The Role of Pirh2 E3 Ligases in Ubiquitination and p73 Regulation

by

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Abstract

Cancer and tumor suppressors have been highly associated with many cancer researches (1). The main function of these proteins is to detect any error in DNA that might eventually leads to abnormal cell division and repair these errors; hence eliminating the risk of cancer development (2,3). Many researches focused on the p53 family protein consisting of three proteins: p53, p63, and p73 (4). p53 was the first to be discovered, and many scientists refer to this protein as the "master switch", or the "guardian of genome" because of its critical role in coordinating cellular processes (4,5). p53 acts as a checkpoint in the cell cycle monitoring all the cellular responses. At this level, should any stress signal occur affecting the cellular mechanism, p53 will be highly expressed and will trigger a variety of programs including a series of repressing and activating responses (6). Unfortunately, 50-60% of human tumors show mutations in the p53 proteins and the remainder, despite bearing wild type (WT) proteins, shows a dysfunctional system (6, 7). More attention later on also included p73 proteins after revealing their role in cell cycle arrest and apoptosis (8). The exact mechanism for the tight regulation of these proteins is not fully understood; however ubiquitination has been shown to be the master regulatory process (9, 10). Several ubiquitin E3 ligases such as MDM2, Pirh2, AIP4, UBE4B, etc.; are shown to act as regulators (11). Interestingly among all E3 ligases, Pirh2 is the only one over-expressed in a wide range of human tumors: 41, 64, 61, 70 and 82% in primary breast cancer, hepatocellular carcinoma, head and neck, prostate, and lung cancer respectively (12).

Knowing that Pirh2 is overexpressed, the first objective of this thesis was focused on examining the self-regulatory process of Pirh2 including the optimal conditions and the role of E2 enzymes. Also we mapped the domains of Pirh2 to reveal that in addition to the RING domain, commonly known for the catalytic activity, portions of the C terminal domain are also essential for Pirh2 self-ubiquitination mechanism. Besides, lysine chains (K48, K63,

and KO), known for their role in determining the substrate's fate, were analyzed showing lack of significance on the contrary to p53 ubiquitination, where K48 lysine chains are utilized.

The second objective of this thesis was to analyze Pirh2 and p73 correlation knowing that p73 share up to 80% homology with p53 at the DNA binding sequence (13). Interestingly, Pirh2 has been shown to bind, ubiquitinate and down-regulate p73 tumor suppressor function without altering p73 protein level. Lack of degradation was explained with the use of lysine chains 63 that do not trigger proteosomal degradation post ubiquitination.

The third objective of this thesis focused on Pirh2-p73 regulatory pathway especially when studies have shown that the same substrate, p73 in our case, has been shown to be regulated by other ligase: AIP4 (14). In this study, we reveal for the first time a novel correlation between two ligases within the same signalling pathway where Pirh2 physically interacts with AIP4 and significantly down-regulates its expression. This down-regulation is shown to involve the ubiquitination of AIP4 by Pirh2. At the p73 level, we showed that Pirh2 ceased the AIP4-p73 negative regulatory pathway. In-vivo and in-vitro ubiquitination analyses regarding p73 isoforms (α & β) confirmed the decrease of p73-AIP4 induced ubiquitination when Pirh2 is introduced. At the translation level and regarding p73 cell cycle arrest function in specific, Pirh2 secured p73 G1 arrest role despite overexpressing AIP4.

Overall, our findings added valuable knowledge over the E3 ligase self-regulation and also the substrate regulation. Also we introduced a novel concept correlating two independent signalling pathways. This opens a gateway to explain how E3 ligases differentiate between regulating multiple substrates that may belong to the same family of proteins as it is the case for p53 and p73 proteins; hence, providing a new framework for the development of novel anti-cancer targeting strategies.

Preface

This thesis represents collaborative work, led by Dr. Roger Leng at the University of Alberta.

Chapter 2 of this thesis has been published as:

Rami Abou Zeinab, Hong Wu, Consolato Sergi, Roger Leng. 2013. Residues 240-250 in the C-terminal of Pirh2 protein complement the function of the RING domain in self ubiquitinating Pirh2 protein. PlOS ONE, 8 (12): e82803, 1-8. I was first author of this manuscript, and contributed to the practical work, manuscript writing, and data analysis as outlined in chapter 2.

Chapter 3 of this thesis has been published as:

Hong Wu, Rami Abou Zeinab, Elsa R. Flores, Roger Leng. 2011. Pirh2, a Ubiquitin E3 ligase, inhibits p73 Transcriptional Activity by promoting its Ubiquitination. Molecular Cancer Research, 9 (12): 1780-1790. I was second author of this submitted manuscript and did the in vivo and in-vitro ubiquitination analysis as outlined in chapter 3.

Chapter 4 of this thesis is to be submitted as:

Rami Abou Zeinab, Hong Wu, Consolato Sergi, Roger Leng. 2014. Pirh2 E3 ligase inhibits the AIP4-p73 negative regulatory pathway by mediating AIP4 expression and ubiquitination. In progress to be submitted by December 2014. I am the first author of this manuscript, and contributed to all practical work, manuscript writing, and data analysis as outlined in chapter 4.

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Abbreviations

AIP4- atrophin-1-interacting protein 4

ATM- ataxia telangiectasia mutated

ATR- ataxia telangiectasia and Rad3 related

Apaf-1- apoptotic protease activating factor 1

BER- base excision repair

BRCA1- breast cancer 1, early onset

CaMKII- calmodulin dependent kinase

C-terminal- carboxy terminal

CHIP- c-terminus Hsc70 interacting protein

CHK 1- checkpoint kinase 1

CHK 2- checkpoint kinase 2

CKD- cyclin dependent kinase

COP-1- constitutively photomorphogenic-1

CTD- C-terminal domain

CXCR-4- C-X-C chemokine receptor type 4

DBD- DNA binding domain

DISC- death inducing signalling complex

DUB- deubiquitinating enzyme

E.coli- Escherichia coli

EGFR- Epidermal growth factor receptor

FADD- Fas -associated protein with death domain

HDAC-1- histone deacetylase 1

HECT- homologous to E6 associated protein carboxy terminus

HPV- human papilloma virus

HSP 70- heat shock protein 70

HSP 90- heat shock protein 90

hTERT- human telomerase reverse transcriptase

IGFBP3- insulin growth factor binding protein 3

IP-immunoprecipitation

IPTG- isopropyl-β-D-thiogalactoside

IR-ionizing radiation

K chains- lysine chains

KDa- kilo daltons

MDM2- mouse double minute 2 homolog

MEF- mouse embryonic fibroblasts

miRNA- micro RNA

NER- nucleotide excision repair

NMR- nuclear magnetic resonance

NP40- nonyl phenoxypolyethoxylethanol

NTD- N-terminal domain

NTKL - N-terminal kinase-like protein

OD- oligomerization domain

PBS- phosphate buffer saline

PCR- polymerization reaction

PERP- p53 apoptosis effector related to PMP-22

PHD- plant homeodomain

PI- propiduim iodide

PI3K- phophatidylinositide 3-kinase

Pirh2- human p53-induced RING-H2 protein

PMSF- phenylmethanesulfonyl fluoride

PTEN- phosphatase and tensin homologue

PUMA- p53 upregulated modulator of apoptosis

PVDF- polyvinylidene difluoride

RT- reverse transcription

SAM- sterile alpha motif

SDS-PAGE- sodium docedyl sulfate-polyacrylamide gel electrophoresis

Ser- Serine

siRNA- small interfering RNA

TA- transactivation domain

TET- tertamerization domain TRP- transient receptor potential

TGF-β- transforming growth factor β

Thr- threonine

TRP- transient receptor potential

Tyr- tyrosine

UB- ubiquitin

UBE4B- ubiquitin E4 binding protein

UBH- ubiquitin carboxy-terminal hydrolases

UBP- ubiquitin processing

UV- ultra Violet

WB- western blot

WT- wild type

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Chapter One

GENERAL INTRODUCTION

1.1 Cancer and tumor suppressors

According to Health Canada, cancer is considered a life threatening disease and the major cause of mortality among the Canadian population (15, 16). In 2004, the number of cases diagnosed with cancer was 145000, and in 2013 it was estimated that 187,600 Canadians developed cancer (17). Statistics has shown that two in five Canadians will develop cancer in their life time, where one out of four will eventually die of cancer. Worldwide, cancer is considered a major health problem. Cases diagnosed yearly exceed 12 million with more than 50% death rate (18). The reason that makes cancer such a complex disease is that it is not one disease by itself. It is a group of diseases characterized by uncontrollable cell division and the spread of abnormal cells that fail to respond to normal growth mechanisms. There are many different types of cancer as many as human body organs and cell types (19). Despite the fact that cancer is complex, the human body system harbours a web of tumor suppressor genes and oncogenes that mediates the tumorigenesis and terminates cancer progression (3, 19). The transformation of a normal cell to a malignant phenotype starts with different genetic changes, which make a cell resistant to anti-growth signals and cell growth programs. When such changes are not repaired and involve a disruption in the system conferring an imbalance between pro and anti-growth signals, cells will be malignant and lead to tumor formation (18-21). Cancer and tumor suppressors have been highly associated. Among different tumor suppressor families, the p53 network has taken a wide range of attention in cancer research since around 80% of human tumors show mutations in the p53 system and those bearing wild type (WT) proteins still show a dysfunctional system (3, 6).

1.2 The p53 network

The p53 family is composed of three members: p53, p73 and p63 (4). p53 was the first to be identified and characterized as a tumor suppressor (2, 3, 22). Several years after p53 characterization, two genes p63 and p73 were found to encode proteins of similar structure

and function; yet not identical to p53 (23, 24). Studies have shown that three members share very significant homology at the genomic and protein level as well (4, 25-28). Each of these members is composed of major domains. They share a DNA binding domain (DBD), an oligomerization domain (OD), and a transactivation domain (TA). Further analysis revealed the existence of a new domain only found in p73 and p63 known as SAM (Sterile Alpha Motif) motif at the C-terminus. In terms of homology, the highest level is detected at the DBD reaching 63% between p53 and p73, and 60% between p53 and p63 (Figure 1.1) (4, 27, 28).

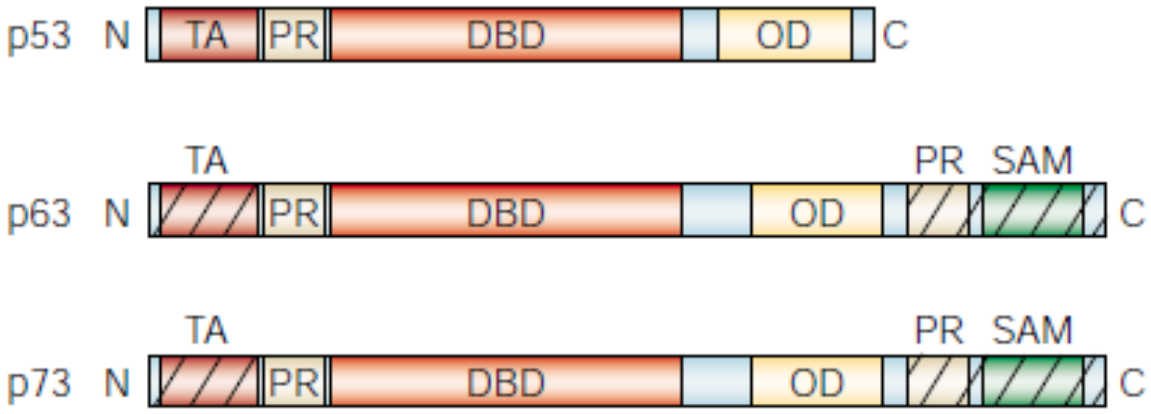

Figure 1.1. The p53 family. The p53 family includes three genes that encode p53, p63 and p73. The overall domain structure of p53, p63 and p73 is conserved. In contrast to p53, p63 and p73 have many different isoforms with distinct amino (N) and carboxyl (C) termini. Fulllength isoforms contain the transactivation domain, so are designated TA; amino-terminal-deletedisoforms are designated ΔN. Dashed lines indicate different isoforms.

Adapted with permission from [p73: friend or foe in tumorigenesis. Melino G, De Laurenzi V, & Vousden KH. Nature Reveiws Cancer 2(8). Copyright ©2002 Nature Publishing Group] (license number 3497760562481).

This homology described above was a gateway to investigate the binding and correlation between these members, and also the transactivation of similar promoters. Initially it was anticipated that members of this family form hetero-oligomers, yet studies have shown the exclusive formation of homo-oligomers between wild types (29). Also in terms of structure, p53 family proteins have different isoforms due to mRNA splicing occurring at the 3' end terminal (24). In terms of tumor suppressor function, more attention has been drawn to p53 as a tumor suppressor compared to the other two members. The p53 gene is the most frequent site for genetic alterations including all types of human tumors (1, 3, 7, 10). Investigations on animal models also showed that mice expressing mutant p53 or with knockout p53 gene are prone to tumor formation (30). Also, patients diagnosed with Li-Fraumeni syndrome that carry a mutant p53 allele have increased risk of developing cancer (31). Later, it was shown that 90% of p53 mutations are missense mutations that only block p53 transcriptional activity (32, 33). Nowadays, many scientists refer to the p53 protein as the "master switch", or the "guardian of genome", or even "cellular gatekeeper" because of its critical role in coordinating cellular processes. However, the situation was totally opposite when p53 was initially discovered.

