas and Rose, 1982; Hershko *et al.*, 1983). Residues within the E1 active site position the ATP molecule such that the C-terminal Gly76 residue can attack the ATP α-phosphate and activate ubiquitin, resulting in the formation of a ubiquitin adenylate intermediate (Walden *et al.*, 2003). This intermediate then serves as a ubiquitin donor to an E1 active site cysteine (Cys; C) residue which, in a similar nucleophilic mechanism, attacks the ubiquitin adenylate, resulting in the formation of an E1-Ub

thioester intermediate (Pickart, 2001). The E1 ubiquitin-activating enzyme is fully activated when it is bound to active ubiquitin both covalently as a ubiquitin thioester within its active site and non-covalently as a ubiquitin adenylate intermediate (Pickart, 2001; Passmore and Barford, 2004). The E1-Ub thioester intermediate next serves as a ubiquitin donor to an E2 ubiquitin-conjugating enzyme (Glickman and Ciechanover, 2002).

The organization of the ubiquitin enzyme cascade is hierarchical with only two E1 enzymes providing activated ubiquitin to about 40 different E2 ubiquitin-conjugating enzymes (Deshaies and Joazeiro, 2009). All E2 enzymes share a core globular domain of about 150 amino acids, and structural studies revealed that a Cys residue sits in a shallow groove in the E2 active site, ready to attack the E1-Ub thioester intermediate, resulting in the formation of an E2-Ub thioester intermediate and regenerating the E1 active site cysteine (Pickart, 2001; Glickman and Ciechanover, 2002). The E2 enzyme preferentially binds Ub-loaded E1 enzymes over either free E1 or ubiquitin molecules, and it is the E1 enzyme that brings ubiquitin and the E2 enzyme together to mediate ubiquitin transfer from itself to the E2 active site in a transesterification reaction (Pickart, 2001). The E2-Ub thioester intermediate acts as the ubiquitin donor for the third step of the reaction catalyzed by the E3 ubiquitin-ligating enzymes (Glickman and Ciechanover, 2002).

The E3 ubiquitin ligase family of enzymes consists of several hundred to a thousand members and are key enzymes that impart substrate specificity to the ubiquitination reaction (Pickart, 2001; Glickman and Ciechanover, 2002). E3 enzymes are thought to mediate substrate specificity through specific protein-binding domains that participate in recruitment of the substrate, while other domains are responsible for the recruitment of the Ub-loaded E2 enzyme

(Pickart and Eddins, 2004; Glickman and Ciechanover, 2002; Deshaies and Joazeiro, 2009). E2 enzymes dock on a shallow hydrophobic groove within the E3 ligase structure after which the E3 enzyme mediates the transfer of an activated ubiquitin moiety from the E2 enzyme to an amino group within the substrate protein, resulting in a ubiquitin-conjugated protein and regenerating the catalytic Cys residue in the E2 active site (Hershko *et al.*, 1983; Pickart, 2001; Deshaies and Joazeiro, 2009). This final ubiquitin transfer reaction results in the conjugation of a ubiquitin monomer to a protein, and the generation of oligo- and poly-ubiquitinated substrates involves repetition of the aforementioned mechanism (Pickart, 2001). Figure 1 illustrates the ubiquitination mechanism. The E3 ligase family of proteins are subdivided into two families based on the structure of their catalytic domain: the (a) homologous to E6-Associated Protein (E6-AP) C-terminus domain (HECT) domain E3 subfamily and the (b) Really Interesting New Gene (RING) domain E3 subfamily (Passmore and Barford, 2004; Pickart, 2001).

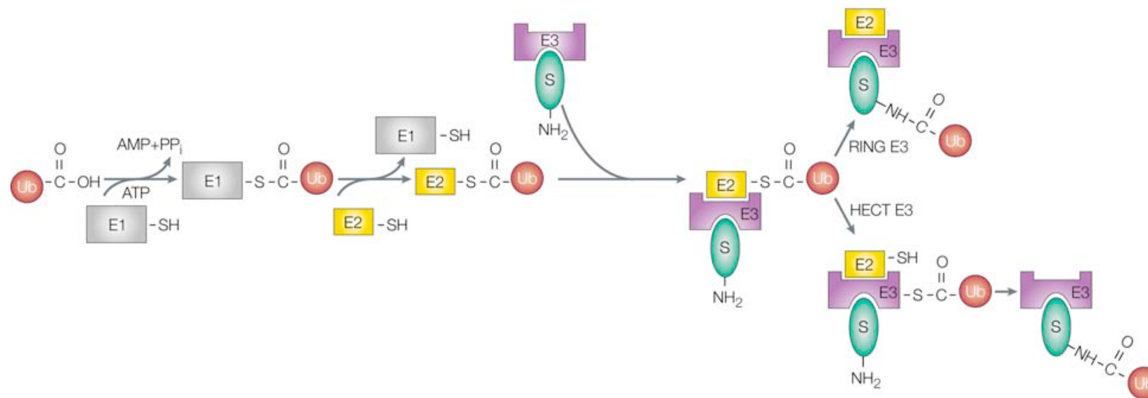

FIGURE 1: THE UBIQUITINATION MECHANISM. Free ubiquitin (Ub) is conjugated through its C-terminus to a Cys residue in the E1 active site in an ATP-dependent reaction. Ub-loaded E1 then transfers its conjugated ubiquitin to an active site Cys residue in the E2 enzyme, regenerating the E1 active site and resulting in the formation of Ub-loaded E2 enzyme. This enzyme can interact with members of the HECT family of E3 ligases, which form an E3-Ub thiol intermediate prior to attachment of ubiquitin to the substrate, or with members of the RING family of E3 ligases, which act as molecular scaffolds to mediate direct transfer of ubiquitin from the E2 to the substrate. Reproduced from Weissman (2001).

The E3 ubiquitin ligase family

Members of the HECT and RING subfamilies of E3 ligases both catalyze the final step of the ubiquitination reaction, the conjugation of the ubiquitin moiety to a target substrate, albeit by different mechanisms. While HECT domain E3 ligases participate catalytically in the ubiquitination reaction by acting as the final ubiquitin thioester donor, RING domain ligases are thought to act as molecular scaffolds to allow the direct transfer of ubiquitin from the E2 enzyme to the substrate, as discussed below.

(a) HECT domain E3 ligases

This family of E3 ligases consists of members who share high homology to the C-terminus domain of the founding member, the E6-AP E3 ligase (Fang and Weissman, 2004). HECT E3 ligases are modular proteins: a highly conserved HECT domain of about 350 amino acids lies at the C-terminus of the ligase, while a substrate-interacting module lies at the N-terminus (Pickart, 2001). A catalytic Cys lies about 35 amino acids upstream of the C-terminus domain, and this Cys is thought to attack the E2-Ub thioester intermediate, in a transthiolation reaction, to result in the formation of an E3-Ub thioester intermediate and regeneration of the catalytic Cys in the E2 active site (Pickart, 2001; Scheffner *et al.*, 1995; Fang and Weissman, 2004; Glickman and Ciechanover, 2002; Figure 1). A protein substrate, bound by the N-terminus substrate binding domain, has it amino group poised for attack on the Ub-loaded E3 intermediate such that an isopeptide bond forms between the C-terminal Gly residue of ubiquitin and the ε-amino group of a substrate Lys, or less commonly, an α-amino group of a protein, which results in a ubiquitin-conjugated protein (Glickman and Ciechanover, 2002).

(b) RING domain E3 ligases

This family of E3 ligases consists of an estimated 600 members, all of whom share a conserved Cys-rich motif termed the RING domain (Deshaies and Joazeiro, 2009). The RING domain is a globular Zinc (Zn^{2+}) binding motif consisting of the base sequence (Cys-X_2-Cys-$X_{(9\text{-}39)}$-Cys-$X_{(1\text{-}3)}$-His-$X_{(2\text{-}3)}$-Cys-X_2-Cys-$X_{(4\text{-}48)}$-Cys-X_2-Cys, where His is histidine (H) and X is any amino acid), which binds two Zn^{2+} ions in a cross-brace structure (Deshaies and Joazeiro, 2009; Pickart, 2001). Much like HECT domain E3 ligases, RING domain E3 ligases are modular proteins that have a substrate-binding site, which binds target proteins, and a distinct RING domain that is thought to mediate structural, non-catalytic functions through recruitment and binding of the Ub-conjugated E2 enzyme (Pickart, 2001). Unlike HECT domain ligases, RING E3 ubiquitin ligases do not participate in the catalytic mechanism directly, rather, they act as molecular scaffolds that facilitate direct substrate ubiquitination by Ub-conjugated E2 enzymes through proximity, by effectively increasing the local concentration of Lys residues available to attack the E2-conjugated ubiquitin, and through E2 and E3 conformational changes that are induced upon E2-E3 binding (Passmore and Barford, 2004; Deshaies and Joazeiro, 2009; Figure 1). Based on protein structural analysis, members of this family can be further subdivided into two classes: (i) single subunit RING E3 ubiquitin ligases, such as the p53 ubiquitin ligase Mdm2, which have both the RING domain and substrate binding motifs as part of the same polypeptide, and (ii) multi-subunit RING E3 ubiquitin ligases, such as the Skp-Cullin-F-box (SCF) E3 ligases, where the RING domain and substrate binding motifs are provided by distinct proteins and coordinated by adaptor and scaffold proteins (Deshaies and Joazeiro, 2009). Non-catalytic proteins with RING domain structures have been identified and are thought to function as modulators of the

E3 RING ligase enzymes through RING domain dimerization (Fang and Weissman, 2004; Gu *et al.*, 2002; Deshaies and Joazeiro, 2009).

A third, less defined class of RING E3 ligases is defined by proteins with U-box domains, which is believed to be a RING domain variant, that also mediates ubiquitination by acting as a molecular scaffold (Fang and Weissman, 2004; Figure 2). The U-box domain lacks Zn^{2+} coordinating residues, relying instead on conserved intra-domain electrostatic interactions and hydrogen bonds to maintain its structural fold and it is as of yet only present in a handful of proteins but further characterization of E3 ligases may reveal additional family members (Pickart and Eddins, 2004).

Regulation of ubiquitination

As of any complex system that modulates protein function, regulation of ubiquitination is vital for proper cellular function and a number of mechanisms exist that serve to positively and negatively affect substrate ubiquitination, which are next discussed.

(I) *Degradation signal*: Amino acid sequences such as the destruction box, a sequence of 9 amino acid residues with invariant arginine and leucine at positions 1 and 4, respectively, and protein structural motifs, such as the hydrophobic face of an amphipathic helix, have both been identified as signals that promote ubiquitination and degradation (Ciechanover, 1998**;** Pickart, 2001). However, the frequency with which these signals are found in protein substrates is quite low, and thus the selection of a specific Lys residue within a substrate is thought to be non-specific, although Deshaies and Joazeiro (2009) suggest that a basic microenvironment around

specific Lys residues may target them for deprotonation, thus functionally acting as degradation signals.

(II) ***Formation and topology of polyubiquitin chains*****:** Certain E3 ligases can only catalyze monoubiquitination reactions, and thus another E2-E3 ligase pair is needed to elongate the ubiquitin chain and form polymers, which may be referred to as an E4 polyubiquitin ligase or E4 ubiquitin chain assembly factor (Grossman *et al*., 2003; Shi *et al*., 2009). As an example, the p53 tumor suppressor is multi-ubiquitinated by the Mdm2 E3 ligase but is subsequently polyubiquitinated by the E3 ubiquitin ligase activity of another enzyme, p300/CBP (Lai *et al*., 2001; Grossman *et al.*, 2003; Shi *et al*., 2009). In addition, a number of ubiquitin-binding protein motifs have been identified in different proteins including the Ubiquitin-Associated Domain and Ubiquitin-Interacting Motif, and these may function as E4 ubiquitin chain assembly factors to enhance polyubiquitination, may stabilize polyubiquitin chains and prevent their de-ubiquitination, or may serve to cap the growing ubiquitin chain after the addition of a specific number of residues to favor mono- or oligo-ubiquitination (Koegl *et al.*, 1999; Flick *et al.*, 2006; Fang and Weissman, 2004). The specific linkage between the ubiquitin monomers within a polyubiquitin chain is determined by both the E2 and E3 enzymes, and it is thought that each of these enzymes may non-covalently bind ubiquitin monomers and orient them in such a way that only a specific Lys residue is poised for attack (Deshaies and Joazeiro, 2009; Passmore and Barford, 2004).

(III) ***Protein stability and proofreading*****:** Polyubiquitinated proteins consisting of 4 or more ubiquitin moieties are targeted for destruction by the 26S proteasome (Ciechanover, 1998). The 26S proteasome is a cellular organelle that performs controlled, ATP-dependent proteolysis of

proteins. It is composed of a barrel-shaped 20S core particle, shaped by 4 stacked rings in the order αββα, where the 2 β rings make up the proteolytic core which harbor trypsin-, chymotrypsin- and peptidyl-hydrolase-like protease activities (Herrmann *et al*., 2007). The α rings serve to stabilize the proteolytic sites, to regulate entry into the catalytic core and to bind, on both ends of the barrel, to two 19S regulatory particles (Glickman and Ciechanover, 2002). The regulatory 19S particle serves many functions: it associates with ubiquitin-binding proteins that help recognize and recruit polyubiquitinated substrates to the proteasome; it contains a number of ATPase subunits that participate in the ATP-dependent unfolding and translocation of protein substrates; and it stabilizes and gates the entry of proteins into the proteolytic core (Glickman and Ciechanover, 2002). Finally, associated with the proteasome are de-ubiquitinating enzymes that catalyze the recycling of ubiquitin monomers, both from polyubiquitinated proteins and peptides released from the proteasome, and that function in the editing and proofreading of ubiquitin-conjugated proteins to ensure that only proteins meant for destruction are targeted to the proteasome (Fang and Weissman, 2004; Ciechanover, 1998).

(IV) ***Post-translational modifications*****:** Post-translational modifications of both E3 ligases and substrate proteins are involved in the regulation of ubiquitination and include phosphorylation of the substrate, as in the case of IκBα, which must be phosphorylated at both Serine (Ser) 32 and Ser36 residues prior to ubiquitination, and of the E3 ubiquitin ligase, as in the case of Mdm2, which is phosphorylated by Protein Kinase B which results in enhanced interaction with its substrate (Chen *et al.*, 1995; Ogawara *et al.*, 2002). Additionally, sumoylation and neddylation of E3 ligases have been reported to enhance the enzymatic activity of both the Mdm2 and SCF protein complex, respectively, while sumoylation of both Mdm2 and IκBα are thought to

compete with ubiquitin for binding to specific Lys residues and thus interferes with their ubiquitination (Buschmann *et al.*, 2000; Duda *et al.*, 2008; Desterro *et al.*, 1998). Ubiquitination itself targets many E3 ligases for degradation, while oligomerization of RING and SCF E3 ligases have been reported to be important for their enzymatic activity (Deshaies and Joazeiro, 2009). Finally, other post-translational modifications may influence ubiquitination, such as the oxidation of the Hypoxia Inducible Factor-1α transcription factor which targets it for degradation, and the acetylation of the p53 transcription factor at various Lys residues which competes with its ubiquitination (Jaakkola *et al.*, 2001; Weissman, 2001).