1.3 p53 overview

1.3.1 Discovery of p53

The discovery of p53 goes back to 1970 when cancer research was focusing on tumor viruses and oncogenes. Scientists noticed the overexpression of a protein in the majority of human tumors. The size of this protein was 53kDA (393 amino acids), and that was the reason why this protein was named p53 (34, 35). Being overexpressed in human tumors made scientists anticipate that p53 acts as an oncogene. With further investigations, it was shown that p53 cooperates with Ras oncogene. Also, it forms complex with SV40 virus oncoprotein and several other oncoproteins produced by tumor viruses such as: HPV (E6) and E1B 55k protein.

Interestingly, this was totally opposite to the exact role of p53 which was revealed couple of years post its identification (36-38). The consequence of p53 binding to the oncoproteins revealed the degradation of p53 or its inactivation; hence loosing its function. Also, to resolve the contradiction of p53 overexpression in human tumors, studies analyzed these proteins to reveal that mutant or missense p53 and not WT are the ones overexpressed in tumors. Early thoughts about acting as a tumor suppressor instead of oncogenes were highlighted once studies revealed that DNA errors, which normally induce tumor suppressor expression, affected the cellular level of p53. This was detected in response to DNA damage induced by Ultraviolet (UV) radiation, Ionization radiation (IR), and chemical treatments. Since then, numerous studies analyzed the role of p53, the input that triggers its active action, the regulation of p53, the downstream events post p53 activation, and the cellular outputs (39-43).

1.3.2 Role of p53 as tumor suppressor

The role of p53 as a tumor suppressor is summarized with several cellular functions including: cell cycle arrest and DNA repair, senescence, and apoptosis all of which contribute to the anti-proliferative effect of p53 function (7, 44). Many studies in cultured cells and mice models have revealed the p53 target genes involved in every pathway. To start with, cell cycle arrest is considered one of the major tumor suppressive properties of p53. The cell cycle is a multifaceted process characterized by DNA replication and segregation of replicated chromosomes into two daughter cells from a mother cell. It is composed of checkpoints (G1/S and G2/M) that ensure the fidelity of cell division (45). At any stage, when an error occurs, progression of proliferation is blocked. p53 has been shown to transactivate components of the cell cycle checkpoints (20). p21 is a p53 induced gene that can abrogate the cell cycle at both check points through regulating the cyclin dependent kinases (CDKs). CDKs are a family of Serine/threonine kinases. More than 5 CDks in complex with cyclins have been identified to

be activated at different stages of the cell cycle. Cyclin E/CDK2 are associated with G1 arrest; whereas Cyclin B/Cdc2, essential for mitotic division, are associated with G2/M arrest. Also, p21 has the ability to activate RB tumor suppressor pathway. The consequence of inhibiting cyclin E/CDK2 results in RB hypo-phosphorylation; hence binding E2F and inhibiting the cell cycle genes mediated by E2F transcription (46, 47). Regarding G2/M, p53 has been shown to transcriptionally activate 14-3-3σ proteins after DNA damage inducing G2/M arrest (48-50). 14-3-3σ inactivates Cdc25 and Cdc2 through sequestering them to cytoplasm and disrupting their nuclear localization (51, 52). Also p53 induces microRNAs, miR34 in specific, which in turn down regulates CDK4 and MET both essential for cell cycle progression (53-55). Not only does p53 up regulate genes that promote arrest, but also it down regulates genes that promote the cell cycle, as it is the case with c-Myc (56). In many cases, the DNA error can be repaired and cells can be programmed back to cellular division and replication. p53 has also been shown to be involved in DNA repair mechanism through enhancing the excision repair mechanism. This mechanism involves the nucleotide excision repair (NER) or the base excision repair (BER). Studies have shown that p53 induced genes differ between the NER and BER. GADD45 induces NER, while BER is associated with AP endonuclease and DNA polymerase β. Other DNA repair mechanism induced by p53 involves TFIH and p53 R2 reductases (45, 57-59). p53 role is also associated with senescence, which is a permanent state of G1 cell cycle arrest. This is also accompanied by morphological changes (large and flattened cells) that are used as an indication to differentiate between a normal arrest and a senescence state. Clear evidence for p53 contribution was the lack of senescence when p53 was inactivated in MEF cells. Also, the reactivation of p53 in deficient tumors resulted in tumor regression. Further analysis revealed that senescence is the cellular process accompanied with this regression (7, 44, 60). Since RB tumor suppressors were known for their senescence effect, the p53-p21-RB correlation involving E2F gave more insight into p53 role in senescence. Also p53 can down regulate the human telomerase reverse transcriptase subunit (hTERT). hTERT prevents telomerase shortening and facilitates cell replication (61-

63). However, it remains unclear how p53 differentiates between inducing cell cycle arrest or senescence in response to DNA damage.

The major role of p53 as tumor suppressor is reported in apoptosis. In definition, apoptosis is a programmed cell death or programmed suicide triggered when the DNA damage is not reparable or when cells are in uncontrolled growth. It is considered a physiological form of cell death. At the cellular level, it is associated with several changes in cytoplasm, nucleus, and plasma membrane. Morphologically cells shrink, flatten, and round up. In the nucleus, chromatin aggregates are formed and fragmented by endonucleases; this has been used as an indication at the molecular level to confirm apoptosis. As a result, apoptotic bodies will be formed due to plasma membrane blebbing and will be later recognized and eliminated by phagocytes (64-66). WT p53 and apoptosis were first connected when studies were done on temperature sensitive mutant p53 that acquires the WT confirmation and function upon temperature shift to 32^{o}C. The detected cell death with functional p53 confirmed their role in apoptosis. Also, when the apoptotic activity was analyzed in transgenic mice with defective RB function, tumor formation rate was slow and not aggressive unless p53 was eliminated by crossbreeding with p53-null mice (67, 68). Many p53 induced genes have been identified in both the intrinsic and extrinsic apoptotic pathway, also known respectively as the mitochondrial pathway and the cell death receptor pathway. In brief, in response to DNA damage the intrinsic pathway is activated. It involves the depolymerisation of the mitochondria and releasing, into the cytosol, apoptotic inducing factors and cytochrome c, which binds apoptotic protease activating factor (Apaf-1) and procaspase 9 forming a complex known as apoptosome (Figure 1.2) (64-66). Then caspase 9 is activated and promotes a cascade of caspase activation (caspase 3, 6, and 7 all known as executioners). Members of Bcl-2 family proteins have been widely studied in the intrinsic pathway and are classified in two categories: pro-survival proteins (Bcl2 and BclX_L) and pro-apoptotic proteins (Bax, Bak, PUMA, etc.) (69-71). Extensive researches have confirmed the role of p53 in

activating apoptotic genes (PUMA, Noxa, Bax) in response to cellular stress (72-76). Also p53 represses Bcl-2 expression; hence hindering its oncogenic effect (77, 78). Interestingly, p53 induced apoptosis can be induced in a transcription independent manner (79). Such findings were initially highlighted when mutant p53s that are transcriptionally inactive still induced apoptosis (33, 80). In response to DNA damage p53 can be localized to the mitochondria, where it directly activates Bax proteins or physically interacts with Bcl-X_L (81, 82). Consequently, cyctochrome c is released and apoptosis is initiated. Regarding the extrinsic pathway, it is initiated when cell surface receptors bind their ligand transmitting the death signal from cell surface to intracellular signaling that triggers a sequence of caspase events starting with caspase 8 resulting in programmed cell death (65, 66). With respect to p53, it has been shown that p53 induces genes encoding the three transmembrane proteins: Fas, DR15 and PERP (83-86). Numerous studies have linked the intrinsic and extrinsic pathway in response to p53 function and transcriptional activation especially that they share the same execution pathway.

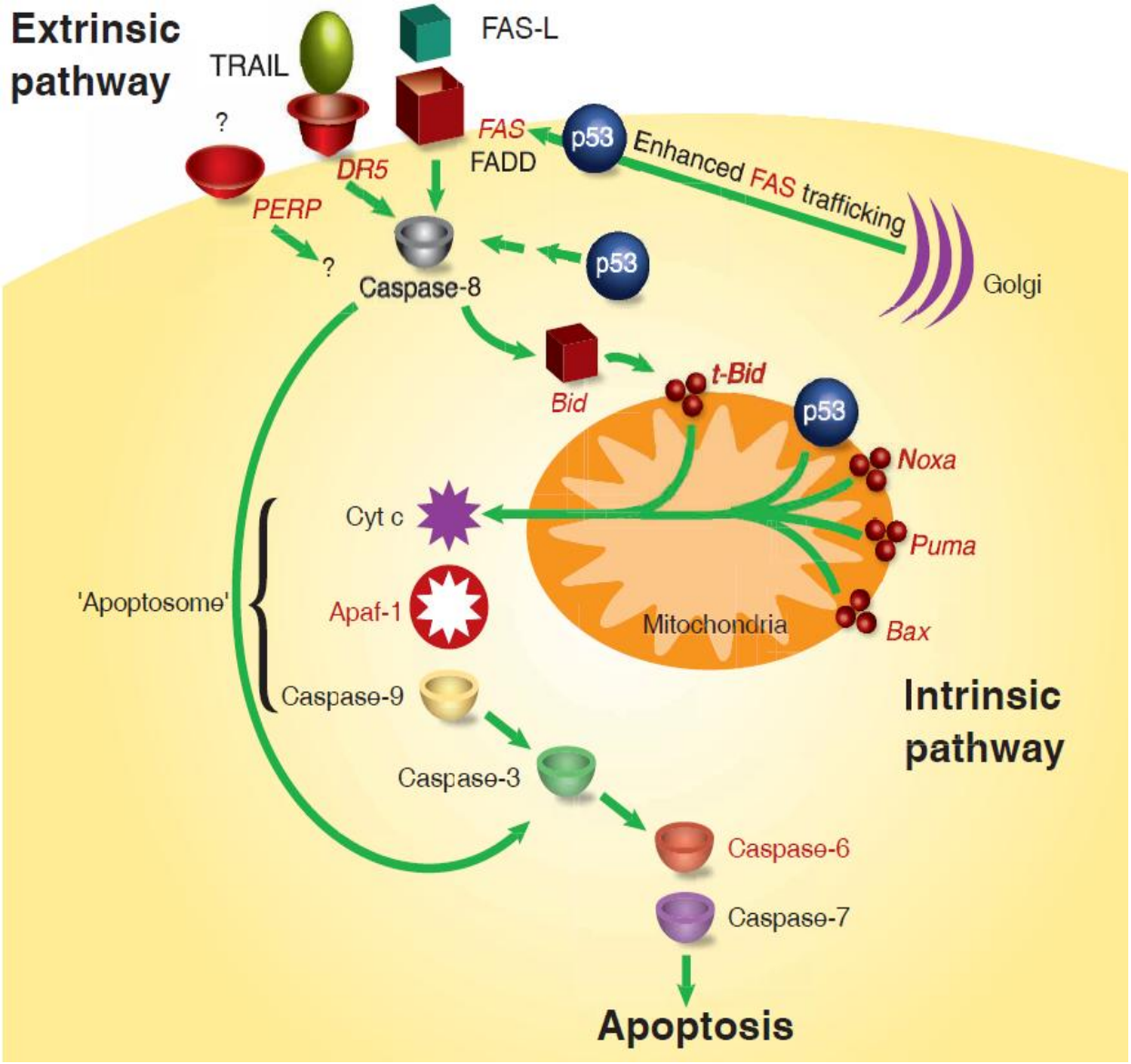

Figure 1.2. A model for p53 mediated apoptosis. This model depicts the involvement of p53 in the extrinsic and the intrinsic apoptotic pathways. P53 target genes are shown in red. The convergence of the two pathways through Bid is shown.

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1.3.3 Regulation of p53

After explaining the role of p53 as a tumor suppressor, it was expected to know that p53 should be kept under strictly controlled low levels to avoid any unnecessary cell killing (2). It has been shown that constant overexpression of p53 or lack of regulation is lethal (3, 6, 7). For this reason, in normal unstressed cells, p53 is down regulated where it exists in latent form and at very low levels to promote normal cell growth and division. Once cells sustain any type of cellular stress (UV and Gamma radiation, DNA Depurination, DNA crosslinking, hypoxia, reaction with oxidative free radicals, etc.) that damages the DNA integrity of a cell, the levels of p53 and its transcriptional activity significantly increases eliminating the risk of tumor development (43, 87). For this reason, the half-life of p53 under normal conditions is around 6-20 minutes, whereas it increases to several hours under stressed conditions (6). Regulation of p53 proteins is very complex composing of a network of several key factors that contribute to this mechanism. This includes 3 major steps: stabilization of p53, sequence specific DNA binding, and transcriptional activation of target genes (7). Although the precise mechanism of p53 regulation (stabilization or termination) is not fully understood, yet the interaction of proteins with cofactors and the post-translational modification (88, 89) such as: phosphorylation, acetylation, methylation, sumoylation, ubiquitination, etc., are considered vital in terms of p53 activation or repression. Such events may result in inhibitory or stimulatory effects. For example, p53 binds BRCA1 or 14-3-3σ and many others all of which stabilize p53 proteins and stimulate their cell cycle arrest or apoptotic function (90-92). In terms of post-translational modifications, p53 bears several acetylation lysine sites (Lys320, 373, 382) over its domains, where those on C-terminus are identified as activating sites post acetylation (61, 93, 94). Similarly, methylation can occur on multiple residues. Studies have shown the activation of p53 as a consequence of methylation (Lysine 372), while others demonstrated the loss of p53 post methylation at residue 370 (95). Also sumoylation of p53, a mechanism where a SUMO-2/3 is added to a lysine residue (Lys386), demonstrated the

induction of p53 transcriptional activity (96-100). Furthermore, p53 harbors multiple serine and threonine sites (Ser 15, 20, 37, 46, 392; Thr 18, 81, etc.) for kinase induced phosphorylation. Phosphorylation is known to stabilize p53 through ceasing the binding and the negative regulation of p53 by its regulators. It has been shown that the type of DNA damage activates different enzymes. Each enzyme induces a specific p53 post-translational modification at different residues, hence alerting p53 stability and binding affinity to DNA sequences that promote further gene transcription in the signalling pathway (101-103). However, among the post-translational modification pathway, ubiquitination is thought to be the master regulatory process (104-106). Since ubiquitination pathway may induce the proteosomal degradation of the substrate, treatment of cells with protease inhibitors was accompanied by high levels of ubiquitinated p53 (104-106). Based on that, studies analyzed with depth the p53 ubiquitination mechanism and its cofactors.

1.4 The ubiquitin system

Ubiquitin is a protein tag consisting of 76 amino acids that is highly conserved in eukaryotes and is recruited through the ubiquitination mechanism to result in protein tagging. Ubiquitin tagging signals the proteosomal degradation through the 26S proteasome complex, subcellular localization, or DNA repair of the substrate (107, 108). There are three types of ubiquitination: monoubiquitination (single ubiquitin monomer attached to the substrate), multi-ubiquitination (more than one ubiquitin monomer each attached at a different site on the substrate), and polyubiquitination (polymer of ubiquitin molecules all attached to the same site on the substrate) (Figure 1.3) (107-110). In the ubiquitination system, proteins destined for ubiquitination are covalently tagged with an ubiquitin chain in which the terminal residue of one ubiquitin molecule is linked through an isopeptide bond to a specific lysine residue within another (9-11). Seven different lysine residues can be used for chain formation and each may have a distinct effect on the substrate's fate (108, 109). Much less is known about the functions of chains with other topologies. Monoubiquitination of proteins may result

in (109, 110): intracellular location, endocytosis, budding, histone regulation, etc. On the other hand, poly-ubiquitinated substrates are mostly targeted for proteosomal degradation (111-113). More details regarding the substrate fate will be discussed in the upcoming section. Also, ubiquitin like molecules including SUMO and NEDD8 used in similar mechanisms also play a role in protein tagging, localization, stabilization but not degradation (114-116). In principle, ubiquitination is a reversible process where deubiquitinating enzymes (DUB) can release the ubiquitin from precursors, remove ubiquitin tags from substrates, and also prevent accumulation of ubiquitin in proteasome complex (102, 117). More than 100 DUBs exist in the human body system.

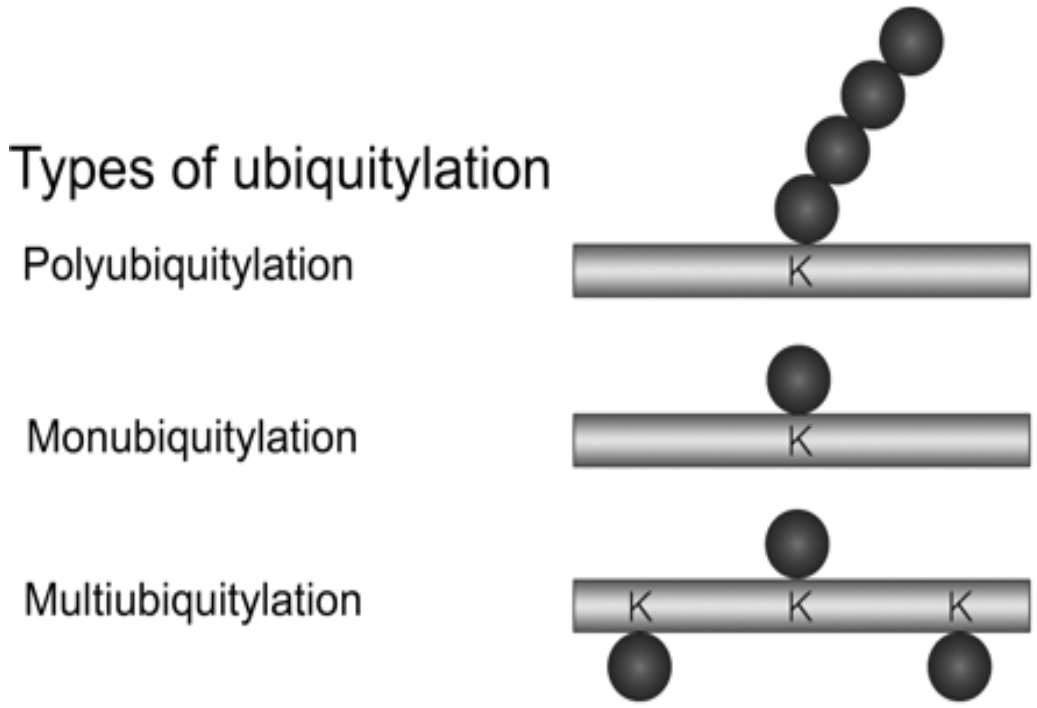

Figure 1.3. Ubiquitin modifications. Monoubiquitylation is the modification of a protein with a single ubiquitin molecule on a single Lys residue. Multiubiquitylation refers to the modification of several Lys residues with one ubiquitin. Polyubiquitylation is the modification of a protein, on one or more Lys residues, with an ubiquitin chain

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1.4.1 Ubiquitination enzymes

The ubiquitination pathway involves the role of three major enzymes: E1, E2, and E3; respectively known as: ubiquitin activating enzyme, ubiquitin conjugating enzyme / ubiquitin carrier enzyme, and ubiquitin ligase enzyme (107-109). In some situations an additional enzyme identified as E4 is required to aid E3 in a poly-ubiquitin chain assembly when more than four Ub moieties are added (118, 119). As indicated in Figure 1.4, Adenosine TriPhosphate (ATP) is needed to form a thiol ester bond between E1 and ubiquitin. E1 adenylates the C-terminus of ubiquitin and then forms a thioester between the cysteine residues of E1 and C-terminus of ubiquitin. The E2 then carries the activated ubiquitin moiety to form a complex with E3 and the substrate. It is the E3 role to transfer the ubiquitin from E2 to the substrate. The final ubiquitin results in an isopeptide bond between the amino group of a substrate lysine and ubiquitin C-terminal (113).

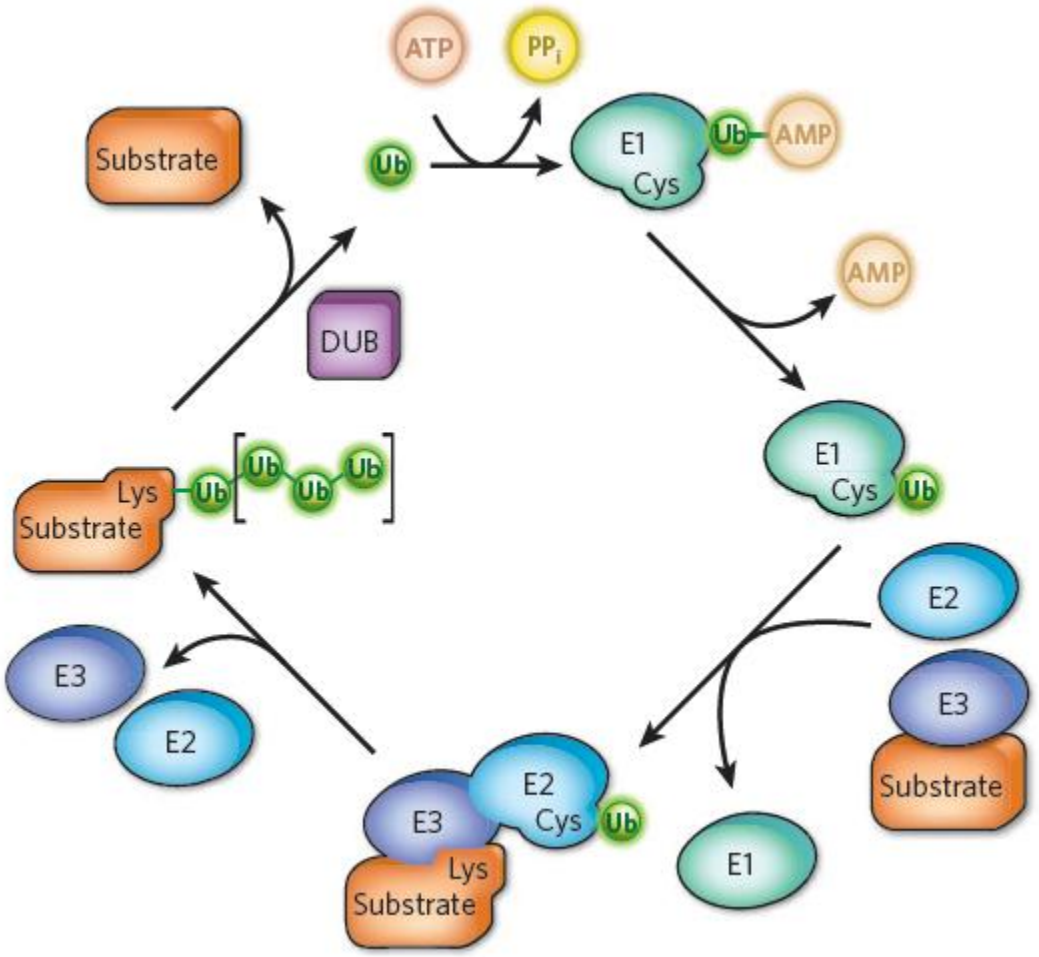

Figure 1.4. A schematic representation of the ubiquitination pathway and the ubiquitin enzymes. E1, E2, and E3 are respectively the activation, conjugating, and ligating enzymes. Abbreviations: Lys- Lysine, Cys- Cysteine, DUB-deubiquitinating enzyme.

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Unlike E1, where two E1 enzymes exist in the human body system, around 45 different E2 enzymes have been identified. All E2 enzymes are characterized by a highly conserved ubiquitin conjugating domain (UBC) consisting of 150-200 amino acids in addition to possible N and C-terminal extensions. Within this domain, a catalytic cysteine responsible for accepting the activated ubiquitin and interacting with E3 enzymes is embedded (120, 121). The role of E1 is limited to activating the ubiquitin moiety; whereas it has been detected that E2 enzymes have a more significant role in ubiquitination. Initially, E2 enzymes were thought to be important in determining the type of ubiquitination (mono vs poly) and the ubiquitin linkage involved in polyubiquitination. This idea was supported with the fact that the same E3 ligase may utilize different chains on different substrates (122, 123). Recent studies on different types and classes of E2 ligases showed that the attachment of ubiquitin to specific sites on the substrate, the type of ubiquitination, and the selection of lysine chains are determined by the E3 enzymes as well. The exact mechanism remains unclear (124-126). Since E3 enzymes confer the specificity of the substrate, more than 600 different E3 enzymes were identified in the human body system (127). E3 enzymes were shown to be functional only when binding specific E2 enzymes. Also it has been shown that ubiquitin binding to E2 does not overlap with the E3 binding site (125). Since they serve as the specific substrate recognition factor of the ubiquitination mechanism, the focus was placed on E3 ligases that were later classified into three major groups (127, 128): the RING-H2 (really interesting new gene), HECT (homologous to E6 associated protein carboxy terminus), and U-box family. The RING-H2 family protein was named after the RING domain that consists of series of histidine and cysteine residues that coordinates two zinc ions in a cross-brace structure. The most famous members of this family are: MDM2, Pirh2, COP1, BRCA1, Rbx1, etc. RING-H2 E3 ligases are distinguished from other groups by lacking the ability to bind ubiquitin yet successfully transferring the moiety from E2 to the substrate. Analysis of E2-E3 complex shows that the RING domain is not positioned adjacent to the catalytic cysteine of E2 and is unlikely to be involved in catalysis (129). However, deletion of the RING domain in many E3

ligases significantly ceases the ubiquitination and alters the lysine chains utilized. Studies regarding the exact role of the RING domain are still under investigation. On the contrary, HECT E3 ligases (AIP4, E6AP, and WWP1) with a conserved 350 amino acid HECT domain have the ability to covalently bind ubiquitin before transferring it to the substrate through an active cysteine site. Accordingly, the E3 cysteine and not the E2 cysteine would be the last step for activating ubiquitin (130, 131). The third group is the U-box which was first identified in yeast Ufd2. Members of this group: CHIP, Prp19, UBE4B, etc., display E4 like activity. At this point it's worth mentioning that in the absence or lack of E3 substrate binding, ubiquitin molecules are attached to the E3 enzyme. This process is identified as self ubiquitination. E3 enzymes such as MDM2, Pirh2, AIP4, CHIP, etc., have all shown to harbor the self-regulatory mechanism (132).