Ubiquitination, being a complex but essential cellular mechanism, is involved in a number of pathologies and malignancies where E3 ligases have been reported to be mutated or aberrantly expressed. The E3 ubiquitin ligases, a family of enzymes that is reported to outnumber kinases, thus present themselves as potential disease biomarkers and highly valuable drug targets, due to their physiological and clinical significance.

This thesis project was aimed at understanding the physiological role of the p53-induced RING-H2-containing E3 ubiquitin ligase Pirh2.

THE Pirh2 E3 UBIQUITIN LIGASE

The *PIRH2* gene was first identified as a p53 target gene by Leng *et al.* (2003) through a differential display assay using the mouse erythroleukemia cell line DP16.1/p53ts, which encodes a temperature-sensitive p53 that assumes a wild-type conformation at 32°C and a mutant conformation at 37°C. The p53 tumour suppressor protein is a central player in the cellular

response to stress. In response to diverse stress signals including DNA damage, nutrient deprivation and oncogene activation, p53 protein becomes activated and functions as a sequence-specific DNA-binding transcription factor to regulate the expression of both protein-coding and non-coding RNA genes that collectively result in cell cycle arrest, senescence or apoptosis. Due to the integral role of p53 in the cellular stress response, p53 protein expression is lost or its function otherwise impaired in almost all cancers (Vousden, 2006).

Characterization of the *PIRH2* gene product revealed that it encodes a 261 amino acid protein with a central RING-H2 domain, named the p53-induced protein with a RING-H2 domain, and that the protein functions as a single subunit, E3 ubiquitin ligase that targets both itself and p53 for ubiquitination. Pirh2 was shown to regulate the steady state levels of p53 protein by interacting with and ubiquitinating p53 both *in vivo* and *in vitro*, thus participating in an auto-regulatory feedback loop, much like Mdm2 (Leng *et al.*, 2003). Subsequently, Pirh2 was found to preferentially regulate protein levels of active p53 protein, targeting it for polyubiquitination and proteasome-mediated degradation, specifically under cellular stress conditions (Sheng *et al.*, 2008; Hakem *et al.*, 2011). However, the functional significance of having yet another E3 ligase target the p53 protein for degradation is still unclear, and this high level of functional redundancy, coupled with the ability of Pirh2 to induce ubiquitination in the absence of p53 protein both *in vitro* and *in vivo*, led many to believe that Pirh2 mediates the degradation of additional cellular substrates (Leng *et al.*, 2003). Indeed, a number of additional substrates have been identified including the p27/Kip1 cyclin-dependent kinase inhibitor, c-myc, checkpoint kinase 2 (Chk2) and DNA Polymerase η (Hattori *et al.*, 2007; Hakem *et al.*, 2011; Bohgaki *et al.*, 2013; Jung *et al.*, 2011). Pirh2 protein levels are regulated both at the

transcriptional level, through p53-induced gene expression, and post-translationally through self-ubiquitination, which destabilizes the protein and tags it for degradation, and through protein-protein interactions with the Tat-interactive protein of 60 kDa (Tip60), which functions to stabilize Pirh2 protein in the cell (Leng *et al*., 2003; Sheng *et al*., 2008; Logan *et al*., 2004). Finally, Pirh2 can be phosphorylated by two distinct protein kinases: the Calmodulin-dependent kinase II (CaMK II), which phosphorylates the threonine (Thr)-154 and Ser-155 residues, and the Cyclin-Dependent Kinase 9 (Cdk9), which phosphorylates the Ser-211 and Thr-217 residues (Duan *et al*., 2007; Bagashev *et al*., 2013). Both phosphorylation events abrogate the E3 ligase activity of Pirh2 towards p53 and shorten Pirh2 half-life by enhancing self-ubiquitination (Duan *et al*., 2007; Bagashev *et al*., 2013).

Previous research in our lab identified a putative interaction of Pirh2 protein with Protein Kinase Cδ (PKCδ). Purified Glutathione-S-Transferase (GST) or GST-hPirh2 fusion proteins were used as bait to fish for interacting partners using Pirh2 null (Pirh2-/-) immortalized kidney fibroblast lysates in a GST-pulldown assay, and bound proteins were resolved by Sodium Dodecyl Sulphate-Poly-Acrylamide Gel Electrophoresis (SDS-PAGE) (Tai, 2009). Protein bands that were exclusive to the GST-hPirh2 lane were excised and analyzed by Matrix-Assisted Laser Desorption Ionization (MALDI) Mass Spectrometry (MS)/MS, which identified a fragment of the Protein Kinase C δ N-terminus as an interacting partner of Pirh2 (Tai, 2009).

THE PROTEIN KINASE C SUPERFAMILY

Protein phosphorylation is a key protein modulation event that is mediated by over 500 distinct kinases within the cell. Members of the Protein Kinase C (PKC) family were first identified almost 30 years ago and are thought to participate in diverse cellular responses including cellular proliferation, apoptosis and immunity (Mackay and Twelves, 2007; Griner and Kazanietz, 2007).

The PKC family

The PKC family of enzymes consists of ten serine/threonine kinases that belong to the AGC family of protein kinases (Mackay and Twelves, 2007; Steinberg, 2008). PKC was first identified as an enzyme that requires both calcium (Ca^{2+}) and diacylglycerol (DAG), a membrane-bound second messenger product of phosphatidylinositol (PI) hydrolysis, for its activity, however, subsequent studies revealed that different isozymes are activated by different combinations of cofactors (Takai *et al*., 1979; Breitkreutz *et al*., 2007). The isozymes are subdivided into three subfamilies based on structural differences and cofactor requirements: classical PKCs (cPKCs: PKCα, the alternatively spliced PKCβ isoforms PKCβI and PKCβII, and PKCɣ isozymes), which require both Ca^{2+} and DAG binding for activation; novel PKCs (nPKCs: PKCδ, PKCθ, PKCε and PKCη isozymes), which require DAG binding for activation; and atypical PKCs (aPKCs: PKCζ and PKCλ/l), which do not require either of Ca^{2+} or DAG binding for activation, relying on protein-protein interactions instead (Griner and Kazanietz, 2007; Newton, 1995; Steinberg, 2008; Mackay and Twelves, 2007). All family members are activated by binding to

phosphatidylserine, an anionic phospholipid restricted to the cytoplasmic side of the plasma membrane (Steinberg, 2008).

Structural properties of PKC enzymes

PKC enzymes are modular enzymes that consist of an N-terminal regulatory domain and a C-terminal catalytic domain separated by a hinge region (Steinberg, 2008). Structural features of PKC enzymes are defined by four distinct protein domains: two membrane-targeting domains, the lipid-binding C1 domain and the phospholipid- and Ca^{2+}-binding C2 domain, and the highly conserved ATP-binding C3 and substrate-binding C4 domains, which together make up the kinase domain (Mellor and Parker, 1998; Steinberg, 2008; Figure 2). Conserved amongst all three PKC subfamilies is an N-terminal pseudosubstrate domain that closely resembles PKC substrates which maintains the enzyme in a closed, inactive conformation until an appropriate signal is received (Newton, 1995; Mellor and Parker, 1998). A brief explanation of the PKC functional domains follows.

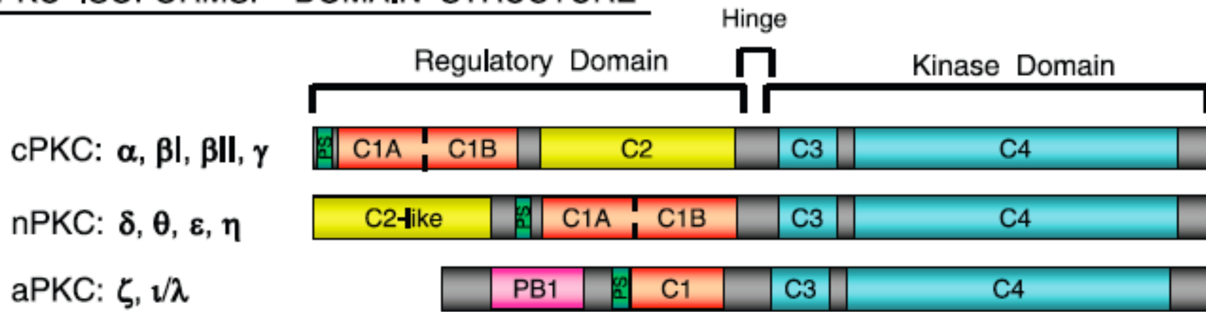

FIGURE 2: DOMAIN STRUCTURES OF THE PKC FAMILY MEMBERS. Representative structures from each PKC subfamily are illustrated. Regulatory and kinase domains and their constituent motifs are shown. The C1 domain of cPKCs and nPKCs is made up of two tandem motifs designated C1A and C1B, while only a single C1 motif is found in aPKcs. The pseudosubstrate domain (PS) is shown in green, and a unique Phox and Bem 1 (PB1) protein domain, implicated in mediating protein-protein interactions, is found in aPKCs. Reproduced from Steinberg (2008).

(a) ***C1 domain*****:** The C1 domain serves to anchor cPKCs and nPKCs into the plasma membrane by mediating interactions of PKCs with both DAG and anionic phospholipids such as phosphatidylserine (Newton, 1995; Steinberg, 2008). The C1 domain consists of two tandem cysteine-rich Zn^{2+} binding motifs, termed C1A and C1B (Mellor and Parker, 1998). The C1A/B motifs both adopt similar tertiary structures in which the upper third of each motif is a largely hydrophobic surface that binds DAG, the middle third is an exposed basic surface that binds anionic phospholipids, and the bottom third is made up of the two Zn^{2+}-binding domains that maintain the C1 fold (Steinberg, 2008). DAG binding to the C1A/B motifs caps a hydrophilic groove within a largely hydrophobic surface, thus providing a contiguous hydrophobic surface

that serves to increase membrane affinity of PKCs (Newton, 1995; Steinberg, 2008). A single C1 motif is fully capable of anchoring PKCs into the plasma membrane, and the two C1 motifs are thought to be non-equivalent, binding DAG with differing affinities in different PKC isozymes (Mellor and Parker, 1998; Steinberg, 2008). The single C1 domain in aPKCs can still recognize anionic phospholipids that help localize it to the membrane, however, this motif has different structural properties that render it unable to bind DAG, and is thought to mediate nuclear localization of aPKCs by housing a nuclear localization signal (Steinberg, 2008). Finally, the C1A domain mediates the interaction of PKC isozymes with the RING-finger protein that interacts with C kinase (RINCK) E3 ubiquitin ligase (Chen *et al*., 2007).

(b) ***C2 domain*****:** The C2 domain is another membrane-targeting domain found in cPKCs, which binds Ca^{2+} ions in a highly cooperative manner at five conserved aspartate (Asp) residues within its structure (Newton, 1995; Steinberg, 2008). This domain binds anionic phospholipids weakly and transiently in the absence of Ca^{2+}, however, upon Ca^{2+} binding, a conformational change is triggered that increases the affinity of the enzyme for the plasma membrane, allowing the docking of the enzyme at the membrane where it encounters DAG and becomes fully active (Newton, 1995). The C2-like domain of nPKCs conserves structural residues required for maintaining the C2 fold, but lacks the conserved Asp residues required for Ca^{2+} binding, and as such cannot be activated by Ca^{2+} (Mellor and Parker, 1998). The C2 domains of of the nPKCs PKCδ and PKCθ were characterized as phosphotyrosine binding domains, which may help recruit these isozymes to the plasma membrane and allow for their activation during agonist-induced cellular signaling (Benes *et al*., 2005; Stahelin *et al*., 2012). aPKCs lack the structural residues required for maintaining the C2 fold and this domain is thus absent (Steinberg, 2008).

(c) ***Kinase domain (C3 and C4)*****:** The C3 and C4 domains form the nucleotide binding and substrate binding domains, respectively, in the PKC active site (Steinberg, 2008). These domains are highly conserved in the AGC family and consists of a glycine-rich ATP binding motif that anchors the non-transferable phosphate groups in the active site, a conserved Lys residue that orients the ATP molecule for catalysis through coordination of its α and β phosphate groups, and an activation loop that orients Mg^{2+} and the peptide substrate for optimal enzyme catalysis (Steinberg, 2008). The activation loop is the site of a crucial phosphorylation event that primes kinases for activation (Newton, 1995). A mutation in the aforementioned Lys residue abolishes enzymatic activity in cPKCs and nPKCs and is used to generate kinase-dead enzyme mutants in the laboratory, however, this mutation is well-tolerated in aPKCs hinting that their ATP-binding motifs are distinct from those in other PKCs (Steinberg, 2008; Spitaler *et al*., 2000).

(d) ***The pseudosubstrate motif*****:** The pseudosubstrate motif is an amino acid sequence that highly resembles the optimal PKC substrate sequence but where an alanine residue is found in lieu of phosphoacceptor amino acids such as Ser and Thr (Mellor and Parker, 1998). PKC active sites are constitutively occupied by the pseudosubstrate motif in inactive enzymes, however, upon enzyme activation, the accompanying conformational changes displace this motif from the active site to allow binding of specific substrates (Newton, 1995). This motif is conserved across all three subfamilies and is usually found N-terminal to the C1 motif (Steinberg, 2008).

(e) ***The hinge region*****:** The hinge region is a region that varies between the different PKC isoforms which separates the N-terminal regulatory domain from the C-terminal catalytic domain. In some isozymes, including PKCδ, θ, ε and ζ, this domain acts as the recognition site for caspase-mediated cleavage during apoptotic signaling (Steinberg, 2008).

***(f) The V_5 domain*:** This is another region in the PKC structure that varies between members of the PKC family, located at the extreme C-terminus of the protein (Figure 2). This domain is the site of two crucial phosphorylations that prime enzymes for activation: the turn motif and hydrophobic motif phosphorylations, which are thought to participate in stabilizing the enzymatic structure and are essential for enzymatic activity (Newton, 1995; Steinberg, 2008). It is at this site that the alternatively spliced PKCβI and PKCβII isozymes differ, which allows these isozymes to localize differently and mediate separate functions within the cell (Chalfant *et al.*, 1995). A nuclear localization signal has also been identified in the V_5 domain of PKCδ, which targets this isozyme to the nucleus during apoptosis, where it encounters its substrates and participates in the apoptotic response (DeVries *et al.*, 2002).

The PKC activation mechanism

PKC structure elucidation allowed the formulation of an activation mechanism for PKC enzymes in response to agonist binding at the cell surface. A general mechanism for activation of cPKCs is explained next, and deviations from this mechanism in nPKC enzyme activation are noted. aPKCs are thought to be activated by protein-protein interactions mediated through their PB1 domain (Parker and Murray-Rust, 2004).