1.4.2 Ubiquitination signalling and function

As mentioned earlier, the type of ubiquitination and lysine chains (6, 11, 27, 33, 48, and 63) utilized determine the fate of the ubiquitinated substrate (Figure 1.5).

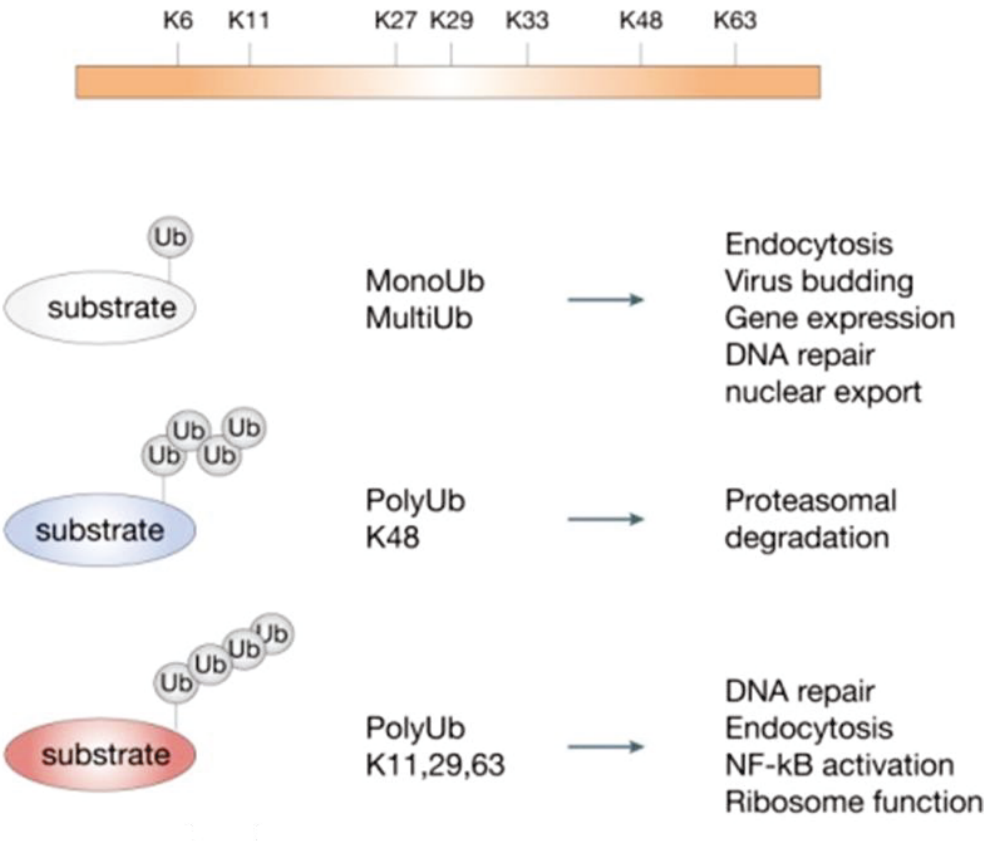

Figure 1.5. Ubiquitin lysine chains (K) and the substrate's fate post ubiquitination.

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Based on that, the ubiquitination system is categorized in two major groups according to their signalling function: proteasome dependent function and proteasome independent function. The proteasome dependent function confers the degradation of ubiquitinated substrates by the 26S proteasome (133). The 26S proteasome is a multi-catalytic protease (2-MDa) that degrades poly-ubiquitinated proteins to short peptides. It is composed of two sub complexes: the 20S core that carries the degrading catalytic activity, and the 19S which in turn regulates the pathway through recognizing the poly-ubiquitinated proteins. Also the 19S sub complex has been shown to be responsible for the unfolding of the proteins, which is an ATP dependent step and is required to channel the protein into central active site chamber for degradation (134). Interestingly, after degradation the short peptides are released and will further be degraded by amino and carboxy-peptidases present in the cytosol. In parallel, reusable ubiquitin moieties are released as well by the ubiquitin processing (UBP), and ubiquitin carboxy-terminal hydrolases (UBH) (133-135). K48 lysine chains are recognized by the 26S proteasome system in vivo and induce substrate degradation post ubiquitination. On the other hand, K63 chains, commonly utilized by many E3 ligases, are known to function in signal transduction, DNA repair, inclusion formation, endocytosis, lysosomal degradation, etc. (136, 137). Also, K63 linked chains are involved in protein kinase activation and DNA damage tolerance through recruiting additional factors or translocating proteins to regions where they can be functional. This highlights the role of proteasome independent ubiquitination function. Interestingly, structural analysis of protein conformation post ubiquitination using K63 versus K48 showed remarkable difference. The extended conformation in case of K63 compared to closeted one for K48 provides a possible explanation why K63 is not recognized by the 26S proteasome system. Replacing the lysine residue at this position ceases the proteosomal degradation and turns to be lethal in yeast. This is not the case with K63. Also mutated K63 inhibits repair without detectably inhibiting proteolysis (138-142). In some cases K29 was also reported to induce the proteosomal degradation of the ubiquitinated substrate, however the mechanism is less studied since K48 and K63 are more commonly used by the E3 ligases

(139). Knowing that ubiquitination is the regulatory mechanism for many tumor suppressors or oncogenes, this pathway became a target pathway for cancer therapy (143, 144).

1.5 E3 ligases

1.5.1 RING-H2 ligases (MDM2, Pirh2 and COP1)

As stated above, the RING-H2 family protein was named after the RING domain that consists of series of histidine and cysteine that coordinates two zinc ions in a cross-brace structure. Despite successfully ubiquitinating the substrate in the presence of E2, E3 ligases belonging to this family lack the ability to bind ubiquitin. The RING domain possesses the ubiquitin catalytic activity. When the RING domain is deleted, the ubiquitination mechanism is inhibited. Studies regarding the exact role of the RING domain are still under investigation. The most famous members of this family are: MDM2, Pirh2, COP1, etc.

1.5.1.1 MDM2

MDM2 is a member of the RING-H2 family proteins consisting of 491 amino acids. It contains several conserved domains including: N-terminal bearing the DNA binding domain, the central region that contains the nuclear localization and export sequences, an acidic domain plus a zinc finger domain, and a RING finger C-terminal domain (145, 146). Many reports describe the overexpression of MDM2 in several types of human tumors. MDM2 has been widely studied and associated with the regulation of several genes transactivating substrates including: NUMB, Arrestin, Insulin Growth Factor Receptor-1 (IGFR-1), etc. However, the MDM2-p53 pathway has taken the widest attention since both genes demonstrated a strong correlation in tumors (147, 148). To start with, MDM2 null mice died at embryonic stage early after implantation. Interestingly when accompanied with p53 knockdown, the mice were rescued from embryonic lethality. Investigations has shown that MDM2 itself is a p53 inducible gene which is kept at low concentration under unstressed conditions. MDM2 was

later identified as a negative regulator that binds and mono-ubiquitinates p53 (at low doses in unstressed condition) and poly-ubiquitinates p53 at high doses leading to degradation. Investigations revealed that association of MDM2 with other cofactors has led to p53 polyubiquitination and induced proteosomal degradation (149-151). Also MDM2 binding to p53 mediated the latter's translocation to the cytoplasm. Exporting p53 from nucleus ceases its tumor suppressor function. Under stressed condition, accumulation of p53 in nucleus was detected. However, it is worth mentioning that the degradation of p53 has been detected in both the nucleus and cytoplasm indicating that nuclear export is not a limitation for proteasome degradation (152, 153). Interestingly when p53 function is required, MDM2 is regulated by self ubiquitination mechanism. This is also known as auto-ubiquitination where ubiquitin moieties are attached to MDM2 leading to self proteosomal degradation (154). In depth, MDM2, through its N-terminus hydrophobic pocket domain, binds the p53 transactivation domain located at its N-terminal and mono-ubiquitinates p53. p53 mono-ubiquitination blocks its transcriptional activity. Residues 14, 19, 22, 23, and 26 are most critical in p53 for MDM2 binding. However, binding does not guarantee successful ubiquitination of p53 (155-157). The RING domain of MDM2 is essential to perform the E3 ligase activity. Mutant MDM2 at the RING domain that can still bind p53 cannot perform the E3 ligase activity. Studies showed that blocking MDM2 binding, such as using Nutlin-3a, activates p53 and suppresses tumor growth in vivo. Similar data was obtained when deactivating MDM2 catalytic activity as an E3 ligase (158, 159). Under cellular stress conditions, post-translational modification of p53 and in specific p53 phosphorylation blocks its interaction with MDM2. This terminates MDM2 induced regulation. Also MDM2 itself is under tight regulation through post-translational modifications such as acetylation of RING domain residues (159). This stops the catalytic activity and consequently up regulates p53 expression. Also under stressed conditions, MDM2 is phosphorylated at multiple sites by a wide range of pathways. Generally, this phosphorylation ceases MDM2-p53 binding releasing p53 from the negative regulation. For example in response to IR, MDM2 is phosphorylated by

ATM kinases blocking its ability to bind p53 which is phosphorylated as well. Under UV exposure, ATR protein kinases are activated and both MDM2 and p53 are phosphorylated. As a result of binding inhibition, the export mechanism is ceased and p53 is accumulated in the nucleus (160). The MDM2 pathway has also been linked to ARF tumor suppressor which binds and inhibits MDM2 and hence activates p53. A model was proposed suggesting that ARF sequesters MDM2 into the nucleolus or nucleoplasm away from p53. On the contrary, MDM2 is stabilized through AKT pathway. MDM2 is a substrate of AKT that induces MDM2 phosphorylation. AKT induced phosphorylation stabilizes MDM2 expression and facilitates p53 degradation after localizing MDM2 to the nucleus (151). Many investigations revealed the role of other cofactors that in turn regulate the AKT expression such as: PIP3, PTEN, 14-3-3 all of which down regulate AKT and enhance p53 activity. Interestingly, AKT activity is elevated in many human tumors (151, 161). Other post-translational modifications have been shown to alter MDM2 status which is regulated in response to cell cycle transition, ribosomal stress, microRNAs, etc...

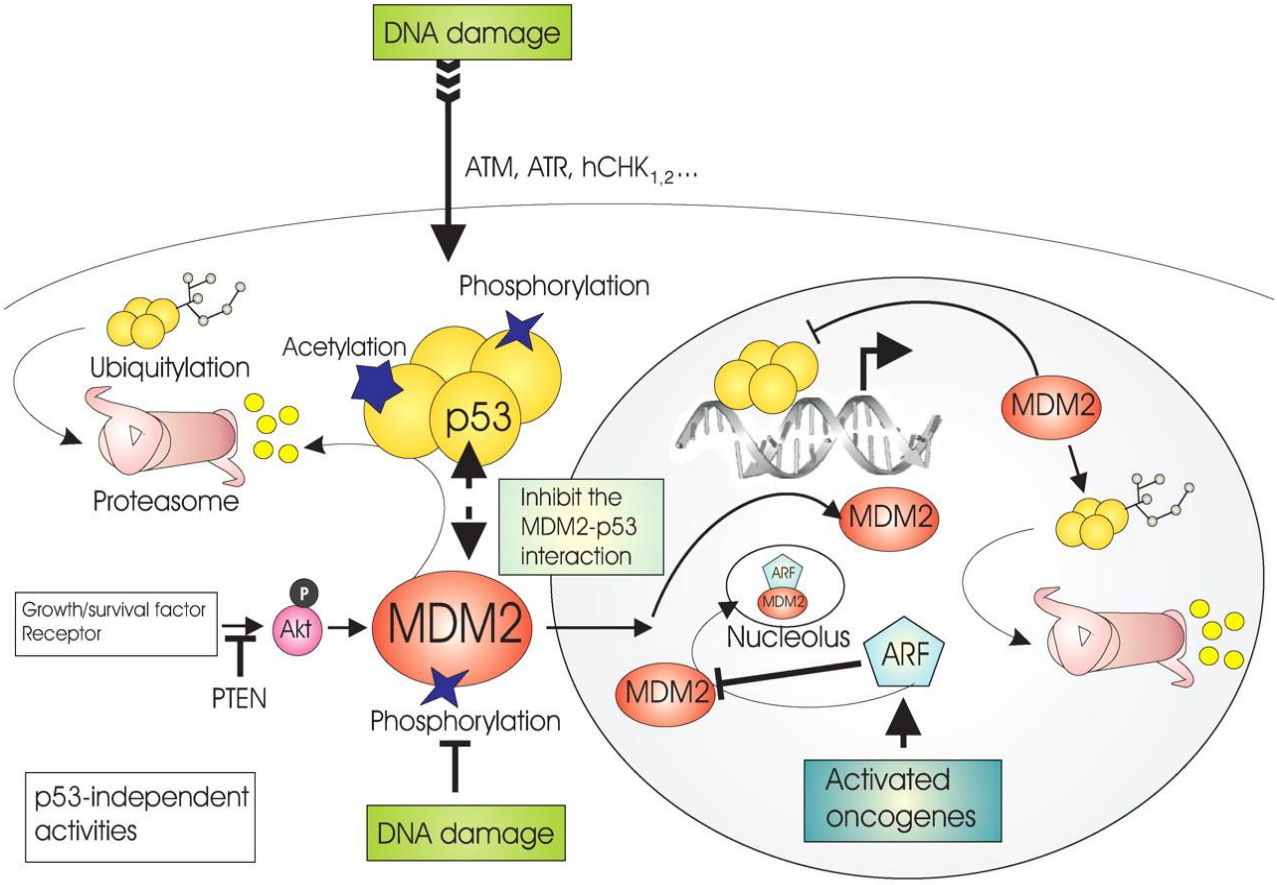

Figure1.6. Regulation of p53 by MDM2. p53 and MDM2 form an auto-regulatory feedback loop. p53 stimulates the expression of MDM2; MDM2 inhibits p53 activity because it blocks its transcriptional activity, favours its nuclear export and stimulates its degradation. Different cellular signals, such as DNA-damage or oncogene activation, induce p53 activation. DNA damage favours p53 phosphorylation, preventing its association with MDM2. Activated oncogenes activate the ARF protein, which prevents the MDM2-mediated degradation of p53. Similarly, inhibitors of the p53–MDM2 interaction should activate p53 tumour-suppressor activity in tumour cells that express wild-type p53. These compounds, because they bind to MDM2, could also affect the p53-independent activities of MDM2.

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A few years after MDM2 was identified, a MDM2 homologue, known as MDMX or MDM4, was identified to be essential for complementing MDM2 role in regulating p53. MDMX is overexpressed in many cancers including: colon, lung, breast, lymphoma, etc. (162). MDM4 possess a p53 binding domain at the N-terminus and RING finger domain through which it hetero-dimerizes with MDM2. Interestingly, MDMX showed no ability to act as an E3 ligase ubiquitinating p53. However, deletion of MDMx leads to p53 dependent lethality (163). Conversely when compared to MDM2 knock out lethality, it is noticed at later mid-gestation stage rather than preimplantation. Studies demonstrated that MDM2 can mono-ubiquitinate p53 in the absence of MDMX whose presence is required for p53 polyubiquitination. In vivo studies confirmed that the role of MDM2 and MDMX is not overlapping and neither can compensate for the role of the other. MDM2 in vivo ligase activity requires MDMX. Also, the heterodimer of MDM2 and MDMX is very stable. In vitro analysis in terms of ubiquitination proposed that MDMX stabilizes MDM2 through interfering in the self ubiquitination mechanism. Upon forming the heterodimer, auto-ubiquitination will shift to substrate ubiquitination (164-166). On the other side, phosphorylation of MDM2 and MDMx leads to accelerated auto-regulation. However some findings were contradictory as some studies showed the ubiquitination and degradation of MDMX by MDM2. They added that MDMX interfere with MDM2-p53 binding and regulation. Phosphorylation of MDM2 causes MDMx degradation; hence leading to p53 stabilization (Figure 1.7) (167,168).

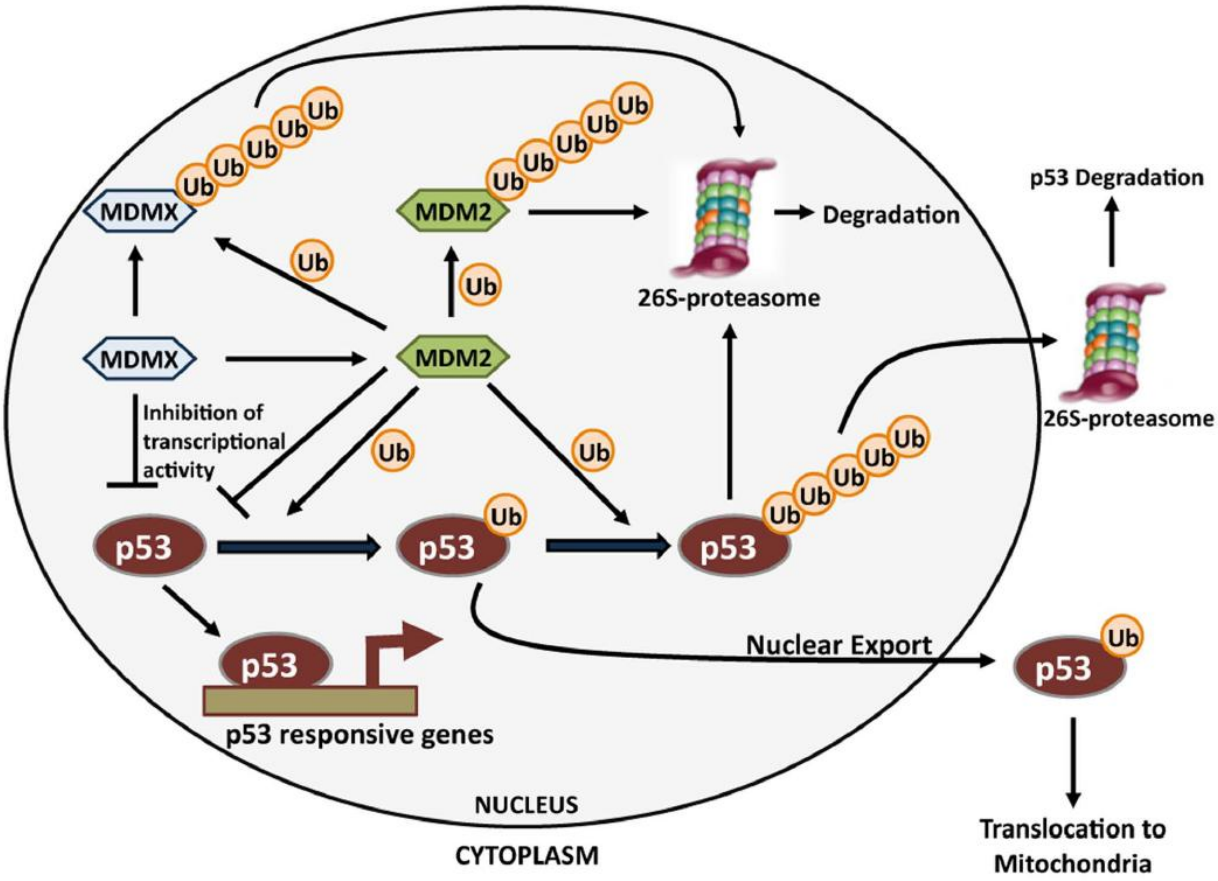

Figure 1.7. Complex control of p53 protein levels. Mdm2 as an example: An intricate regulatory mechanism controls ubiquitination of p53 and dictates its activity. MDM2 in partnership with MDMX poly-ubiquitinates p53, which is targeted for proteasomal degradation. MDM2 also directs self-destruction and MDMX degradation, mediated by its RING-domain. Mono-ubiquitination of p53 by MDM2 leads to its nuclear export. Direct binding with MDMX may also render p53 transcriptionally inactive. p53 up regulates transcriptional activation of MDM2 to complete a negative feedback loop.

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1.5.1.2 Pirh2

Pirh2 is another member of the RING-H2 family. It is a human 261 residue protein that contains three major domains: N-terminal domain (1-137 residues), the central RING domain (138-189 residues), and the C-terminal domain. The three domains are independently folded and do not interact with one another (169, 170). It was initially identified as an androgen receptor N-terminal interacting proteins that bears a RING domain responsible for its E3 ligase activity (171). Also WT Pirh2 proteins have a Plant Homeoddomain (PHD), which is thought to possess an E3 ligase activity. This domain is characterized by two interleaved zinc fingers and cysteine architecture. Little is known about Pirh2 PHD domain and its role in binding and ubiquitination (172, 173). The best characterized mechanism was Pirh2-p53 pathway where Pirh2 binds, ubiquitinates, and proteosomaly degrades p53. Interestingly, Pirh2-p53 correlation was demonstrated to be independent of MDM2 (174, 175). More attention was shifted to Pirh2 pathway when detecting its overexpression in a variety of human tumors regardless of p53 status. For example, Pirh2 is overexpressed in 84% of 32 human lung neoplasms in a study evaluating Pirh2 expression in lung cancer. p53 ubiquitination was detected at high levels in the same tumor tissues highly expressing Pirh2 compared to normal lung tissues (176). Likewise Pirh2 is overexpressed in prostate cancer (82%), hepatocellular carcinoma (78%), head and neck cancer (35%), and breast cancer (51%) (176-181). Similar findings were detected in mouse tumors. Mouse models of Pirh2 overexpression promoted tumorigenicity; hence not only confirming its essential role for p53 functions and regulation but also its possible association with other oncoproteins (12). In depth, Pirh2 was shown to be a p53 inducible gene that directly binds p53 in vivo and in vitro. It utilizes residues 120-137 thus excluding the role of RING domain in p53 binding. Residues utilized in p53 for binding were shown to be from 82-292. Shortly afterwards, binding investigations revealed the role of other different regions in each protein where the Pirh2 NTD

binds p53 DBD and the Pirh2CTD binds p53 TET domain. However the latter was thought to be more strong and essential for regulatory mechanism (169, 170).

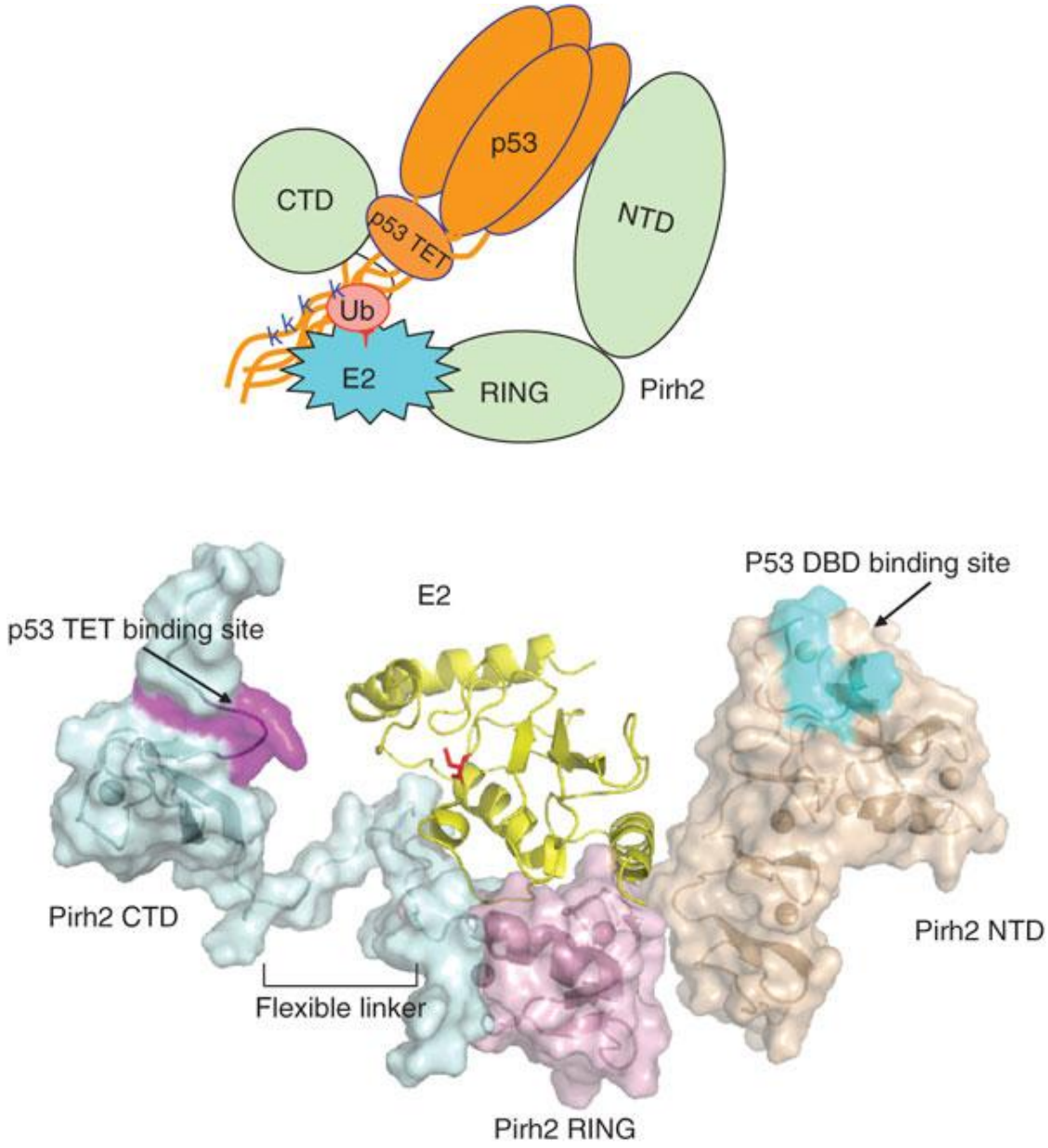

Figure 1.8. Model of a potential ternary complex between Pirh2, p53. And the E2 enzyme UBE2D2. The top panel illustrates schematically how domains of tetrameric p53 interact with those of Pirh2. The UBE2D2-Pirh2 ring domain orientations are based on the crystal structure of the homologous complex formed between UBCH7 and the c-Cbl ring domain. The active site cysteine of E2D2 which is charged with ubiquitin is shown in red.