PKC activation is downstream of signaling cascades that result in PI hydrolysis (Newton, 1995). When an agonist engages a membrane-bound G-protein coupled receptor (GPCR) or a receptor tyrosine kinase (RTK), either of phospholipase C (PLC) β or PLCɣ, respectively, are recruited and activated to catalyze the hydrolysis of PI(4,5) biphosphate (PIP_2) to yield inositol triphosphate (IP_3) and DAG (Mackay and Twelves, 2007; Parker and Murray-Rust, 2004). IP_3

acts as an intracellular messenger that mobilizes Ca^{2+} from cellular stores such as the endoplasmic reticulum (ER), while DAG acts as a membrane-bound second messenger that activates membrane-targeted PKC isozymes (Koivunen *et al*., 2006). As aforementioned, in the absence of Ca^{2+}, cPKCs only bind the plasma membrane weakly, however, Ca^{2+} binding initiates a conformational change in the C2 domain that increases the affinity of this domain for the plasma membrane, bringing the C1 domain in close proximity to DAG, and allowing for full activation of PKCs (Steinberg, 2008). Upon DAG binding, the enzyme becomes stabilized and releases the pseudosubstrate motif from its active site, and is now ready to mediate the phosphorylation of its substrates or otherwise associate with different targeting proteins that alter its subcellular localization (Newton, 1995; Mackay and Twelves, 2007; Koivunen *et al.*, 2006). The activation mechanism of nPKCs is similar, however, the C2-like domain of nPKCs has an increased affinity for the plasma membrane, and as such, binding of DAG is sufficient to activate nPKCs (Steinberg, 2008). The C2-like domain of certain nPKCs, being a phosphotyrosine binding domain, may also help recruit the novel PKCδ to the membrane upon receptor tyrosine kinase activation, where it can encounter DAG and become fully activated (Benes *et al*., 2005; Stahelin *et al*., 2012). The activation mechanism of PKC is illustrated in Figure 3.

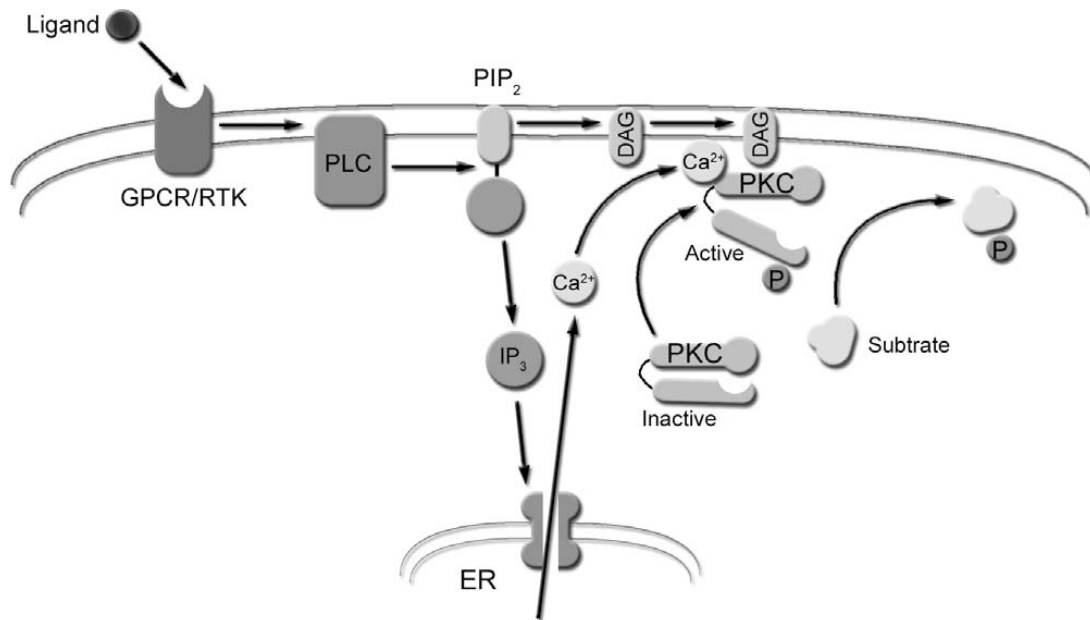

FIGURE 3: ACTIVATION OF PROTEIN KINASE C. Agonist binding to either of G-protein coupled receptors (GPCR) or receptor tyrosine kinases (RTK) initiates a signaling cascade that leads to phosphatidylinositol hydrolysis and generation of the DAG second messengers, which serves to localize PKC enzymes to the membrane and results in their activation. Reproduced from Koivunen *et al*. (2006).

Regulation of PKC activity

The regulation of PKC isozyme activity is achieved by a number of mechanisms including phosphorylation of Ser/Thr and tyrosine (Tyr) residues, site-specific proteolysis, ubiquitination, specific interactions with proteins, lipids and phorbol esters such as phorbol 12-myristate 13-acetate (PMA), and all of these may modulate protein function or help target the different

isozymes to specific subcellular locations (Steinberg, 2008; Griner and Kazanietz, 2007; Parker and Murray-Rust, 2004; Newton, 1995).

(I) *Phosphorylation*:

(a) Serine/Threonine priming phosphorylations: Phosphorylation of Ser/Thr residues at the activation loop of the enzymatic active site is required for the activity of all PKC isozymes except for PKCδ, which has a glutamate residue that can act as a phospho-mimetic active site phosphorylation in lieu of a phospho-Thr505 residue, but can be phosphorylated at the Thr residue nonetheless (Steinberg, 2008). Newly synthesized enzymes are targeted by the phosphoinositide-dependent kinase 1 (PDK1) protein kinase for phosphorylation at a conserved Ser/Thr residue within the active site, and this phosphorylation helps align the active site residues for optimal catalysis, generating an active enzyme (Newton, 1995; Griner and Kazanietz, 2007). The active enzyme then autophosphorylates at two residues located within the proline-rich "turn" motif and within the hydrophobic motif, both localized to the V_5 region of the enzyme (Steinberg, 2008). These phosphorylations help stabilize the active form of the enzyme, and serve to keep it in a stable, closed conformation with the pseudosubstrate domain occupying the active site, after which the enzyme relocalizes to the cytosol (Freeley *et al*., 2011). The enzyme is thus said to be "primed" for activation and remains in this inactive, but catalytically competent state, until activated by its specific cofactors when an appropriate signal is received (Newton, 1995). The active loop and C-terminus phosphorylations can also be mediated by other kinases as evidenced for PKCδ in which the PKCε isoform was found to phosphorylate the activation loop, and PKCζ and the mammalian target of rapamycin

(mTOR) were found to mediate phosphorylation of the C-terminal hydrophobic motif (Rybin *et al*., 2007; Parekh *et al*., 1999; Zeigler *et al*., 1999).

(b) Tyrosine phosphorylations: Phosphorylation at a number of Tyr residues in PKCδ has been reported to increase its activity, alter its substrate specificity and even activate the enzyme independently of DAG under oxidative stress conditions (Steinberg, 2004). Tyrosine phosphorylations have also been reported to enhance the membrane affinity of a phosphorylated C2 domain in PKCθ and to modulate the enzymatic activities of aPKCs ζ and λ/ι (Liu *et al*., 2000; White *et al*., 2002).

(II) *Proteolysis*: Caspase-dependent cleavage of PKC isozymes δ, θ, ε and ζ has been reported as part of the apoptotic signaling cascade, and this cleavage frees the catalytic domain from the influence of the regulatory N-terminal domain. The nPKC isoforms δ, θ and ε remain catalytically active following caspase cleavage and can then mediate pro-apoptotic functions within the cell while the aPKC isozyme is presumed to be either inactivated after cleavage or thought to mediate kinase-independent functions in promoting apoptosis (Steinberg, 2008).

(III) *Subcellular localization*: A number of targeting signals are found within the functional domains in PKC enzymes that direct the enzymes to specific organelles such as the nucleus, ER and the Golgi apparatus (Steinberg, 2008). Receptors for activated C kinase (RACK) proteins are also present in the cell where each RACK protein interacts with its specific kinase and helps localize the activated kinase in close proximity to its unique substrates (Parker and Murray-Rust, 2004; Steinberg, 2008).

(IV) *Ubiquitination*: PKC isozymes are thought to be become ubiquitinated and destined for degradation following their activation. The identities of the E3 ligases mediating PKC ubiquitination remain elusive (Griner and Kazanietz, 2007). Recently, RINCK has been identified as a RING-containing E3 ubiquitin ligase that targets PKC isozymes from all three subfamilies for ubiquitination and proteasome-mediated degradation (Chen *et al*., 2007). RINCK was found to localize to the plasma membrane, to bind the C1A domain of PKC isozymes directly, and to regulate the basal levels of PKC enzymes in the cell, independently of their activation states.

(V) *Phosphatidylserine binding*: Phosphatidylserine is bound by both the C1 and C2 domains of PKC enzymes and this binding is thought to increase the enzyme's affinity for the plasma membrane and help stabilize its structure possibly by interfering with intramolecular C1-C2 interactions (Steinberg, 2008).

(VI) *Phorbol esters*: Phorbol esters such as PMA are DAG analogs that can bind the C1 domains of cPKCs and nPKCs with high affinity (Mellor and Parker, 1998). Unlike DAG, phorbol esters cannot be metabolized and thus result in the constitutive activation of DAG-responsive PKC isoforms, and this activation may result in downregulation of certain isozymes (Griner and Kazanietz, 2007; Mackay and Twelves, 2007). Phorbol esters have been reported to induce tumor formation in mouse models, but it is unknown if tumorigenesis is promoted by ester-mediated activation or ester-mediated downregulation of PKC enzymes (Mackay and Twelves, 2007; Griner and Kazanietz, 2007).

Physiological relevance of the PKC family of kinases

Substrates of the PKC family enzymes are not well-defined, possibly owing to the highly conserved structural features amongst the isozymes, rendering the identification of isozyme-specific substrates a difficult task. Nonetheless, knockout mouse models have been useful in identifying isozyme-specific roles. PKCα, δ, and η have been implicated as tumor suppressors, and disruptions in these enzymes have been shown to enhance tumorigenesis and/or increase resistance to apoptosis (Oster and Leitges, 2006; Humphries *et al.*, 2006; Chida *et al.*, 2003). Transgenic mice overexpressing PKCε in the skin were found to be sensitized to UV-induced carcinogenesis (Reddig *et al.*, 2000). Both PKCθ and PKCι knockout mice have implicated these isozymes as regulators of insulin function (Kim *et al.*, 2004; Farese *et al.*, 2007). Finally, the knockout mouse model of PKCɣ isozyme shows cognitive defects, implicating this enzyme as essential in the central nervous system function (Abeliovich *et al.*, 1993). Apart from the PKCι knockout mice, none of the PKC knockout mice are embryonic lethal and display subtle phenotypes (Farese *et al.*, 2007). It is thus evident from the near-normal phenotypes of these mice that some level of functional redundancy exists between the different PKC isozymes (Griner and Kazanietz, 2007). Understanding how each of these enzymes mediates their isozyme-specific function is contingent upon elucidation of isozyme-specific substrates and the mechanisms governing these functions are presently unknown. Aberrant expression of these isozymes has been reported in a number of cancers, and anti-cancer drug trials that target PKC isozymes have been undertaken with little success (Griner and Kazanietz, 2007; Mackay and Twelves, 2007). The elucidation of isoform-specific roles and substrates, as well as their

regulation, will clarify the role that they play in tumorigenesis and provide basis for rational, isoform-specific anti-cancer drug design.

THE PROTEIN KINASE C δ

PKCδ is a 78 kilo-Dalton (kDa), pro-apoptotic member of the novel subfamily of PKC enzymes. When a ligand engages its cognate GPCR or RTK at the cell surface, activation of phospholipase C ensues which results in PI hydrolysis and generation of the DAG second messenger (Parker and Murray-Rust, 2004; Figure 3). DAG then recruits an inactive PKCδ to the membrane and triggers a conformational change that dislodges the pseudosubstrate motif from the active site, resulting in an active PKCδ enzyme (Newton, 1995; Figure 3).

PKCδ-mediated signaling is preferentially activated in response to DNA damage-induced apoptosis and participates in the intrinsic apoptosis pathway, however, its precise role in the apoptotic response is ill-defined although it is known to elicit loss of the mitochondrial membrane potential and result in cytochrome *c* release, both hallmarks of mitochondrial apoptosis (Brodie and Blumberg, 2003). Only a few PKCδ substrates have been identified to date which include the DNA-dependent Protein Kinase (DNA-PK), phosphorylation of which results in the dissociation of DNA-PK from DNA and blockage of DNA repair, and lamin B, where phosphorylation promotes lamin B degradation by Caspase-6 and aids in the disassembly of nuclear lamina in apoptosis (Bharti *et al.*, 1998; Cross *et al.*, 2000). Furthermore, PKCδ-mediated phosphorylation of the induced myeloid leukemia cell differentiation protein Mcl-1 may help target the latter for proteasome-mediated degradation while that on Ser46 of p53

protein commits p53 to apoptotic signaling through upregulated expression of the pro-apoptotic p53 target genes *PUMA* and *NOXA* (Sitailo *et al*., 2006; Yoshida *et al.*, 2006; Li *et al*., 2005; Ichwan *et al*., 2006). PKCδ can also be proteolytically activated during apoptotic signaling by Caspase-3, which targets the hinge region of the enzyme and separates the C-terminal kinase domain from the N-terminal regulatory region, which results in the release of a constitutively active PKCδ enzyme (Emoto *et al*., 1995; Ghayur *et al*., 1996). Strikingly, a PKCδ-/- knockout mouse model and two patients with a homozygous, loss-of-function or deletion mutations in the PKCδ gene display signs of a lymphoproliferative disorder with B-cell hyperproliferation and splenomegaly, supporting an *in vivo* anti-proliferative, pro-apoptotic role for this kinase (Miyamoto *et al*., 2002; Kuehn *et al*., 2013; Salzer *et al*., 2013). Finally, the apoptotic response is severely impaired in cells isolated from both the knockout mice and from an aforementioned patient, and enforced expression of PKCδ in these cells helps restore their apoptotic competence, further highlighting the role of this isozyme as an important mediator of the apoptosis pathway *in vivo* (Humphries *et al.*, 2006; Kuehn *et al*., 2013).

Regulation of PKCδ function takes on many forms. Like other PKC isozymes, the maturation of the enzyme involves priming Ser/Thr phosphorylations at the turn and hydrophobic motifs while a phosphorylation at the active loop is not required for enzyme maturation but does enhance catalytic activity (Stempka *et al*., 1997; Rybin *et al*., 2003; Steinberg, 2004). In addition, regulatory phosphorylation on multiple tyrosine sites have been identified and are thought to be mediated by Src family kinases and the Abl tyrosine kinase, and the *in vivo* function of these modifications may be to activate the enzyme independently of ligand-induced signaling, to serve as docking sites for other proteins or to increase Caspase-3-

mediated cleavage of PKCδ (Blake *et al*., 1999; Yuan *et al*., 1998; Steinberg, 2004; Freeley *et al*., 2011; Blass *et al*., 2002). It is believed that pools of differentially phosphorylated PKCδ protein may exist in the cell and that these phosphorylation codes may target different forms of PKCδ to different substrates and/or cellular locales (Steinberg, 2004). PKCδ can be shuttled to different cellular compartments in response to different stimuli; it can localize to the plasma membrane, upon DAG or phorbol ester binding to its C1 domain, to the nucleus, due to a nuclear localization signal housed in its C-terminus, and to the mitochondria (Brodie and Blumberg, 2003; DeVries *et al.*, 2002). Finally, DAG- and phorbol ester-mediated activation of PKCδ is known to induce its ubiquitination and subsequent 26S proteasome-mediated degradation, however, the identity of the E3 ubiquitin ligase mediating this step is still elusive (Lu *et al*., 1998). Recently, RINCK has been identified as an E3 ubiquitin ligase that targets PKC isozymes from all three subfamilies for proteasomal degradation (Chen *et al*., 2007). However, RINCK does not participate in the PMA-induced degradation of PKC isozymes and the levels of the PKCδ isozyme were less sensitive to *in vivo* manipulations of RINCK protein expression, suggesting that RINCK primarily functions in the regulation of PKC enzymes' basal cellular levels independently of their activation status (Chen *et al*., 2007).