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When monitoring the endogenous expression of p53 in response to Pirh2 overexpression, low p53 levels were detected (12). Analysis revealed the regulation of p53 through Pirh2 induced ubiquitination that leads to polyubiquitination of p53 (169). Interestingly, this ubiquitination was not dependent on MDM2 confirming that the E3 ligase activity of Pirh2 and MDM2 is unconnected (12, 174, 175). In vitro ubiquitination assays confirmed the intrinsic E3 ligases activity of Pirh2 that efficiently ubiquitinates p53 or Pirh2 itself (self-ubiquitination in the absence of the substrate) with degradation post ubiquitination (169). Emphasis on the roles of the domains confirmed that the RING domain is essential for ubiquitinating and degrading p53 proteins (169, 174, 182). To further elucidate the consequence of ubiquitination on p53, its transcriptional activity, growth inhibitory function, and cell cycle arrest were monitored. In addition to decreasing p53 half-life from 240 min to 80 min in response to Pirh2, results also confirmed that Pirh2 decreased p53 tumor suppressor function (169). Also Pirh2 and in contrary to other E3 ligases, has the ability to bind phosphorylated p53 (183). As described earlier, post-translational modifications and in specific phosphorylation of p53 inhibits MDM2 binding.This is not the case with Pirh2 that has the ability to bind both: phosphorylated and non-phosphorylated p53. Residue Ser15, which is phosphorylated in response to cellular stress and that ceases the binding of MDM2 to p53, is not effective in terms of Pirh2 binding to p53 (149, 184, 185). On the other hand, Pirh2 is regulated similar to MDM2 through self ubiquitination mechanism that leads to self proteosomal degradation under cellular stress (169, 170). Further investigations in this mechanism revealed the role of Calmodulin dependent kinase (CamKII) that phosphorylates Pirh2 impairing its E3 ability to bind and ubiquitinate p53. When phosphorylated, Pirh2 self-ubiquitination is induced. In depth, in response to stress CaMKII binds Pirh2 and induces phosphorylation at residues Thr-154 and Ser-155 at the G2/M phase of the cell cycle. This causes the export of Pirh2 to the cytoplasm. Pirh2 mutant construct resistant to CamKII phosphorylation shows unsuccessful self ubiquitination mechanism and nuclear accumulation where Pirh2 normally binds p53. In terms of stability, phosphorylated Pirh2 is more unstable than its unphosphorylated form

(186). This data was supported with findings showing that the majority of Pirh2 is phosphorylated in normal tissues. Pirh2 is present in unphosphorylated form in tumor tissues (12). Also CaMKII is down regulated in human tumor cells (186, 187).

Several isoforms as a result of splicing have been identified regarding Pirh2 including Pirh2 B, C, and D, where A is referred for WT human Pirh2 (188). These findings added knowledge to the role of the domains in Pirh2 proteins. For example, Pirh2 B and C isoforms, lacking residues respectively 171-179 and 180-261 respectively, lost ubiquitin ligase activity when tested in vitro despite successfully binding p53. Interestingly when running in vivo analysis both isoforms (Pirh2 C to a lesser extent than Pirh2B) were able to ubiquitinate and down regulate p53. This highlights the possible role of other cofactors in the cellular ubiquitination mechanism. Further investigations revealed that all isoforms are commonly present in the nucleus and cytoplasm but the exact mechanism remains unclear. Little is known about the role of Pirh2D isoform. Pirh2 D is the smallest isoform consisting only of 75 amino acids lacking the RING and C terminal domain and predicted not to possess any ligase activity (189). Recently, two new isoforms E and F have been identified. E and F isoforms expression is elevated in hepatocellular liver carcinoma cell line. Both isoforms lack residues in the CTD (23-261 residues for isoform E and 227-261 residues for isoform F) yet bear a full RING domain. No ubiquitination analysis regarding these isoforms has been reported in the literature (190).

In comparison to MDM2, Pirh2 display different levels of p53 ubiquitination. However it is worth mentioning that despite that MDM2 is rarely overexpressed whereas Pirh2 is commonly overexpressed in human tumors, Pirh2 knockout mice are not embryonically lethal (12). Based on that, comparing and analyzing the structure and role of the RING domain in both ligases was of high importance. Both ligases showed high similarity in terms of structure; however Pirh2 was shown to act as a monomer where as MDM2 forms a dimer with itself or MDMx. Other studies showed the dimerization of Pirh2 especially with its isoforms, yet

functioning in a monomer state decreased the significance of such investigations (174, 175). In terms of p53 ubiquitination, many lysine residues are ubiquitinated by both ligases with the exception of few residues that were exclusive for each ligase such as: Lys 164 for Pirh2 and Lsy319 for MDM2. This opens up a new gateway since the Lys164 residue of Pirh2 was reported to be acetylated and ubiquitinated altering p53 mediated cell growth and apoptotic function. Also, MDM2 was able to conjugate ubiquitin more efficiently than Pirh2 (174, 175). One reason could be the ability of MDM2 to bind and function with a wider variety of E2 enzymes compared to Pirh2. This was justified by the presence of an extended E3-E2 interaction surface on MDM2. However we shouldn't neglect the fact that in vivo analysis showed that Pirh2, unlike MDM2, requires no additional cofactors and can successfully poly-ubiquitinate its substrate. In summary, it is believed that MDM2 is the primary regulator of p53 under unstressed conditions. In response to stress and when p53 is phosphorylated and dissociated from MDM2, Pirh2 becomes the main regulator.

Interestingly, Pirh2 is also involved with other proteins that are correlated with the p53 regulatory pathway. For example Pirh2 can degrade Histone deacetylase 1 (HDAC-1), which can inactivate p53 transcriptional activity (191). Also HDAC-1 alters the E2F pathway which controls the p53 activity and the expression of other genes required for the entry and progression of the cell cycle (192). Moreover, Pirh2 is a key regulator of the cell cycle kinase inhibitors such as p27 and p21 (193). Pirh2 is also correlated with SCYL-1 like 1 binding protein (194). A study on SCYL-1 showed that it promotes Pirh2 degradation through auto-ubiquitination after exporting it to the cytoplasm. SCYL-1 are also known to accelerate MDM2 self ubiquitination (195). Many of these findings gave more knowledge regarding Pirh2 yet highlighting that Pirh2 might have a dual role as a tumor suppressor or an oncogene. This was further confirmed when Pirh2 was correlated with other proteins aside from p53. Pirh2 was shown to bind, ubiquitinate, and degrade c-Myc. c-Myc is an oncogene highly expressed in cancer. Also when mutating Pirh2, elevated levels of c-Myc are detected and higher risk of

cell hyperplasia and tumorigenic is identified (197). Furthermore, Pirh2 can inhibit apoptosis by binding Keratin 8/18 and altering the release of cytochrome and the progression of the caspace pathway (12). PolH, necessary for translesion DNA synthesis and lead to cancer when deficient, is another substrate of Pirh2. Pirh2 can physically interact with PoLH and promote its degradation (12). Pirh2 can also interact with histone acetyl tip60, hNTKL-BP1, PLAG2, all of which are involved in regulating Pirh2 regulatory mechanism and auto-ubiquitination (12, 94, and 198). Besides, Pirh2 is highly conserved in yeast that are known for lacking p53 like genes raising the possibility of having other roles of Pirh2 in different pathways (12). Accordingly, Pirh2 function and regulation along with its effect on substrates involved in cancer represent an attractive approach for cancer therapy.

1.5.1.3 COP1

COP1 is the third member of the RING family proteins that is associated with p53. COP1 is a well conserved E3 ubiquitin ligase that can poly-ubiquinate and induce the proteosomal degradation of its substrate (199). With respect to cancer, variety of human tumors displayed the overexpression of COP1 reaching 80% in breast adenocarcinomas and 45% in ovarian adenocarcinomas. Other cancers such as: lung cancer, prostate cancer, and acute lymphoblastic leukemia lacked functional COP1 proteins (200). Interestingly and unlike Pirh2, COP1 deletion is embryonically lethal as it is the case for MDM2. At the molecular level, COP1 is self-regulated through ubiquitination. In addition to its cellular localization between the cytosol and the nucleus, the stability of the protein is also affected by DNA damage and cellular stress. p53 is the first mammalian substrate successfully shown to be regulated by COP1 (199, 201). In vivo and in vitro analysis regarding binding and ubiquitination confirmed that COP1 induces proteosomal degradation in an ubiquitin dependent manner modulated by the catalytic RING domain. Remarkably, no physical interaction between COP1 and p53 has been detected. Also COP1 inhibits p53 tumor suppressor function in terms of cell cycle arrest

responsive genes (p21) and apoptosis (201). Studies confirmed that COP1 function is independent of either MDM2 or Pirh2. It is thought to fine tune p53 at low levels in unstressed cells with high resemblance to MDM2-p53 mechanism. Other major substrates of COP1 are oncogenes such as: JUN, ETV proteins, MTA1 highlighting the role of COP1 in tumor suppression and promotion (202). Like other E3 ligases, COP1 became an attractive drug target in cancerous therapeutic approaches.

1.5.2 AIP4 HECT ligases

AIP4 is a human E3 ligase that belongs to the HECT (homologous to the E6-associated protein carboxyl-terminus) family proteins which contains around 30 E3 ligases in mammals. HECT proteins are mostly involved in cellular process and proliferation in addition to immune responses and trafficking (203). Initially, AIP4 was discovered in mice as ITCH. It is characterized by three major domains: N-terminal kinase C2 domain, multiple WW domains, and a C-terminal HECT domain (14, 204). WW domains are commonly known for their role in binding. WW domains mediate the protein-protein interaction through recognizing the rich peptide motifs and the phosphorylated sites such as serine and threonine (205, 206). AIP4 bears two sets of WW domains: WW1-2 and WW 3-4. Each WW domain consists of 35-40 amino acids and folds into three stranded antiparallel B sheets with two ligand binding grooves. They have been characterized into 4 groups depending on the substrate binding (205, 206). HECT domain comprises of 350 amino acids and is located at the C terminus. HECT domain is bi-lobed consisting of N-terminal lobe that interacts with E2 enzymes. Unlike RING-H2 family ligases, HECT has the ability to bind ubiquitin before transferring them to the substrate. Based on that, the RING ligases are referred to as adaptors in the ubiquitination pathway. The last 60 amino acids of the HECT domain modulate the ubiquitin chain linkage specificity (131). The figure below is a schematic representation comparing RING to HECT family proteins in terms of ubiquitination.

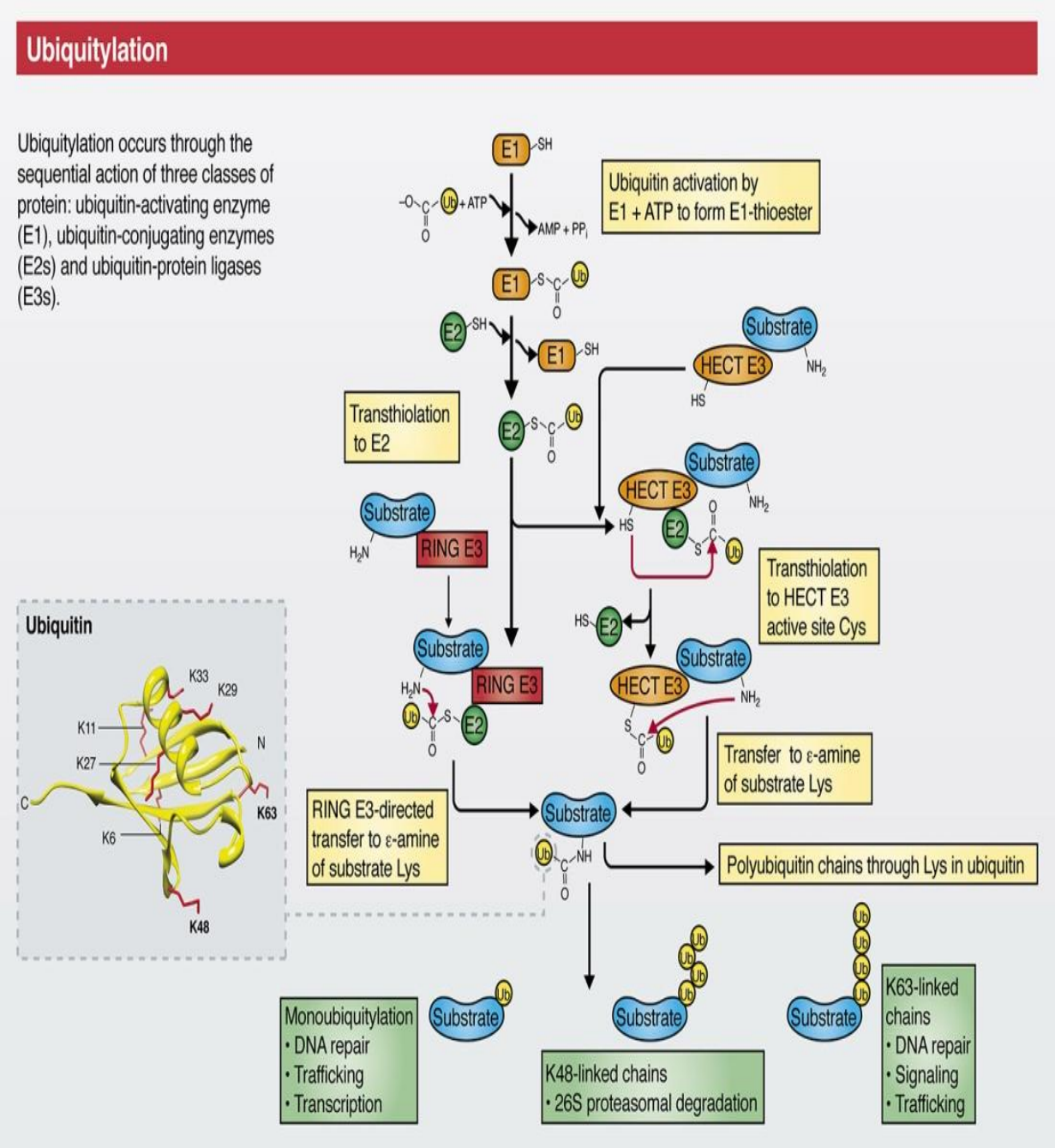

Figure 1.9. Differences with respect to the ubiquitination process between RING and HECT domain in the RING-H2 and HECT family ligases. Abbreviations: Cys- Cysteine, Lys and K- Lysine.