THESIS RATIONALE

In addition to p53, the Pirh2 E3 ubiquitin ligase targets a number of cellular substrates for ubiquitination including c-myc, p27/Kip1 and DNA polymerase η. A GST-pulldown assay using Pirh2 as bait had been previously employed to identify novel substrates and interacting protein partners of Pirh2, resulting in the identification of an N-terminal fragment of PKCδ as an interacting partner of Pirh2 (Tai, 2009). Activated PKCδ enzyme is known to be targeted for ubiquitination and subsequent proteasome-mediated protein degradation, however, the identity of the E3 ligase mediating this ubiquitination remains elusive (Lu *et al.*, 1998). The objective of this research project was to validate a putative interaction between the Pirh2 and PKCδ proteins, to characterize this interaction and elucidate its significance. I tested the hypotheses that (i) PKCδ is a novel substrate of the Pirh2 E3 ubiquitin ligase; and that (ii) Pirh2 targets PKCδ protein for ubiquitination and subsequent protein degradation. Pirh2 may thus serve to functionally inactivate PKCδ and interfere with the PKCδ-mediated apoptotic response.

MATERIALS AND METHODS

Cell culture. Immortalized kidney fibroblast cell lines derived from wild-type (WT) and Pirh2 null (Pirh2-/-) mice were previously generated by Elizabeth Tai in the Benchimol lab (Tai, 2009). Briefly, mouse kidney was harvested and separated into cells by passing through a 70 μm strainer. Cells were incubated in α-minimum essential medium (α-MEM) supplemented with 10% fetal calf serum (FCS) at 37°C, 5% CO_2 overnight, after which, cellular debris and media were removed and replaced with fresh α-MEM, 10% FCS. Adhered cells were washed with phosphate-buffered saline (PBS) and maintained in α-MEM, 10% FCS for one week, and were then immortalized using the 3T3 protocol (Xu, 2005). Immortalized wild-type and Pirh2-/- cells were maintained in α-MEM supplemented with 10% fetal bovine serum (FBS) at 37°C, 5% CO_2. H1299 cells in which endogenous Pirh2 protein expression can be inducibly knocked down (H1299-Pirh2-KD) were provided by Drs. Xinbin Chen and Rasqallah Hakem (Jung *et al.*, 2010). Both parental H1299 and H1299-Pirh2-KD were maintained in RPMI-1640 medium supplemented with 10% FBS at 37°C, 5% CO_2. The MCF-7 cell line was maintained in α-MEM supplemented with 10% FBS at 37°C, 5% CO_2. MCF-7 cells stably transfected with Pirh2 expression vectors encoding either the wild-type protein, the C164A and H169A mutants, both of which disrupt the RING domain of Pirh2 by mutating the Cys and His at positions 164 and 169, respectively, to Alanine (A; Ala), or the ΔRING Pirh2 mutant which lacks the RING domain motif, were all previously generated by Roger Leng in the Benchimol lab (Leng *et al.*, 2003).

Mouse organs. Mouse kidney, lung and liver from matched WT and Pirh2-/- mice were kind gifts from Dr. Rasqallah Hakem (Hakem *et al.*, 2011). Organs were minced and lysed in 2X SDS Lysis Buffer (0.136 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol) and the lysates were aliquoted and frozen at -80°C.

Cell Lysis, SDS-PAGE and immunoblotting. Cells were washed once with PBS and lysed directly in 2X SDS Lysis Buffer on ice. Lysates were then heated at 99°C for 5 minutes, followed by centrifugation at 14000 x *g* for 10 minutes; to pellet cellular debris. Protein concentration was determined using the BCA Protein Assay (Pierce). A pre-determined amount of total protein was mixed with 0.04% bromophenol blue and 100 mM dithiothreitol (DTT), loaded onto a 12% polyacrylamide gel containing SDS, resolved by electrophoresis, and transferred onto polyvinylidene difluoride (PVDF) membranes.

The PVDF membrane was blocked using TBST (20 mM Tris-HCl, pH 7.5, 0.3 M NaCl, 0.05% Tween-20) containing non-fat milk and/or bovine serum albumin (BSA). Primary and secondary antibodies were prepared in TBST containing non-fat milk and/or BSA and incubated with the membrane for pre-determined time periods. TBST was utilized for all intermediate washes of the membrane.

Enhanced chemiluminescence (ECL) (Amersham Biosciences) was used to detect proteins of interest on the PVDF membrane. Equal protein loading of each sample was confirmed by re-probing of membranes with β-actin antibody.

Antibodies. The following antibodies and their respective dilutions were used for immunoblotting: rabbit anti-human PKCδ (C-20 (sc-937), Santa Cruz, 1:5000), rabbit anti-human PKCδ (CS-#2058, Cell Signalling, 1:1000), rabbit anti-human Pirh2 (A300-357A, Bethyl Laboratories, 1:5000), GST-purified rabbit anti-mouse Pirh2 (serum, Benchimol lab, 1:5000), crude rabbit anti-mouse Pirh2 (serum, Benchimol lab, 1:10,000), mouse anti-human p53 (DO-1 (sc-126), Santa Cruz, 1:1000), mouse anti-β-actin (A2228, Sigma Aldrich, 1:60,000), sheep anti-mouse horse-radish peroxidase-(HRP-) conjugated antibody (515-035-003, Jackson

ImmunoResearch Inc., 1:10,000-1:50,000) and goat anti-rabbit HRP-conjugated antibody (111-035-003, Jackson ImmunoResearch Inc., 1:30,000).

shRNA-mediated Pirh2 protein knockdown. Treatment of the H1299-Pirh2-KD cell line with tetracycline (Tet) induces shRNA-mediated knockdown of endogenous Pirh2 expression (Jung *et al.*, 2010). Both parental H1299 and H1299-Pirh2-KD cells were treated with tetracycline for 72 hour to completely knockdown endogenous Pirh2 expression. Following treatment with Tet for 48 hours, cells were treated with PMA, a DAG analog that is known to activate PKCδ and induce its ubiquitination and subsequent degradation, for 24 hours (Lu *et al.*, 1998). Cells were lysed in 2X SDS Lysis Buffer and lysates were assayed by immunoblotting.

Transient overexpression of Pirh2 protein. MCF-7 cells were transfected for 48 hours using Lipofectamine 2000 (Invitrogen), as per manufacturer instructions, with empty vector or with Pirh2 expression vectors encoding wild-type protein (5 or 10 μg, as indicated) or mutant Pirh2 proteins with defective E3 ligase activity (C164A and H169A Pirh2 expression vectors, 5 μg; Leng *et al.*, 2003). Cells were either left untreated or treated with PMA for an additional 24 hours prior to lysis in 2X SDS Lysis Buffer. Lysates were assayed by immunoblotting.

Stable overexpression of wild-type, C164A, H169A and ΔRING Pirh2 proteins. Both parental MCF-7 cells and MCF-7 cells stably transfected with Pirh2 expression vectors C164A, H169A and ΔRING were treated with PMA for time periods ranging from 30 minutes to 48 hours to induce activation and degradation of PKCδ protein. At the appropriate time point, cells were lysed in 2X SDS Lysis Buffer and lysates were assayed by immunoblotting.

RESULTS

Pirh2 interferes with steady state levels of PKCδ protein in immortalized kidney fibroblasts. The levels of PKCδ protein were assessed in extracts of immortalized kidney fibroblasts from wild-type (WT) and Pirh2 null (Pirh2-/-) mice under normal growth conditions. If Pirh2 is indeed the E3 ligase responsible for ubiquitin-mediated degradation of PKCδ, then the endogenous levels of PKCδ protein should be elevated in the Pirh2-/- cells. Indeed, expression of the full length PKCδ protein, seen as a band around 72 kDa, was elevated in the Pirh2-/- kidney cells compared with WT kidney cells (Figure 4). After the membranes were incubated with anti-Pirh2 antibody, I confirmed that the Pirh2-/- cells lacked Pirh2 protein expression, and thus the differential levels of PKCδ protein expression were likely a result of the altered Pirh2 protein expression (Figure 4). β-actin was used as a protein loading control (Figure 4).

Since the kidney fibroblasts cell lines were immortalized, we wondered whether this increase in PKCδ levels might be an artifact of the immortalization procedure, and wanted to confirm the results in a more physiologically relevant setting. Thus, the same experiment was repeated using mouse organs from matched WT and Pirh2-/- mice.

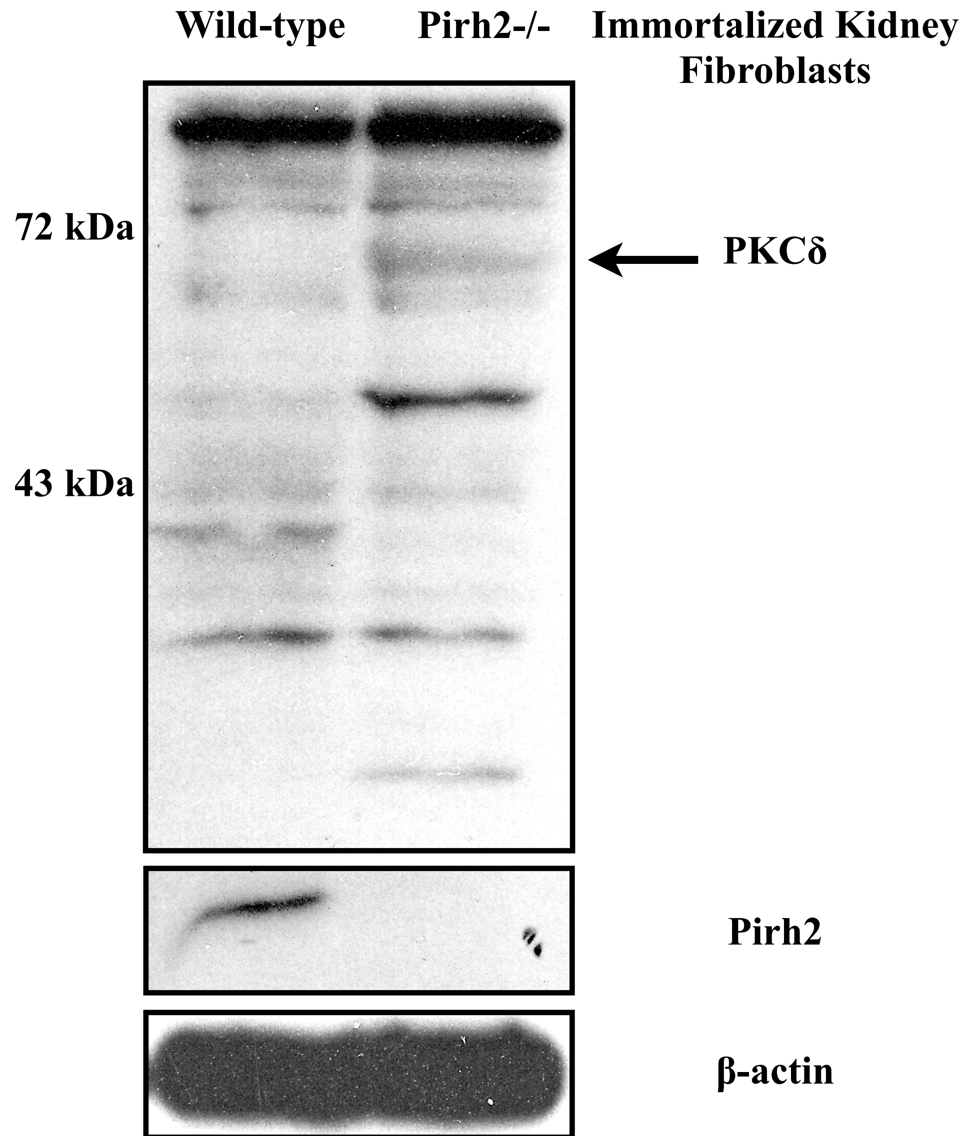

FIGURE 4: PIRH2 INTERFERES WITH THE STEADY STATE LEVELS OF PKCδ IN IMMORTALIZED KIDNEY FIBROBLASTS. Protein extracts from WT and Pirh2-/- immortalized kidney fibroblasts were resolved by SDS-PAGE and analyzed by immunoblotting with antibodies to PKCδ (sc-937) and Pirh2. β-actin was used as a protein loading control. Arrow indicates full-length PKCδ.

Pirh2 interferes with steady state levels of PKCδ in mouse kidney and lung tissues. PKCδ protein expression in mouse kidney, lung and liver organs from WT and Pirh2-/- mice was assessed by immunoblotting (Figure 5). In both the kidney and the lung, full-length PKCδ protein levels were elevated in the Pirh2-/- tissues compared with WT tissues, although the effect was more pronounced in the lung (Figure 5). An additional band at 34 kDa, unique to tissues derived from Pirh2-/- mice, was also observed, however, the identity of this band remains unclear, but may represent a fragmented PKCδ (Figure 5). The C-terminus catalytic domain, at 40 kDa, is not expected to run at this level in the protein gel. Pirh2 protein was expressed in both WT kidney and lung, and absent in the matched Pirh2-/- organs (Figure 5). β-actin was used a protein loading control (Figure 5).

A unique banding pattern was observed in the Pirh2-/- liver and not in the WT tissue (Figure 5). Of note, higher expression was also observed of 34 kDa band in the Pirh2-/- liver compared to the WT tissue, and at 43 kDa, where the C-terminus catalytic fragment is expected to run, however, no such differential is observed at 72 kDa, where a band corresponding to full-length PKCδ protein would be expected (Figure 5). However, both organs seemed to lack Pirh2 protein expression, and thus, results from this organ were not considered. It seems likely that the observed band at 34 kDa in all Pirh2-/- organs may represent a non-specific interaction with the antibody. In summary, these findings show that Pirh2 protein expression interferes with steady state protein levels of PKCδ both *in vitro* and *in vivo* in select tissues.

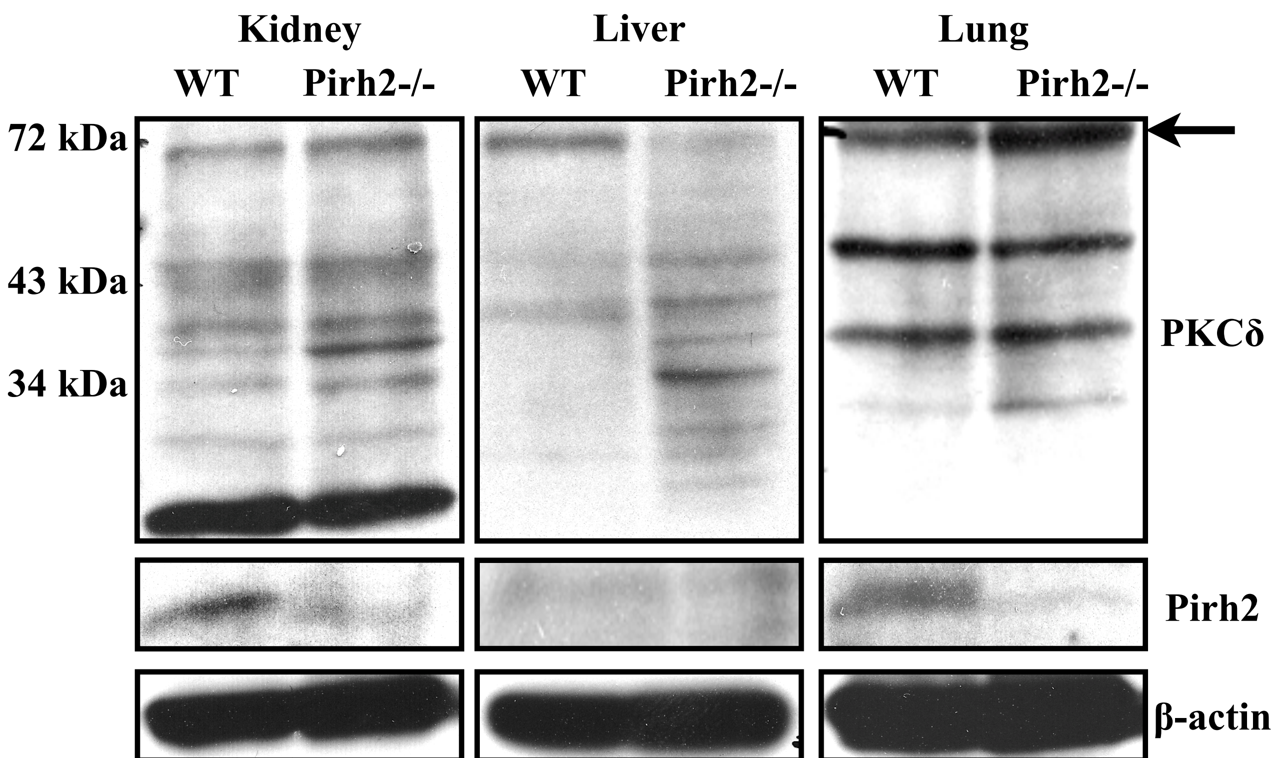

FIGURE 5: PIRH2 INTERFERES WITH THE STEADY STATE LEVELS OF PKCδ IN MOUSE KIDNEY AND LUNG TISSUES. Kidney, lung and liver tissue extracts from matched WT and Pirh2-/- mice were resolved on SDS-PAGE and assayed by immunoblotting with antibodies to PKCδ (sc-937) and Pirh2. β-actin was used as a protein loading control. Arrow indicates full-length PKCδ.

The effect of shRNA-mediated knockdown of Pirh2 expression on protein levels of endogenous and activated PKCδ. To further confirm the effects of Pirh2 protein abrogation on levels of PKCδ protein, we next used a cell system in which Pirh2 protein expression can be inducibly downregulated. H1299-Pirh2-KD cells were successfully used by Jung *et al.* (2010) to identify DNA polymerase η as a target for Pirh2 protein. We wanted to investigate the effects of Pirh2 protein abrogation on the steady state levels of PKCδ in cells with endogenous expression of both proteins.

H1299-Pirh2-KD cells were treated with and without tetracycline as described in Materials and Methods. To control for tetracycline-mediated effects on protein expression, we included the parental H1299 cell line to act as a baseline control as well as a positive control for PMA-mediated PKCδ degradation. To test whether prior activation of PKCδ was necessary for its association with Pirh2 and subsequent degradation, I treated cells with PMA, a DAG analog and PKCδ activator that induces its ubiquitination and subsequent degradation (Lu *et al.*, 1998). In the parental H1299 cell line, treatment with tetracycline should not interfere with levels of either Pirh2 or PKCδ proteins, and indeed, it did not (Figure 6, middle panel; lanes 1 and 2). Furthermore, treatment of the parental H1299 cell line with PMA should induce downregulation of full-length PKCδ protein, reflected on an immunoblot as a decrease in its protein levels, which was observed in Figure 6 (Upper panel; compare lanes 3 and 4 with lanes 1 and 2). Treatment of the H1299-Pirh2-KD with Tet should induce complete downregulation of Pirh2 protein levels, however, only a mild decrease in Pirh2 protein expression was observed (Jung *et al*., 2010; Figure 6, middle panel; compare lanes 5 and 6). This mild decrease was not accompanied by an increase in endogenous PKCδ protein levels, contrary to what we had seen in both the

immortalized kidney fibroblast cell lines and the mouse organs where Pirh2 protein levels were abrogated completely (Figures 4 and 5).

If Pirh2 is the E3 ligase mediating PMA-induced ubiquitination of PKCδ, PMA treatment of cells lacking Pirh2 protein expression should not result in PKCδ degradation. However, treatment with PMA resulted in a decrease in PKCδ protein levels, whether the cells were pre-treated with Tet or not (Figure 6, upper panel; compare lanes 7 and 8 with lanes 5 and 6). Interestingly, treatment of cells with both PMA and Tet led to a mild increase in Pirh2 protein expression, and thus interfered with Tet-induced Pirh2 protein knockdown (Figure 6, middle panel; compare lanes 6 and 8). Since Pirh2 protein expression was not successfully downregulated using the H1299-Pirh2-KD system, this cell line was not useful in identifying whether Pirh2 is the E3 ubiquitin ligase mediating ubiquitination of PKCδ in response to PMA treatment.

A band observed just below 34 kDa that disappeared with Tet treatment in H1299-Pirh2-KD cells was probably an artifact of Tet treatment, as it was not observed in the parental cell line (Figure 6). β-actin served as a protein loading control (Figure 6).

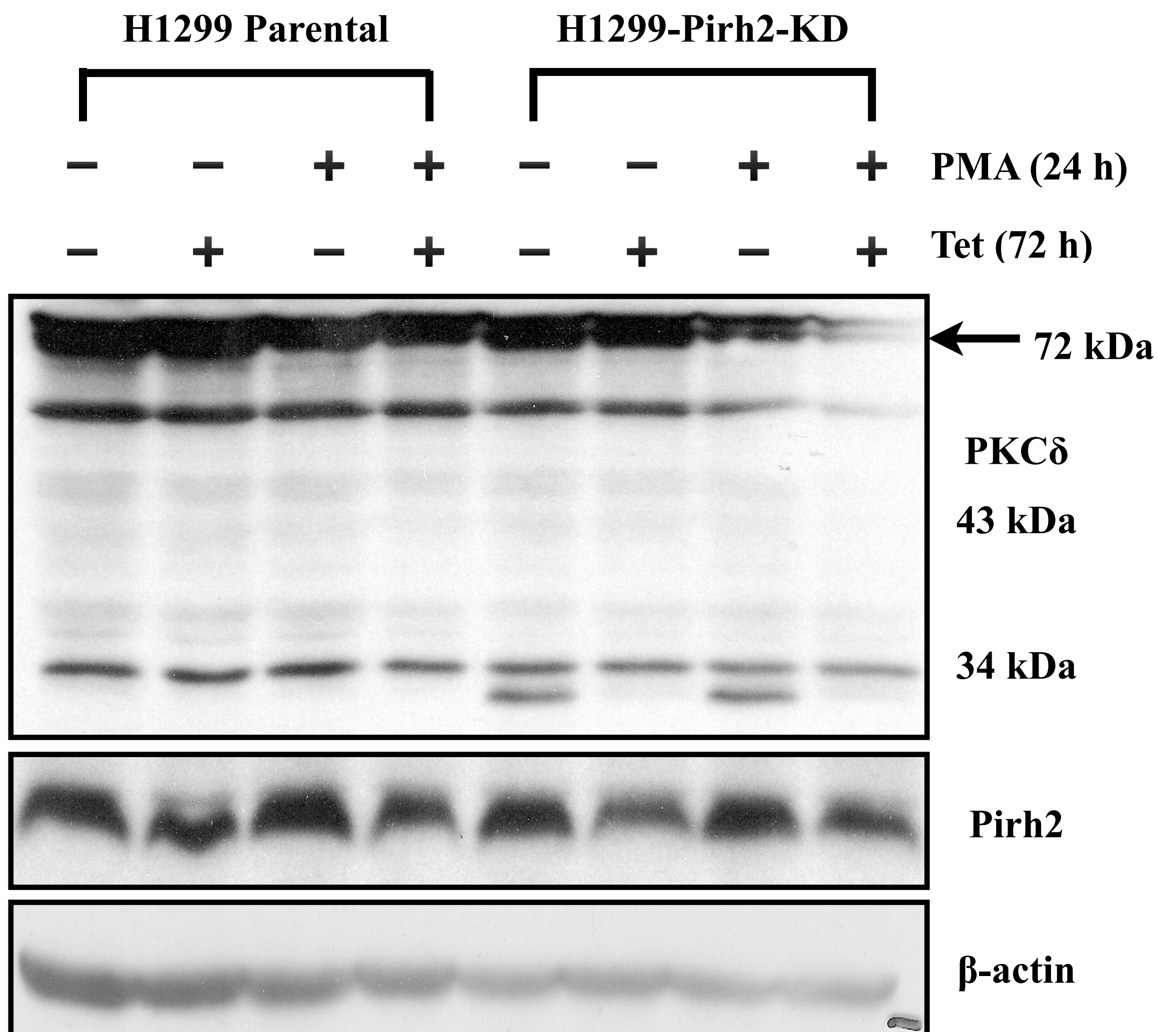

FIGURE 6: THE EFFECT OF shRNA-MEDIATED KNOCKDOWN OF PIRH2 PROTEIN EXPRESSION ON STEADY STATE AND ACTIVATED PKCδ PROTEIN LEVELS. Parental H1299 and H1299-Pirh2-KD cells were treated with Tet for 72 hours, to induce knockdown of Pirh2, and with PMA for 24 hours, to activate PKCδ and stimulate its ubiquitination. Cells were lysed, their lysates resolved by SDS-PAGE, and assayed by immunoblotting with antibodies to PKCδ (sc-937) and Pirh2. β-actin was used as a protein loading control. Arrow indicates full-length PKCδ.

Transient overexpression of Pirh2 protein does not interfere with endogenous PKCδ protein levels. In addition to shRNA-mediated knockdown of Pirh2 protein levels, overexpression of Pirh2 protein can also be used to assess the effect of Pirh2 protein on the steady state levels of PKCδ protein, and was successfully used in identifying p53 as a bonafide substrate for Pirh2 (Leng *et al.*, 2003). Thus, transient overexpression of Pirh2 protein was next attempted to investigate the effects of enforced Pirh2 protein expression on PKCδ protein levels.

MCF-7 cells were transfected with empty vector or with different amounts of Pirh2 expression vectors encoding either the wild-type protein or the C164A and H169A mutants. Both these mutants disrupt the RING domain of Pirh2, by mutating the Cys and His at positions 164 and 169, respectively, to Ala and thus express an inactive Pirh2 protein with no E3 ligase activity (Leng *et al.*, 2003). The protein levels of PKCδ, p53, and Pirh2 were assessed by immunoblotting (Figure 7). β-actin served as a loading control (Figure 7).

Ectopic expression of Pirh2 protein was assessed by comparing endogenous protein levels in cells transfected with empty vector with those in which Pirh2 protein expression was enforced using expression constructs (Figure 7). Enforced expression of wild-type Pirh2 protein did not result in a decrease in protein levels of PKCδ, contrary to what was expected (Figure 7). Steady state levels of PKCδ protein were increased in cells with enforced expression of Pirh2 mutants, compared with both empty vector and wild-type Pirh2, which indicated that the E3 ligase activity of Pirh2 may be necessary for its ability to interfere with PKCδ protein levels (Figure 7). Interestingly, p53 protein levels, expected to decrease in the presence of overexpressed wild-type Pirh2, were also unaffected in spite of the fact that Pirh2 is a validated

E3 ligase for p53 (Figure 7). Successful ectopic expression of Pirh2 protein was verified for both the wild-type protein and the H169A mutant (Figure 7).

I next decided to test whether prior activation of PKCδ was necessary for its association with Pirh2 and thus treated cells with PMA following their transfection with expression plasmids. Since the C164A construct was not efficiently expressed, it was not included in subsequent experiments.

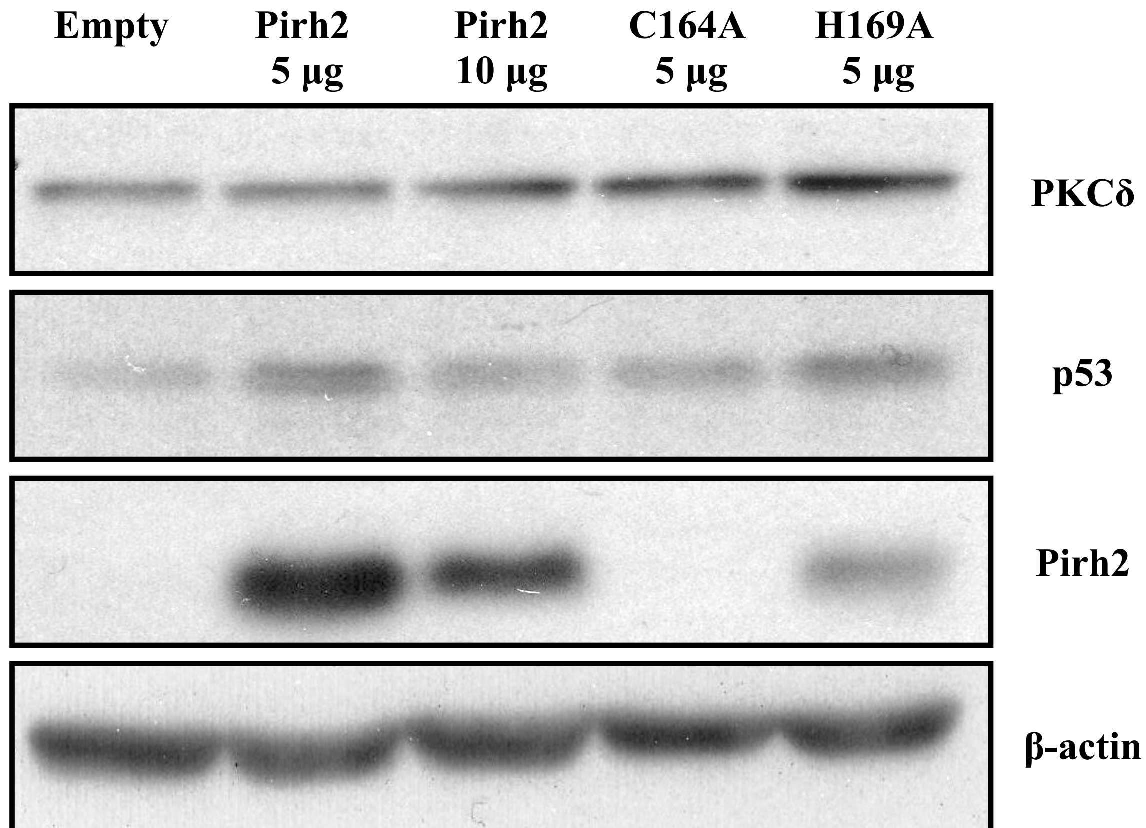

FIGURE 7: EFFECT OF TRANSIENT OVEREXPRESSION OF WILD-TYPE AND MUTANT PIRH2 PROTEIN ON STEADY STATE PKCδ PROTEIN LEVELS IN MCF-7 CELLS. Cells were transfected with empty vector or with Pirh2 expression plasmids encoding either the wild-type protein (Pirh2) or mutants which lack the E3 ligase activity (C164A and H169A). Levels of endogenous PKCδ (CS-#2058) and p53 proteins and of ectopically expressed Pirh2 proteins were assayed by immunoblotting with their respective antibodies. β-actin was used as a protein loading control.