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AIP4 has the ability to bind, ubiquitinate, and degrade a wide range of substrates. It is also regulated by self ubiquitination followed by proteosomal degradation as described above in MDM2 and Pirh2 (14).The first study that revealed AIP4 as an effective E3 ligase studied G-protein coupled receptor CXCR4 (207). CXCR4 are regulated either by redirecting them to plasma membrane or sorting them out for degradation through lysosomal proteolysis. Ubiquitination is involved in the regulation of those receptors as a post-translation modification process. AIP4 successfully ubiquitinates and leads to CXCR4 degradation (207). Also AIP4 acts as an ubiquitin ligase towards transient receptor potential (TRP) proteins: TRPV4 (208). These receptors are under tight regulation through mechanisms of endocytosis and exocytosis. AIP4 ubiquitinates TRPV4 and promotes endocytosis instead of degradation; hence decreasing the amount of channels at the plasma membrane. Also regarding AIP4, a dual role in terms of regulating substrates with opposing functions was detected. One example is the ability of AIP4 to induce ubiquitination and inhibition of Smad7, an intracellular antagonist of transforming growth factor β (TGF-β) (209). However, AIP4 can also stabilize Smad7-TβRI receptor inhibiting the TGF-β signalling pathway. TGF-β is a member of cytokine family involved in proliferation, differentiation, and apoptosis. The exact mechanism remains unclear. Also AIP4 is highly associated with Spartin, a protein involved in degradation of epidermal growth factor receptors and turnover of lipid droplets. Initially, it was thought that AIP4 ubiquitinates and removes Spartin from lipid droplets. Investigations then confirmed that Spartin acts as an adaptor protein and increases AIP4 E3 ligase activity towards lipid droplets by inhibiting the auto-ubiquitination process. AIP4 self ubiquitination is ceased indirectly after the binding between Spartin and AIP4, which disrupts the interaction between HECT and WW domains necessary for auto-inhibition (210).

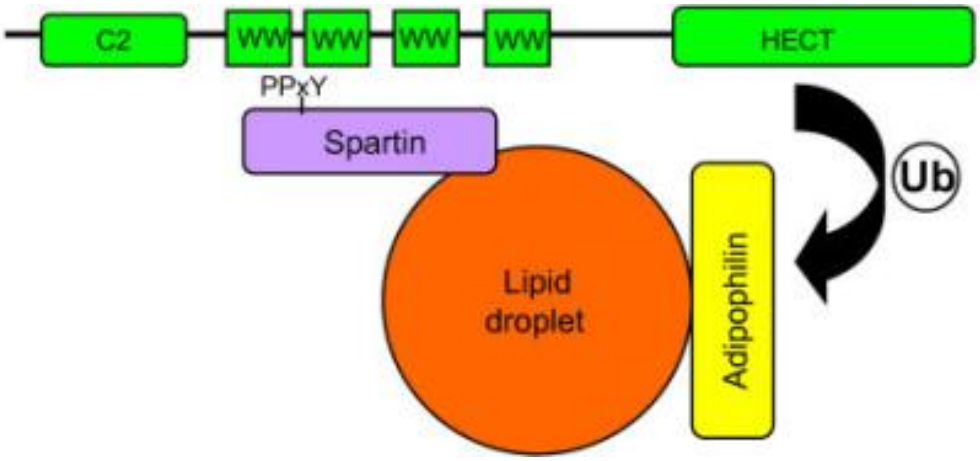

Figure 1.10. A Schematic model of how Spartin binds and enhances AIP4 ubiquitinating function towards lipid droplets.

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Regarding the p53 family proteins, AIP4 was only correlated with p63 and p73 but not p53. With respect to p63, less attention in cancer research is given knowing that p63 is more involved in development. $p63^{-/-}$ mice die at birth with developmental abnormalities such as: limb appendage truncations and epidermis defects (4, 23, 24, 27, 28). Studies showed that AIP4 can bind and ubiquitinate p63. Interestingly, AIP4-p63 binding involves the residues between the PY and SAM motif, both of which are absent in p53. This justifies the lack of AIP4-p53 correlation. Besides, AIP4 reduced p63 half-life and decreased the endogenous expression of p63 in an ubiquitin-dependent manner (211-213). Also with respect to p63 and among the RING-H2 ligases, Pirh2 and not MDM2 was able to bind and ubiquitinate p63. p63 expression was modulated by Pirh2 where Pirh2 overexpression resulted in p63 down regulation and p63 levels were only high when Pirh2 is depleted. Also p63 was poly-ubiquitinated and proteosomaly degraded in response to Pirh2 overexpression (214). The correlation of AIP4 and the remaining ligases with p73 will be discussed in details in section six of chapter one.

1.5.3 Additional groups of E3 ligases (UBE4B and CHIP)

Multiple degradation pathways are active to ensure a proper regulation of p53. Many were tested for possible overlaps in the signalling pathway yet the story remains unclear. Among the new evolving E3 ligases is Chaperon Associated Ubiquitin Ligase CHIP (C-terminus Hsc70 Interacting Protein), which is able to induce proteosomal degradation of p53 (215). This correlation was initially anticipated when mice lacking CHIP developed apoptosis in multiple organs. CHIP mediates substrate ubiquitination in correlation with heat shock proteins HSC 70 and 90. Also CHIP repressed the transcriptional activity of p53, which was restored when CHIP is depleted from cells. A cross talk between MDM2 and CHIP has recently been highlighted in modulating p53. MDM2, p53, and Hsp90 form a hetero-complex. CHIP is suspected to be part of the complex knowing that it physically interacts with Hsp90 (216).

Investigations are still in process to explain the loss of MDM2 function as a result of the hetero-complex formation and the role of CHIP in this pathway.

Recently in 2011, a novel study showed the role of UBE4B, an E3 and E4 ubiquitin ligase belonging to U-box proteins and commonly observed in human brain tumors, in promoting p53 polyubiquitination and degradation via the ubiquitin proteasome pathway (217). UBE4B regulates the stability of p53 in vivo and in vitro with a significant decrease in p53 endogenous levels. In depth, UBE4B interacts with p53 and MDM2 elevating p53 ubiquitination, from mono to polyubiquitination, and enhances its degradation. Interestingly, MDM2 activity was shut down when depleting UBE4B. Analysis confirmed that UBE4B complements MDM2 function in polyubiquitinating and degrading p53. Neither of the two E3 ligases can lead to a successful polyubiquitination when running the in vitro assays. Findings were complementary in terms of regulating p53 dependent transactivation. Also, UBE4B inhibits p53 dependent cell cycle arrest and apoptosis. It was proposed that UBE4B may also cooperate with Pirh2 and COP1 in down regulating p53 (217-219). UBE4B has been associated with p63, and for this reason it is considered a promising regulatory molecule in development (220, 221). Recently, UBE4B has been shown to regulate epidermal growth factor receptor (EGFR) degradation (222).

1.6 p73 overview

p73 is the third member of the p53 family that shares high homology with p53 (4). p73 contains the three typical domains of a transcription factor including: the amino terminal transactivation domain (TAD), the DNA binding domain (DBD), and the carboxy-terminal oligomerization domain (OD) (223). Homology between p53 and p73 reaches 70% at the DNA binding domain (4). However p73 proteins bear a longer C-terminus where the sterile alpha motif (SAM) resides. SAM motif is involved in protein-protein interaction highlighting the possibility that p73 might be associated with more substrates in addition to those linked

to p53. Also the majority of the SAM containing proteins is involved in regulation of development and not cancer (27, 28). The high conservation of the oligomerization domain between the members of the p53 family raises the possibility of forming hetero-oligomers. Studies confirmed homo-oligomers formation for each member. Unlike p53, in normal cells p73 gene gives rise to differentially spliced mRNAs which are translated to 7 different isoforms: α, β, δ, γ, ζ, and ε. Isoforms are spliced at the C-terminal end. p73α is known as the wild type, and the β and δ isoforms are truncated forms of the α isoform. On the other hand the δ isoform lacks the majority of the C-terminal, hence resembling p53 proteins. The ζ isoform has internal deletion where residues 400 to 496 are deleted. On the contrary, the γ contains a longer reading frame that eventually leads to a new 75 residue C-terminal. Figure 1.10 shows the differences at the C-terminal of p73 isoforms (223, 224).

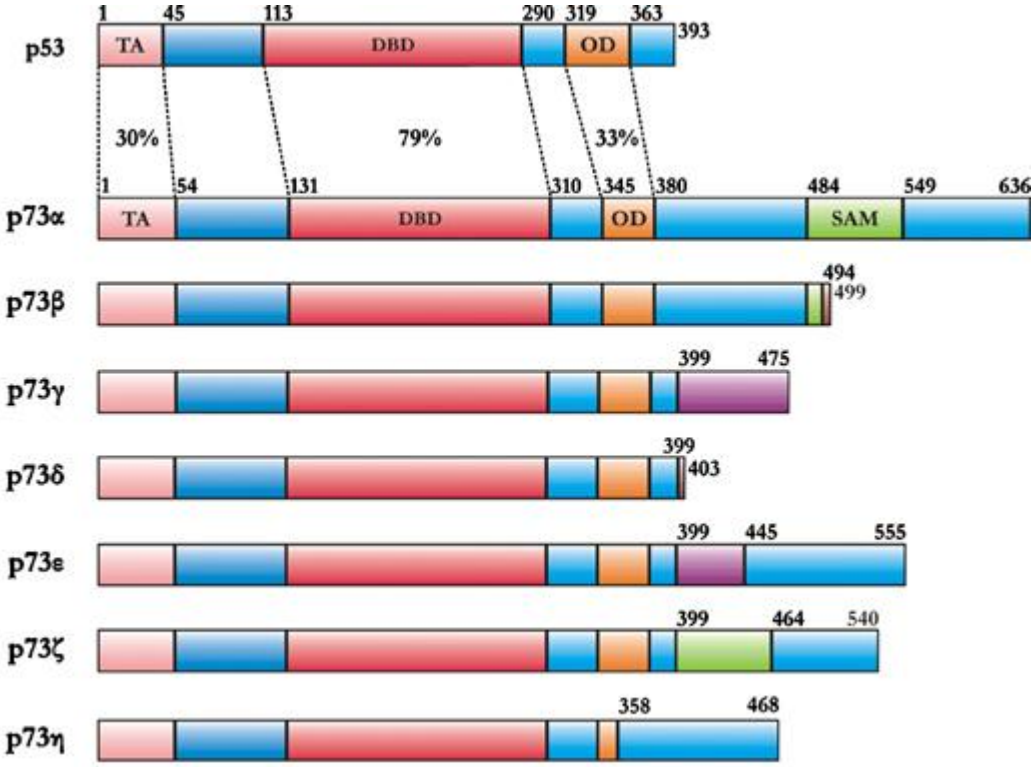

Figure 1.11. Structural similarity between p53 and p73 proteins and organization of different isoforms of p73. Owing to alternative N- and C-terminal splicing of transcripts, P73 gives rise to a variety of isoforms.

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Other short isoforms that lack the C-terminal transactivation domain were identified as η and η1. Both isoforms are less studied and characterized. Also p73 proteins that lack the N-terminal transactivation domain, known as ΔNp73, were identified in human. The exact function of each isoform remains unclear (27, 28). Initially p73 was thought not to be involved in tumor growth and progression because p73 genes are rarely mutated in human cancers. Also the knockout of p73 gene in mice showed no effect on tumor growth. p73 deficient mice were born with defects in neurogenesis (hippocampal dysgenesis and hydrocephalus) and developed chronic infections and inflammation. Also p73 deficient mice were infertile due to disruption of pheromone sensing. The role of p73 in the development of the central nervous system and neuronal survivor was confirmed by many studies (224-226). However, the role of p73 in cancer was highlighted later on when studies focusing on mouse models revealed that mutations in p73 when accompanied with p53 mutants lead to more aggressive tumors. Also, in mice $p73^{+/-}$ deletions developed spontaneous tumors (227-229). Interestingly in many tumor specimens, the loss of p73 heterozygosity was very frequent (230). At the molecular level, it was shown that overexpression of p73 can activate p53 responsive genes and trigger apoptosis (231). Also p73 has been shown to respond to DNA damage and induce cell cycle arrest (232, 233). Despite the stability of p73 protein expression level in normal and tumor tissues, p73 at the mRNA levels was elevated in ovarian cancer, breast tumor, neuroblastomas, prostate, and colorectal cancer (233). Based on that, cancer research considered p73 a new target for investigations.