PMA downregulates PKCδ protein levels in cells with enforced expression of both wild-type and mutant Pirh2 protein. MCF-7 cells were transfected with empty vector or with different amounts of Pirh2 expression vectors encoding either the wild-type protein or the H169A mutant. After transfection, cells were treated with PMA for 24 h. The protein levels of PKCδ, p53, and Pirh2 were assessed by immunoblotting (Figure 8).

Ectopic expression of Pirh2 protein was assessed as aforementioned. Ectopic expression of Pirh2 protein did not result in a decrease in steady-state protein levels of PKCδ (Figure 8). Steady state levels of PKCδ protein were not altered in cells with enforced expression of the H169A Pirh2 mutant (Figure 8). PMA treatment for 24 hours induced downregulation of PKCδ protein regardless of whether Pirh2 protein was overexpressed (Figure 8). p53 protein levels, expected to decrease in the presence of overexpressed wild-type Pirh2, were only mildly decreased in the presence of overexpressed Pirh2 (5 μg) but were unaffected in other lanes (Figure 8). PMA treatment induced downregulation of p53 protein as well, but this was not further investigated (Figure 8). Ectopic expression of Pirh2 protein was not observed in some samples (Figure 8). β-actin served as a loading control (Figure 8).

Based on these data, transient overexpression of Pirh2 in MCF7 cells was not further attempted. Enforced expression of the protein of interest was only seen in some of the cells transfected with expression constructs. Widespread toxicity to the cells, resulting in cell death, was observed and thus yielded a low number of surviving cells for analysis. This toxicity may also impair analysis as it may interfere with p53 protein activation, and thus with Pirh2 protein levels. From this point forward, I decided to use stable MCF-7 cells that stably express either wild-type or mutant Pirh2 protein.

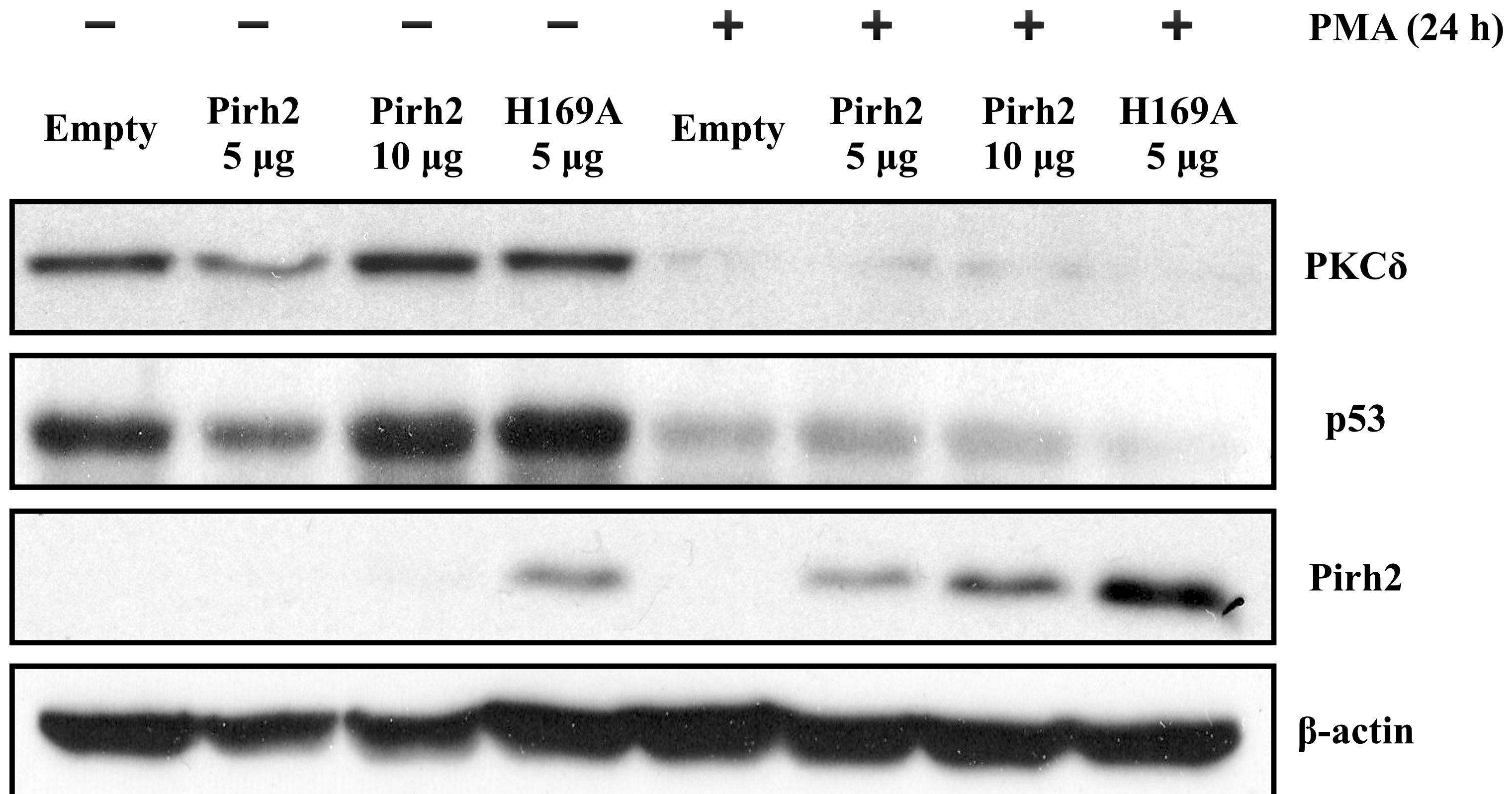

FIGURE 8: THE EFFECT OF TRANSIENT OVEREXPRESSION OF WILD-TYPE AND MUTANT PIRH2 PROTEIN ON ACTIVATED PKCδ PROTEIN LEVELS. MCF-7 cells were transfected with empty vector or with Pirh2 expression plasmids encoding either the wild-type protein (Pirh2) or a mutant lacking E3 ligase activity (H169A). Forty-eight hours following transfection, cells were treated with the PKCδ activator PMA for an additional 24 h. Levels of PKCδ (CS-#2058), p53 and ectopically expressed Pirh2 proteins were assayed by immunoblotting with their respective antibodies. β-actin was used as a protein loading control.

The effect of stable overexpression of wild-type Pirh2 protein and its mutants on PKCδ protein levels. MCF-7 cells stably transfected with Pirh2 expression vectors encoding either the wild-type protein or the C164A, H169A and ΔRING mutants were used. Parental MCF-7 cells served as a control. Cells were treated with PMA for 30 min (Figure 9) and over a time course of 3 to 48 h (Figure 10), to induce the activation and degradation of PKCδ. The protein levels of PKCδ, p53, and Pirh2 were assessed by immunoblotting (Figures 9 and 10).

Levels of endogenous PKCδ protein were unaffected in cells with enforced expression of wild-type Pirh2, compared to the parental line, but were elevated in cells with enforced expression of the Pirh2 mutants (Figure 9). Treatment of the cells with PMA for 30 min did not induce downregulation of PKCδ protein levels (Figure 9). Levels of p53 protein were only slightly increased in cells with enforced expression of the C164A and H169A mutants, but this was barely visible and deemed not significant (Figure 9). Enforced expression of Pirh2 protein was assessed by comparing Pirh2 protein expression in cells stably overexpressing Pirh2 protein with endogenous Pirh2 protein expression in parental MCF-7 cells (Figures 9 and 10). Enforced expression of Pirh2 protein was detected for the wild-type and the ΔRING proteins (Figure 9). Pirh2 protein levels of the C164A mutant were comparable to those seen in the parental cell line while the H169A mutant was not consistently expressed (Figure 9). β-actin served as a loading control (Figure 9).

PMA treatment of the parental MCF-7 cells, with no exogenous expression of Pirh2 protein, did not downregulate protein levels of PKCδ (Figure 10). Strikingly, PKCδ protein levels seemed to increase with PMA treatment in those cells, which was unexpected (Figure 10). PMA treatment of parental cells for 48 hours, however, resulted in complete downregulation of

PKCδ (Figure 10). Levels of endogenous PKCδ were increased in cells with enforced expression of wild-type Pirh2 protein when compared with the parental MCF-7 cell line (Figure 10). PMA treatment of cells with enforced expression of wild-type Pirh2 resulted in a minor decrease in PKCδ levels by the 6 h timepoint, however, PKCδ protein was still detected 48 h after PMA treatment (Figure 10). Levels of PKCδ protein were stable up to 6 h following PMA treatment in cells with enforced expression of the Pirh2 protein mutants, however, PMA still induced complete degradation of PKCδ protein by 48 h in these cells (Figure 10). These results all indicate that Pirh2 is not the E3 ligase responsible for steady state or PMA-induced downregulation of PKCδ. p53 protein levels in cells with stable expression of Pirh2 or its mutants were inconsistent with prior published data, with an increase in p53 protein levels observed in some cells with enforced expression of wild-type Pirh2 (Figure 10). Finally, Pirh2 protein was not consistently expressed while β-actin served as a loading control (Figure 10).

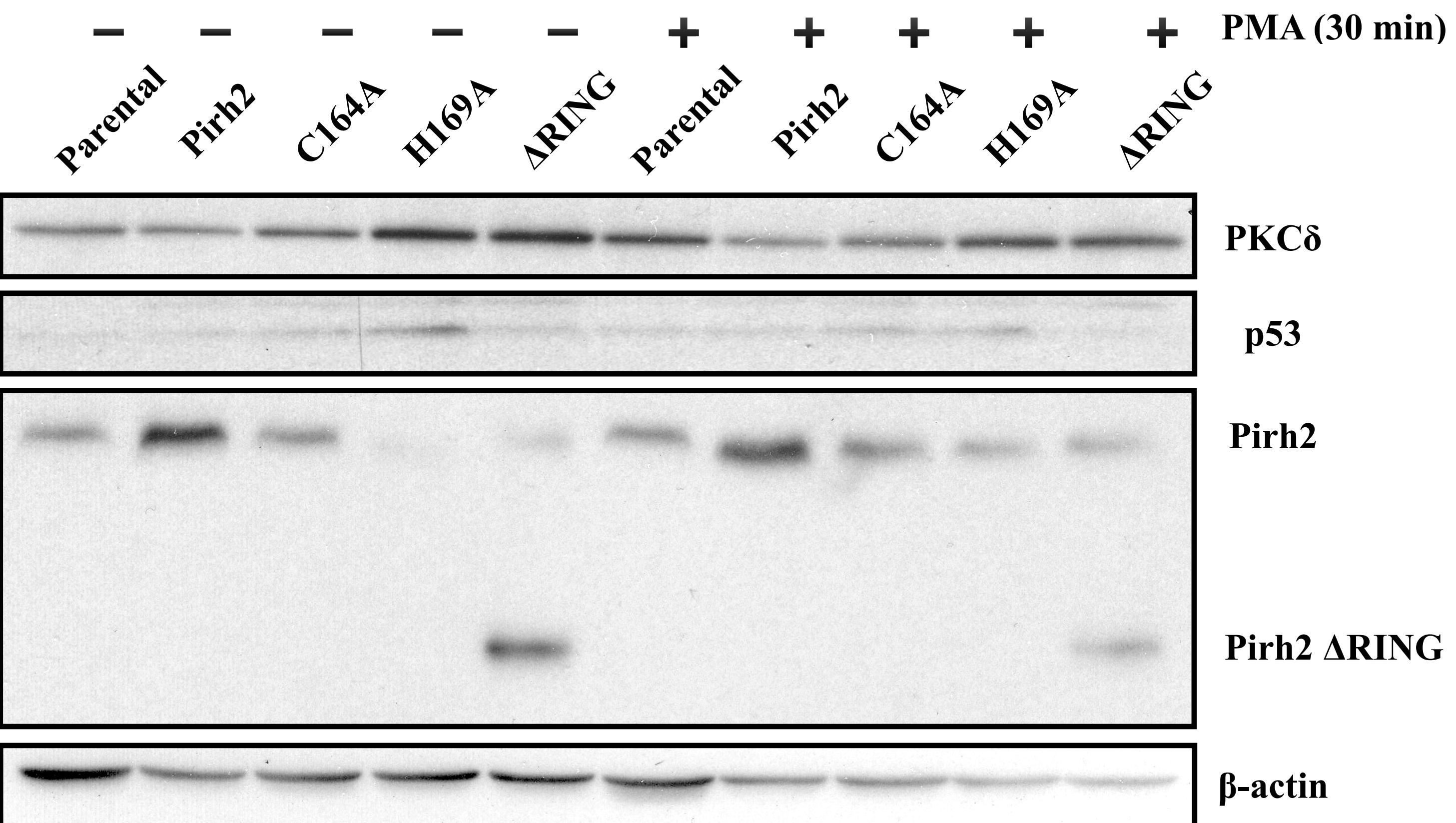

FIGURE 9: THE EFFECT OF STABLE OVEREXPRESSION OF WILD-TYPE AND MUTANT PIRH2 PROTEINS ON THE LEVELS OF ACTIVATED PKCδ PROTEIN. MCF-7 cells stably expressing wild-type Pirh2 (Pirh2) or mutants that disrupt its E3 ligase activity (C164A, H169A and ΔRING) were used (Leng *et al.*, 2003). Stable MCF-7 Pirh2 clones were treated with and without PMA for 30 min to induce activation of PKCδ. Parental MCF-7 cells served as control. Levels of PKCδ (CS-#2058), p53 and Pirh2 proteins were assayed by immunoblotting with their respective antibodies. β-actin was used as a protein loading control.

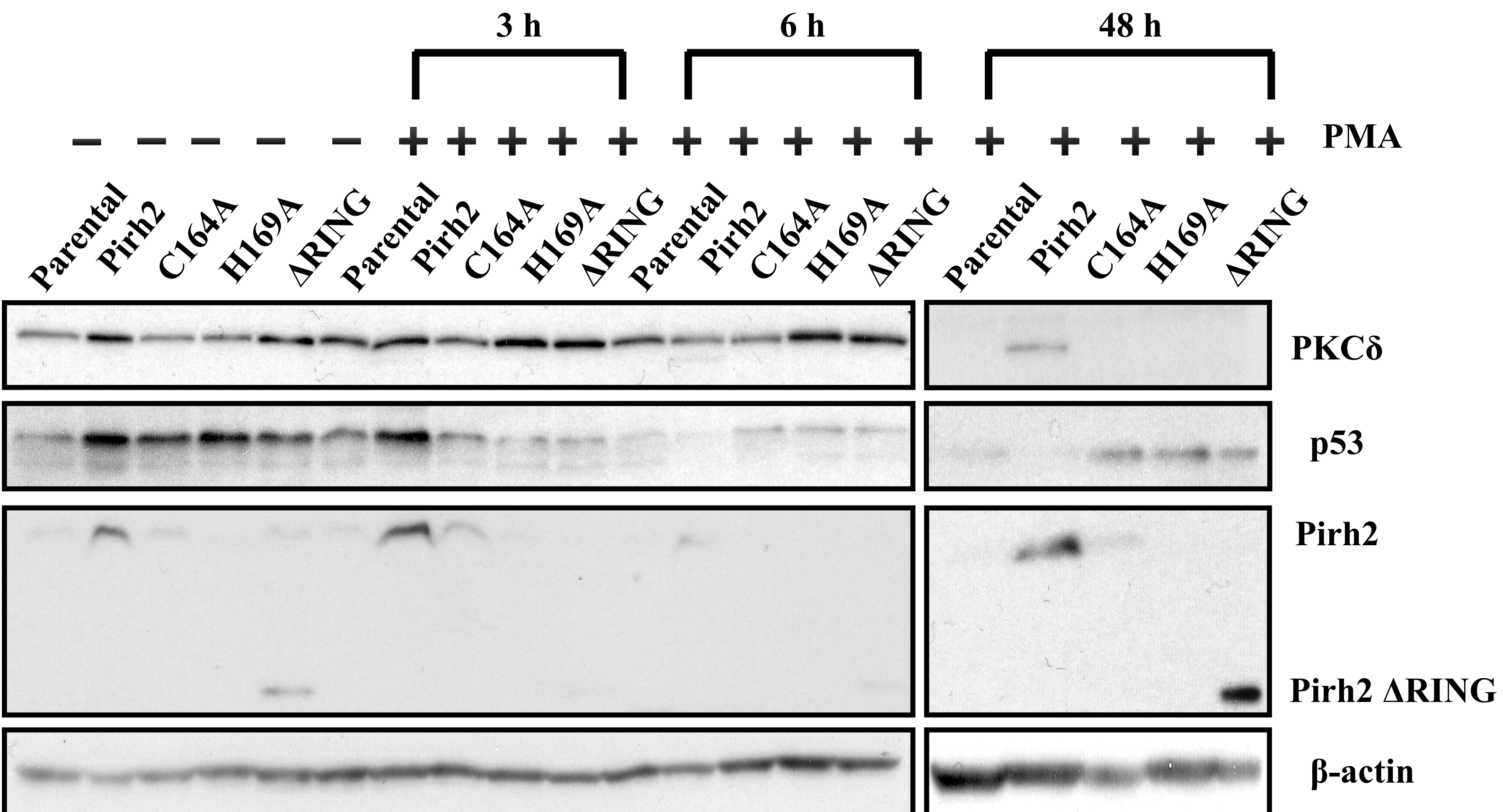

FIGURE 10: THE EFFECT OF STABLE OVEREXPRESSION OF WILD-TYPE AND MUTANT PIRH2 PROTEINS ON THE LEVELS OF ACTIVATED PKCδ. MCF-7 cells stably expressing wild-type Pirh2 (Pirh2) or mutants that disrupt its E3 ligase activity (C164A, H169A and ΔRING) were treated with and without PMA for 3 - 48 h to induce activation and downregulation of PKCδ. Parental MCF-7 cells served as control. Levels of PKCδ (CS-#2058), p53 and Pirh2 proteins were assayed by immunoblotting with their respective antibodies. β-actin was used as a protein loading control.

DISCUSSION AND FUTURE DIRECTIONS

DISCUSSION

The results of this study were suggestive, but not conclusive, of an intracellular regulatory mechanism involving Pirh2 and PKCδ. Following the identification of a novel interaction between Pirh2 and PKCδ, it was exciting to observe that levels of PKCδ protein were higher in immortalized kidney fibroblasts derived from Pirh2-/- mice as compared to those derived from WT littermates (Fig. 4). Further confirmation of the elevated levels of PKCδ protein in select Pirh2-/- tissues led us to speculate that PKCδ was a bonafide substrate of the Pirh2 E3 ligase, as similar results were observed for other, validated targets like p53 and c-myc (Fig. 5). We next investigated if the *in vitro* manipulation of cellular Pirh2 protein levels would affect those of its presumed substrate, however, those efforts were not successful (Figs. 6-10). The tetracycline-induced shRNA-mediated Pirh2 knockdown system had been successfully used in elucidating the effects of Pirh2 protein abrogation on levels of DNA polymerase η and the c-myc proto-oncogene (Jung *et al*., 2010; Hakem *et al*., 2011). The fact that Pirh2 protein expression was not consistently downregulated upon tetracycline treatment of those cells was, as such, unexpected and did not further our understanding of the regulation of PKCδ levels by Pirh2 (Fig. 6).

To circumvent this problem, we decided to assess the effect of enforced Pirh2 protein expression on levels of the PKCδ enzyme, arguing that if PKCδ was a bonafide substrate for Pirh2-mediated polyubiquitination and subsequent proteasome-mediated degradation, PKCδ levels would decrease as Pirh2 protein expression increases (Figs. 7 and 8). Furthermore, we also used expression constructs that encode an inactive Pirh2 enzyme with a destabilized RING domain, to assess whether the E3 ubiquitin ligase function was required for regulation of PKCδ

protein levels (Figs. 7 and 8). In contrast with previous data published using the same constructs, enforced Pirh2 expression was only observed in some of the transfected cellular lysates, and this increased expression did not consistently interfere with steady-state levels of either its presumed substrate, PKCδ, or its bonafide substrate p53 (Figs. 7 and 8; Leng *et al*., 2003). Transient transfection of Pirh2 expression constructs led to widespread cellular toxicity and this may have acted as a negative selective pressure such that, only cells with poor or no uptake of the expression construct were viable and could be used for protein sample collection. In light of this toxicity, MCF-7 cells with stable overexpression of Pirh2 protein or its inactive protein mutants were used to confirm if enforced Pirh2 protein expression interfered with levels of PKCδ protein (Figs. 9 and 10). Enforced Pirh2 protein expression was not observed in all samples, perhaps as result of the cells losing the expression constructs over time (Leng *et al*., 2003; Figs. 9 and 10). Furthermore, no clear conclusion could be made regarding the mechanism of Pirh2-mediated regulation of either PKCδ or p53 levels, in contrast with prior experiments using the same cells (Leng *et al*., 2003; Figs. 9 and 10). It was particularly surprising to observe that the steady state levels of p53 protein were not downregulated by enforced expression of the Pirh2 protein as had been previously published (Leng *et al*., 2003). This may be explained by the fact that subsequent research revealed that Pirh2 may preferentially target active p53 for degradation and that its role in the regulation of steady state levels of p53 may be a minor one (Leng *et al*., 2003; Hakem *et al*., 2011; Sheng *et al*., 2008).

Since it is known that the phorbol ester PMA both activates PKCδ and targets it for ubiquitination and proteasome-mediated degradation, in what has been dubbed a "suicide model", we stimulated cells with and without PMA to ensure that PKCδ can be effectively

downregulated in our cellular systems and to assess if the Pirh2 E3 ubiquitin ligase was involved in PMA-mediated PKCδ protein degradation (Lu *et al*., 1998). It was thought that the absence of Pirh2 protein expression would interfere with or, perhaps altogether impair, PMA-mediated downregulation of PKCδ protein, while the presence of overexpressed Pirh2 protein would perhaps enhance PMA-mediated PKCδ protein degradation. Surprisingly, PMA-mediated downregulation of PKCδ occurred both in the presence of elevated wild-type Pirh2 protein protein expression and in the presence of mutant Pirh2, further indicating that Pirh2 was not involved in PMA-mediated PKCδ protein degradation (Figs. 8, 9 and 10). In particular instances, PMA seemed to interfere with both Pirh2 and p53 protein levels and it is not immediately clear why this was observed (Figs. 6, 8, 9 and 10). In light of these data, it is my view that the Pirh2 E3 ubiquitin ligase is not involved in PMA-mediated downregulation of PKCδ protein levels and that PKCδ is not a bonafide substrate for Pirh2-mediated polyubiquitination and subsequent proteasomal degradation. If a physical interaction between Pirh2 and PKCδ is confirmed, this interaction may induce Pirh2-mediated mono- or multi-ubiquitination of PKCδ, much like Pirh2-mediated regulation of DNA polymerase η, which may influence PKCδ function, subcellular localization or protein interaction (Jung *et al*., 2011). Alternatively, PKCδ may target Pirh2 for phosphorylation to influence Pirh2 function or stability, much like CamKII and Cdk9 (Duan *et al*., 2007; Bagashev *et al*., 2013).

Future experiments to investigate this putative interaction should include another validated Pirh2 substrate, such as p27/Kip1, as a positive control in immunoblots, both to help clarify if the enforced expression is successful and to provide a baseline for Pirh2-mediated protein degradation (Hattori *et al*., 2007). Alternatively, treatment of cells with a DNA damaging

agent such as ɣ- or ultraviolet (UV)-irradiation or etoposide may help stimulate both p53-mediated transactivation of the *PIRH2* gene and the interaction between Pirh2 and any proteins it may bind, perhaps including PKCδ which is known to be activated downstream of such signals (Leng *et al*., 2003; Brodie and Blumberg, 2003). If the interaction is enhanced, it would help elucidate the mechanisms of Pirh2-mediated regulation of PKCδ protein levels and illuminate the physiological contexts under which such an interaction occurs to understand its significance. Under such circumstances, the Pirh2-p53 interaction would also be stimulated and a more accurate p53 positive control signal would be expected (Hakem *et al*., 2011). In fact, such DNA damaging agents have been used during the course of validating c-myc, DNA polymerase η and Chk2 to assess the effect of Pirh2 protein expression on protein levels of its substrates (Hakem *et al*., 2011; Jung *et al*., 2011; Bohgaki *et al*. 2013). Additionally, it is clear that some optimization of the cellular models used in this study is warranted, seeing as the results I have obtained were not consistent with prior published data, and thus the correct dosages of tetracycline, PMA and the Pirh2 expression constructs must be determined (Leng *et al*., 2003; Jung *et al*., 2010). If these experiments reveal an inverse relationship between Pirh2 protein expression and PKCδ protein levels, immunoprecipitation should be attempted using both endogenous and overexpressed proteins to see if the two proteins interact and form a complex *in vivo*. Thereafter, a series of GST-tagged full length and truncated Pirh2 and PKCδ proteins should be generated and used as bait in pulldown experiments to delineate the binding parameters for the putative PKCδ-Pirh2 interaction. Functional studies such as *in vitro* and *in vivo* ubiquitination assays would clarify if the E3 ubiquitin ligase function is involved in Pirh2-mediated regulation of PKCδ protein levels. To assess if Pirh2 interferes with the pro-apoptotic function of PKCδ, cells with endogenous,

enforced and no Pirh2 protein expression should be treated with apoptosis-inducing agents such as etoposide and adriamycin and levels of induced apoptosis should be determined using flow cytometry and Annexin V staining or immunoblotting for cleaved caspases (Brodie and Blumberg, 2003). Finally, the previous GST-pulldown assay using GST-tagged Pirh2 protein as bait to probe protein lysates was limited in the identification of novel Pirh2 protein partners. It required the resolution of eluates from both GST-probed and GST-Pirh2-probed pulldown experiments on a polyacrylamide gel, and only the bands that were visibly exclusive to the GST-Pirh2 eluate were excised and sent for identification by mass spectrometry (Tai, 2009). This may exclude proteins that were not as strongly bound by Pirh2, which may not be visible on a Coomassie-stained gel, and would not positively identify if GST-Pirh2 was found in the eluate, since this band was excluded from analysis based on molecular weight estimation. It is thus recommended that, after a number of stringent washes to eliminate non-specific interactions, mass spectrometry analysis should be performed on entire protein eluates from GST-pulldown experiments to identify both major and minor protein interacting partners of Pirh2. This would additionally help evaluate if the pulldown experiment was successful since validated Pirh2 substrates like p27/Kip1, p53 and c-myc would be expected in GST-Pirh2-probed samples in addition to the GST-tagged Pirh2 bait itself and would reveal a number of new interacting partners that can then be further validated.

FUTURE DIRECTIONS

In the ten years that have elapsed since the characterization of the *PIRH2* gene product as a p53-induced E3 ubiquitin ligase, much has been learnt about its *in vivo* function and the significance of having yet another E3 ubiquitin ligase targeting p53 in a negative feedback loop. A number of additional Pirh2 substrates have been identified including the p27/Kip1 cell cycle inhibitor, c-myc and Chk2 (Hattori *et al*., 2007; Hakem *et al*., 2011; Bohgaki *et al*., 2013). Furthermore, levels of Pirh2 protein have been reported to be overexpressed in lung and prostate cancers and also, surprisingly, downregulated in ovarian and breast cancers, and as such, this protein may function in both oncogenic and tumor-suppressive mechanisms in a tissue-specific manner (Duan *et al*., 2004; Logan *et al*., 2006; Hakem *et al*., 2011). Future investigations into Pirh2 protein function, its substrates and interacting partners, and its regulation will help illuminate its precise *in vivo* role and hopefully reveal the mechanisms by which it may drive tumorigenesis. To this end, we propose a number of questions that need to be addressed to help clarify the physiological function of the Pirh2 E3 ubiquitin ligase.

I. Does Pirh2 engage and complex with additional cellular partners *in vivo*?

Pirh2 has been found to ubiquitinate a number of substrates since it was characterized as a p53-specific E3 ubiquitin ligase including the c-myc proto-oncogene, DNA polymerase η, and p27/Kip1 cell cycle inhibitor (Hakem *et al*., 2011; Jung *et al*., 2011; Hattori *et al*., 2007). Additionally, Pirh2 is known to associate with other cellular proteins such as Tip60, the latter of which stabilizes Pirh2 protein, and the scaffolding protein Axin, with which it associates in an

E3-independent manner, highlighting that we have just scratched the surface on Pirh2 protein partners (Logan *et al.*, 2004; Li *et al.*, 2009). Finally, Pirh2 is known to mediate both mono- and poly-ubiquitination of distinct substrates and it is, as-of-yet, unclear how and under what contexts Pirh2 modulates its E3 ubiquitin ligase activity such that it results in a different ubiquitin outcome on different substrates (Jung *et al.*, 2011; Leng *et al.*, 2003; Bohgaki *et al.*, 2013; Hakem *et al.*, 2011). It is possible that Pirh2 may interact with cofactors and elongation partners, so called "E4s", to mediate polyubiquitination or that it may recruit proteins with ubiquitin binding domains to cap the ubiquitin chain after a certain number of ubiquitin monomers have been attached (Koegl *et al.*, 1999; Flick *et al.*, 2006; Fang and Weissman, 2004). As aforementioned, GST-pulldown experiments using Pirh2 protein as bait can be used to probe whole cell lysates for interacting proteins and the eluates from such experiments should be analyzed by mass spectrometry to generate a number of putative protein targets and cofactors for further validation. The physiological contexts behind Pirh2-target protein engagement will be clarified as the number of its identified interacting partners increases, and this will shed light on both the *in vivo* role of Pirh2 protein and its role in pathophysiological conditions.

II. What factors regulate Pirh2 protein stability and function *in vivo*?

Pirh2 is known to be phosphorylated at four distinct residues by the two protein kinases CamKII and Cdk9, however, cellular signals that direct these post-translational modifications remain elusive (Duan *et al.*, 2007; Bagashev *et al.*, 2013). Furthermore, it is known that Pirh2 can autoubiquitinate and thus downregulate its own protein levels and that polyubiquitination of Pirh2 may be stimulated by its phosphorylation, while association of Pirh2 with Tip60 sequesters

Pirh2 protein and stabilizes it (Leng *et al*., 2003; Sheng *et al*., 2008; Duan *et al*., 2007; Bagashev *et al*., 2013; Logan *et al.*, 2004). Pirh2 has a number of Ser/Thr residues interspersed within its primary structure and it is conceivable that a number of different Ser/Thr sites could be phosphorylated *in vivo*, by different kinases, to modulate its E3 ubiquitin ligase activity, alter its subcellular localization and protein interactions, or regulate its stability, in response to myriad cellular stimuli. In fact, the putative PKCδ-Pirh2 interaction that has been the subject of my thesis may result in PKCδ-mediated phosphorylation of the Pirh2 protein, as opposed to the presumed Pirh2-mediated ubiquitination of PKCδ. Intriguingly, an amino acid sequence that conforms to all but one residue of a PKCδ phosphorylation motif lies at the extreme C-terminus of Pirh2 protein, and it is interesting to speculate that PKCδ-mediated phosphorylation of this site may interfere with the Pirh2-p53 interaction, which is known to rely on the Pirh2 C-terminal domain to contact p53 (Steinberg, 2004; Sheng *et al*., 2008). Mutational analyses of these Ser/Thr residues to Ala and Asp may reveal the functional role of such phosphorylations, while bioinformatic studies of surrounding residues can clarify if these sites represent consensus phosphorylation motifs for particular kinases. Furthermore, other post-translational modifications such as neddylation and sumoylation may regulate Pirh2 protein function and stability and these possibilities remain unexplored (Duda *et al.*, 2008; Buschmann *et al.*, 2000).

III. What is the intracellular role of Pirh2 protein and what cellular pathways does it regulate?

Pirh2 protein function is not required for development and Pirh2-/- mice develop normally and are fertile (Hakem *et al*., 2011). However, the precise physiological role of Pirh2 protein remains

obscure. Insight from recent work indicates that this protein may function as a master regulator of the DNA damage response. Pirh2 is known to preferentially target active p53 protein for degradation and to interfere with p53-mediated transactivation and cell-cycle arrest (Leng *et al*., 2003; Hakem *et al*., 2011). Pirh2 has been also shown to target the DNA polymerase η for ubiquitin-independent, 20S proteasome-mediated degradation, however, it was subsequently found that Pirh2 monoubiquitinated the polymerase and interfered with polymerase η-mediated DNA repair (Jung *et al*., 2010; Jung *et al*., 2011). In another interesting example, Pirh2 polyubiquitinates and downregulates the DNA damage response protein Chk2 following its dephosphorylation, which serves to shut off the DNA damage response (Bohgaki *et al*., 2013). Unlike activation of the *Mdm2* and $p21^{WAF1}$ genes, both of which are maximally induced at 8 h, p53-induced transactivation of the *PIRH2* gene reaches a maximum 16 h after p53 is first activated in the DP16.1/p53ts cell line (Leng *et al*., 2003). This may indicate that: (a) p53 relies on those early genes in driving both cell cycle arrest and regulation of intracellular p53 levels following activation of p53 by DNA damage stress insults, and (b) Pirh2 may then be maximally induced once the DNA repair process is complete to downregulate the protein levels of the key mediators of the DNA damage response: activated p53 protein, DNA polymerase η and Chk2. Thereafter, Pirh2 may result in the ubiquitin-dependent degradation of the p27/Kip1 cell cycle inhibitor such that re-entry into the cell cycle is stimulated (Hattori *et al*., 2007). Figure 11 illustrates a proposed model of intracellular Pirh2 protein function.

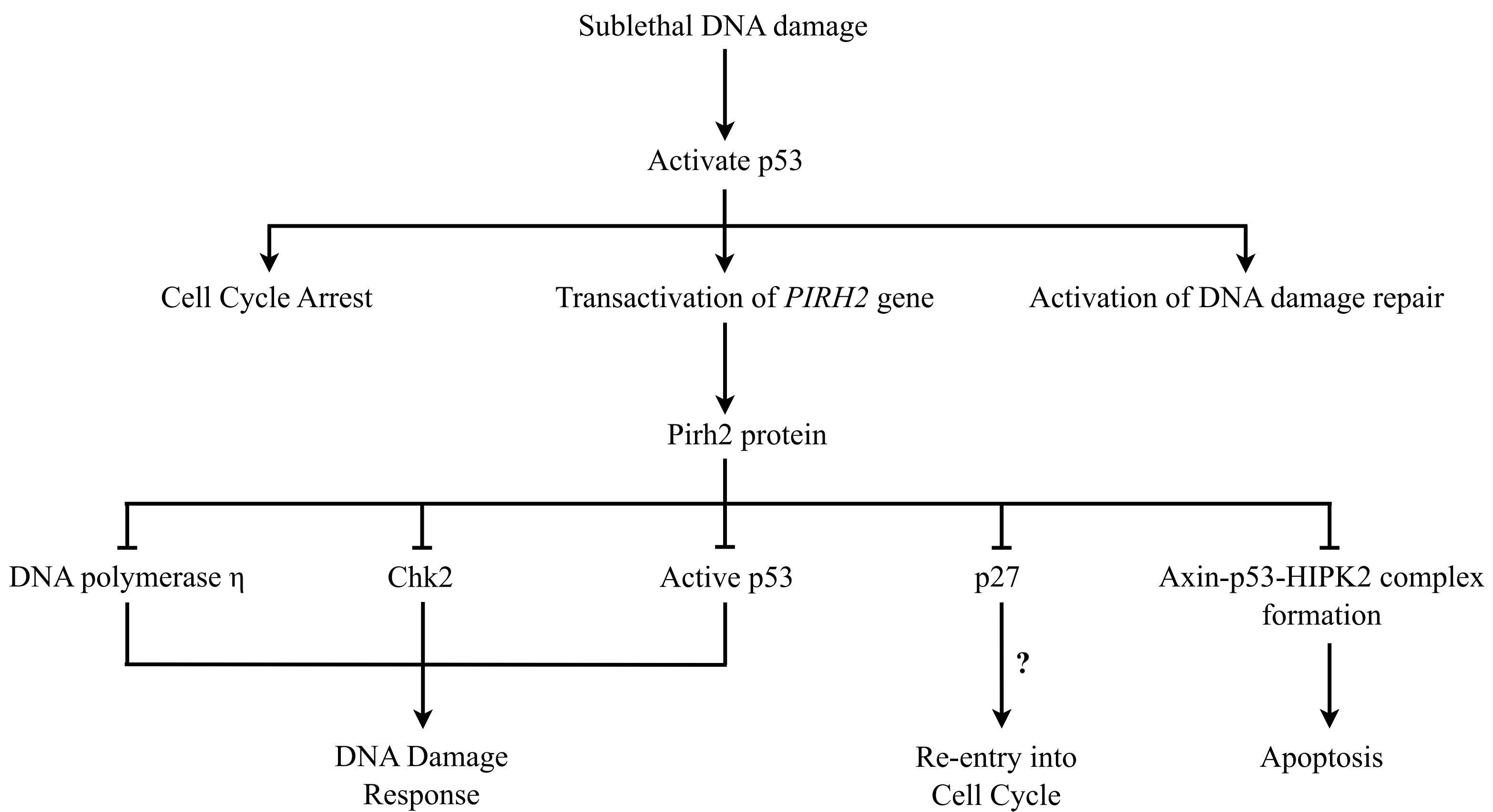

FIGURE 11: PROPOSED MODEL FOR THE INTRACELLULAR ROLE OF PIRH2. Pirh2 protein expression is induced by p53 when the cell experiences sublethal DNA damage. Pirh2 then serves to downregulate effectors of the DNA damage repair process and stimulate re-entry into the cell cycle whilst simultaneously interfering with p53-mediated apoptosis.

Notably, Pirh2 has also been shown to interfere with p53-mediated apoptosis (Leng *et al*., 2003; Hakem *et al*., 2011; Li *et al*., 2009). Pirh2 can function, independently of its E3 ligase activity, as a competitive inhibitor to block the formation of a pro-apoptotic complex between p53, the homeodomain-interacting protein kinase 2 (HIPK2) and Axin, a scaffold protein (Li *et al*., 2009). In unstressed or under sublethal levels of DNA damage, Pirh2 outcompetes the HIPK2-p53 complex from binding to Axin. However, once lethal doses of DNA damage are sensed, recruitment of the Tip60 protein to the Pirh2-Axin complex replaces Pirh2 on Axin and the scaffolding complex is then ready to bind HIPK2-p53, and mediate phosphorylation of p53 on Ser46, which is thought to prime p53 for apoptosis (D'Orazi *et al*., 2002; Li *et al*., 2009; Li *et al*., 2005; Ichwan *et al*., 2006). Strikingly, Tip60 is also known to bind Pirh2 directly and alter its subcellular location, thus the recruitment of Tip60 to the Pirh2-Axin complex may serve two purposes: (i) to outcompete Pirh2 for binding to Axin and form a multi-protein complex with Axin and HIPK2-p53 and (ii) to bind and sequester Pirh2 away from the complex such that it can no longer compete for binding (Logan *et al*., 2004). Interestingly, Mdm2 is also thought to regulate levels of Ser46-phosphorylated p53 in response to non-lethal DNA damage levels by selectively targeting the HIPK2 kinase for ubiquitination and subsequent degradation (Rinaldo *et al*., 2007). Together, these data present a compelling model for why p53 may induce Pirh2 protein expression: sublethal DNA damage insults activate p53, which results in cell cycle arrest. The expression from the *PIRH2* gene is maximally induced 16 h after p53 activation, to allow time for DNA damage repair which, after Pirh2 protein is expressed, is then terminated by Pirh2-mediated degradation of DNA damage repair effectors (Figure 11). At the same time, Pirh2 competitively inhibits the formation of a multi-protein, pro-apoptotic complex and results in the

downregulation of the p27/Kip1 cell cycle inhibitor to re-stimulate the cell cycle, meanwhile Mdm2 targets HIPK2 for degradation and as such, apoptotic signaling is not stimulated (Figure 11). p53 thus upregulates Pirh2 to regulate its own function such that p53-mediated cell cycle arrest and DNA repair is favored and apoptosis is suppressed. p53 also skilfully controls the levels of other DNA damage mediators to terminate DNA repair when needed. Once the cell encounters deadly stress, Tip60 tips the balance towards apoptotic signaling, and Pirh2 is outcompeted and sequestered. It is intriguing to speculate that lethal cellular insults may promote phosphorylation of Pirh2 to destabilize the protein such that the cell is primed for pro-apoptotic signaling. Figure 12 illustrates a proposed model for functionally inactivating Pirh2 under lethal stress conditions.

Finally, it is also notable that PKCδ itself has been shown to phosphorylate p53 on Ser46 (Yoshida *et al.*, 2006). Thus, Pirh2, if proven to target PKCδ for ubiquitination and subsequent degradation, may once again function to block the phosphorylation of the Ser46 residue and interfere with pro-apoptotic signaling by p53, under moderate cellular stress conditions (Figure 12). Alternatively, lethal stress may activate PKCδ, which, in a two-pronged approach, phosphorylates both p53, on Ser46, and Pirh2, and perhaps at the C-terminus, to activate the former and presumably destabilize the latter (Duan *et al*., 2007; Bagashev *et al*., 2013; Figure 12). The phosphorylation of Pirh2 at the C-terminus may also interfere with the Pirh2-p53 interaction, as aforementioned, to functionally inactivate Pirh2 if its stability is not affected (Sheng *et al*., 2008). Thus, PKCδ may be activated separately or in conjunction with the HIPK2-Tip60-Axin complex to stimulate pro-apoptotic signaling by p53.

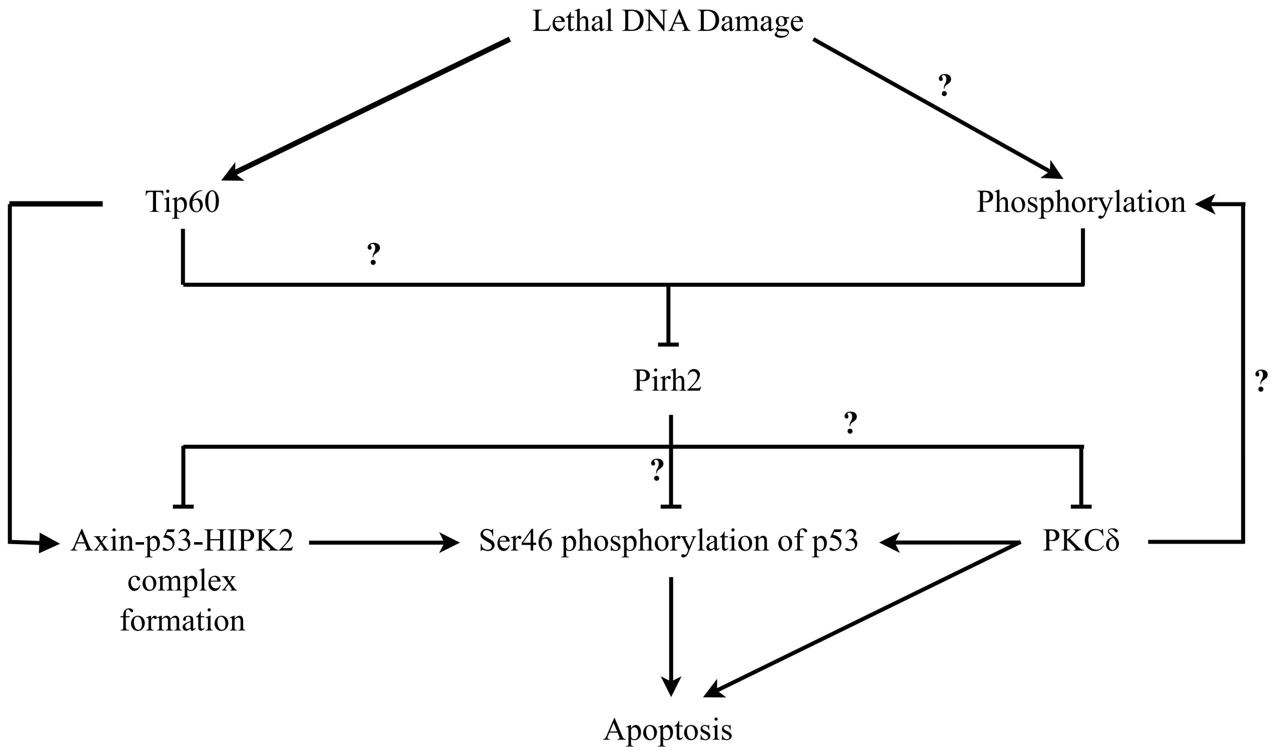

FIGURE 12: PROPOSED MODEL FOR THE FUNCTIONAL INACTIVATION OF PIRH2 BY LETHAL DNA DAMAGE. When lethal DNA damage is sensed by the cell, Pirh2 protein may be functionally inactivated by its interaction with Tip60 or by phosphorylation, which then relieves Pirh2-mediated repression of p53-mediated apoptosis. PKCδ may also participate in regulation of the anti-apoptotic role of Pirh2 or, conversely, its pro-apoptotic function may be regulated by Pirh2.

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