1.6.1 Role of p73 in cancer

Research regarding the role of p73 in cancer opened many gateways; however the story became more complicated when detecting the opposite roles of TA and ΔNp73 isoforms. Each isoform is a product of a different promoter. TAp73 is an inducer of the cell cycle arrest and apoptosis, and highly associated with p53 responsive genes. ΔNp73 is characterized as an oncogene (224, 235). Many studies suggested the down regulation of TAp73 and p53 by

ΔNp73 with an existing auto-regulatory feedback loop where TAp73 and p53 can bind to ΔNp73 promoter and induce transcription. However the picture is not a clear cut for the same reason mentioned above where p73 deficient mice did not show spontaneous tumor formation (8, 233). Later, researchers were more selective in eliminating one of the isoforms and track its consequences in mice. Interestingly, TAp73$^{-/-}$ mice showed an increased susceptibility to tumor formation and carcinogenesis. This was not detected in ΔNp73$^{-/-}$ mice. For this reason, the ratio of TAp73 to ΔNp73 was thought to determine its tumor suppressor versus its oncogenic function. This hypothesis was later supported with findings showing high ΔNp73 RNA levels when TAp73 is knocked down (8, 24, 233-236). Mutant p53 can bind and block the function of p73. In other words and since p53 is highly mutated in cancer, the role of p73 in cancer was suspected to be significant yet blocked (237). This made the p73 signalling pathways of high importance and opened a new gateway for therapeutic approaches that were previously only concerned with p53 function. In depth, p73 was correlated with a wide range of p53 responsive genes involved in cell cycle arrest and apoptosis (232, 237). When overexpressed, p73 binds to p53DNA target sites and transactivates responsive genes in a p53 like manner. To start with, p73 regulates the transcription of Bax, which is a member of the Bcl-2 family involved in apoptosis. Also within the same family, PUMA was shown to be activated by TAp73 and repressed by ΔNp73. Furthermore, Noxa, another mitochondrial apoptotic pathway mediator, was shown to be regulated by p73 (238, 239). The list continues for p53 responsive genes that are as well activated by p73 including: GADD45, IGFBP3, cycling G, etc. (8). The function of p73 as a tumor suppressor also has distinct genes that are not directly associated with p53. For example, p73 strongly induces 14-3-3σ (240). Interestingly, differences between p73 isoforms were detected. Studies showed that p73β is a more efficient inducer of apoptosis and cell cycle arrest function. Interestingly the function of p73β is reduced when overexpressed along the α and ε isoforms suggesting inter-variant association with dependence on oligomerization domain (241-244).

1.6.2 Regulation of p73

Acting as tumor suppressor or oncogene hints for a tight regulation of p73. Many of p53 regulatory genes were suspected to regulate p73 as well (245). E2F1 is one of the major regulators of p73. E2F1 is a member of the E2F transcription factor family that bears both apoptotic and oncogenic properties. E2F1 has the ability to indirectly stabilize p53 without binding through increasing the level of p14ARF proteins. P14ARF proteins cease MDM2 promoted degradation of p53. Unlike p53, E2F1 has the ability to up regulate p73α directly through recognizing and transactivating the p73 promoter that contains E2F1 binding site. In response to E2F1 up regulation, p73 triggered apoptosis is elevated in human tumor cells. At the protein levels, knockdown of E2F1 resulted in significant down regulation of p73α proteins. Surprisingly, other studies in Hela cells confirmed the down regulation of endogenous p73 in response to E2F1 overexpression. Also the use of protease inhibitors confirmed that this degradation mechanism is proteosomaly independent. It was suggested that E2F1-p73 correlation is cell type specific. Also moderate levels of E2F1 act as inducer of p73, and become a negative regulator when overexpressed (62, 192, 246, 247). The correlation remains vague and requires further investigations. c-Ab kinases, previously linked to p53, are also involved in p73 regulation (248). With respect to p53, c-Abl kinases are activated in response to DNA damage enhancing the nuclear accumulation of p53. Similarly, c-Abl stabilizes and enhances the p73 apoptotic activity. c-Abl binds and phosphorylates p73 at residue Tyr-99. p73 phosphorylation is needed for a successful p73 apoptotic activity. The half-life of p73 isoforms α, β, and δ was significantly prolonged when c-Abl tyrosine kinase is overexpressed. c-Abl-p73 enhancement depends on the type of DNA damaging and the stages of cell death (245, 249, 250). For example, p73 accumulation is lacking in response to UV or methylmethane sulfonate treatment. This is explained by the absence or slow c-Abl activation compared to its activation in response to taxol or cisplatin exposure. Also p73 stabilization is maximal when cells are dying. Requiring the presence of c-Abl,

actelytransferase p300 also causes p73 acetylation at Lys 321, 327, and 331 in response to DNA damage. Unlike p53 that binds the C-terminal of p300, p73 recognizes the N-terminal region where the two protein interaction, p73 and p300, is essential for transactivating p73 and inducing apoptosis (250, 251). Negative regulators previously associated with p53 such as Cyclin G mediate p73 degradation independent of ubiquitination; yet the exact details of the regulatory mechanism require further clarifications (252).

On the other hand, p73 regulation is also associated with non p53 correlated genes. One example is checkpoint kinases Chk1 and Chk2. Both kinases are noticed to induce p73 accumulation in response to genotoxic stress conditions. Interestingly, despite being linked to the E2F1 pathway explained above, both kinases showed no correlation to p53 levels or activity when overexpressed or knocked down (253, 254). Moreover, p73 is transcriptionally enhanced with the effect of protein kinase Cδ that phosphorylates p73 at Ser-289 and induces its transcriptional activity. Similarly, p73 apoptotic function is enhanced with E1A and c-Myc regulation (255, 256). As for p73 down-regulation, an F-box proteins known as FBOX45 binds to the SAM motif, thus has the ability to bind TA and ΔNp73. F-box act as an E3 ligase promoting p73 ubiquitination and degradation. Accordingly, all E3 ligases especially those correlated with p53 were of huge importance to p73 regulation (257). E3 ligases: MDM2, AIP4, and Pirh2 were all involved and will be discussed below.

1.6.3 p73 and E3 ligases

Knowing the homology at the DNA binding domain between p53 and p73 and knowing the regulatory mechanism involved between MDM2 and p53, it was of no surprise to know that MDM2 is a p73 inducible gene that binds p73 (258). MDM2 can successfully bind and down regulate the transactivation activity of p73 yet does not lead to p73 degradation or half-life shortening (259, 260). In depth, findings confirmed that MDM2 disrupts the interaction of p73 and p300 by competing/interfering with the binding of the N-terminal domain. Also

MDMx can successfully bind p73. Surprisingly, the lack of p73 degradation by either MDM2 or MDMx was contradicted by the accumulation of p73 proteins in response to protease inhibitors treatment. Many possible explanations correlating the MDM2-p73 pathway to c-Abl were raised especially when noticing the accumulation of p73 in response to DNA damaging stimuli that affect c-Abl levels (261). The same DNA damaging stimuli that affect c-Abl has no consequence on p53 levels hinting for two parallel independent pathways for p53 and p73 with respect to MDM2 regulation (249). This was further confirmed when studies investigated the effect of Nutlin-3a on MDM2-p73 pathways. Nutlin-3a is a MDM2 inhibitor that disrupts MDM2-p53 interaction and restores p53 tumor suppressor function. To investigate its effect on p73, human colon carcinoma cell lines lacking p53 were treated with Nutlin-3a. Interestingly, p73 was stabilized and activated. p73 stabilization as a result of Nutlin-3a treatment involved the role of E2F1 and Chk 1 and 2 described above (13). On the other hand, a study done by Rossi et al. (2005), introduced the significant role of AIP4 ligases in regulating p73. In brief, AIP4 binds, poly-ubiquitinates, and proteosomaly degrades p73 in vivo and in vitro. Also in response to DNA damage, AIP4 is down regulated and p73 levels are consequently elevated (14). In depth, AIP4 can successfully bind TA and ΔNp73 through the WW domains. With respect to ubiquitination, both isoforms of p73 served as ubiquitin substrates in vivo and in vitro; thus concluding that the N-terminal domain is not essential for the AIP4 induced ubiquitination. Also, the lack of AIP4-p53 association and the inhibition of p73δ highlight the role of p73-PY motifs in AIP4 induced ubiquitination. Regarding AIP4 and as expected, the HECT domain was shown to be required for p73 induced ubiquitination. When investigating p73α stability and expression in response to AIP4 regulation, proteosomal degradation was detected post polyubiquitination. Therefore, this degradation was ceased when protease inhibitors were introduced. p73 half-life was also extended when ceasing AIP4 role. Moreover, low TAp73 transcriptional activity detected through monitoring the endogenous levels of p73 responsive genes confirmed the role of AIP4 in repressing TAp73 function. Interestingly, AIP4-p73 signalling pathway is not cell type specific. Results from

different cell lines showed the down regulation of AIP4 in response to DNA damaging stimuli (doxorubicin, cisplatin, etoposide) allowing TAp73 levels to rise. However this is not the case for ΔNp73 that is also rapidly down regulated in response to DNA damage even when AIP4 levels are low hinting for a non-AIP4 dependent regulation (14). The situation is not every different regarding UFD2a that also mediates p73 ubiquitination yet many aspects regarding the regulatory mechanism remains unknown (262).

Last but not least, Pirh2, among the remaining E3 ligases, acts as a potential target for the p73 regulatory pathway. The role of Pirh2 in regulating p53 hints for a possible similar pathway incorporating the other member of the p53 family: p73. A study done by Jung et al. (2011), confirmed that Pirh2 is a p73 inducible gene. According to this study, this relation is restricted to TAp73 and not ΔNp73. With respect to p73 expression, Pirh2 showed no effect on p73 isoforms mRNA levels yet demonstrated a positive feedback loop since TAp73 protein levels were elevated upon Pirh2 overexpression. The story was reversed in case of ΔNp73 that was reduced suggesting that Pirh2 can act as a main regulator in determining TA and ΔN ratios (263, 264). However, it was never understood how Pirh2 could have opposing roles towards TAp73 and ΔNp73 despite the fact that it binds both isoforms in vivo and in-vitro. Also, the stabilization effect was less evident when monitoring the endogenous p73 levels in unstressed cells. In terms of ubiquitination, Pirh2 only ubiquitinates ΔNp73 leading to proteosomal degradation post ubiquitination. In response to DNA damage, TAp73 is increased due to an elevation in Pirh2 levels as a result of DNA damaging agent (etoposide and cisplatin). Surprisingly, this had no impact on cell cycle and apoptotic levels despite the high levels of TAp73 (263, 265). Clearly the correlation remains to be indistinct and further research is required.

1.7 Aims of Thesis

The major focus of my thesis was Pirh2 E3 ligases. My first aim was to study the self-regulatory mechanism of Pirh2. The overexpression of Pirh2 in a wide range of human tumor raises possibilities that defect within the self ubiquitination mechanism is the reason for this overexpression. Based on that aim 1 was:

To reveal the optimal conditions for Pirh2 self ubiquitination, map the domain essential for ubiquitination, reveal the lysine residues utilized for polyubiquitination, and assess the effect of p53 presence or absence on Pirh2 self ubiquitination.

Knowing that Pirh2 binds, ubiquitinates, and degrades p53, we anticipated that Pirh2 is linked as well to p73 regulation. A study by Jung et al. (2011) has shown this correlation yet many questions remained unanswered. Based on that, aim 2 was:

To investigate the ubiquination of p73 by Pirh2 and analyze the lysine chains utilized.

Last but not least, I proposed a novel idea suspecting a possible correlation between two E3 ligases (Pirh2 and AIP4) thought to be independent in regulating the same substrate, p73 in our case. This novel correlation can explain how two proteins belonging to the same family and sharing high homology can be regulated by the same E3 ligase (Pirh2) yet have different fates. Also the proposed correlation may explain how the two E3 ligases coordinate in regulating p73 tumor suppressors. Preliminary data in our lab revealed the binding of Pirh2 and AIP4 using yeast two hybrid screening. This was considered a gateway to speculate the existence of a regulatory mechanism between Pirh2 and AIP4 in terms of p73 regulation. Early insight into this mechanism was detected when findings showed that co-transfection of H1299 cells with AIP4 and increasing doses of Pirh2 significantly inhibits AIP4 expression.

Therefore aim 3 was:

To investigate Pirh2 and AIP4 physical interaction, the regulatory mechanism between them, and the consequence of this correlation on p73 ubiquitination, degradation, and tumor suppressor activity.

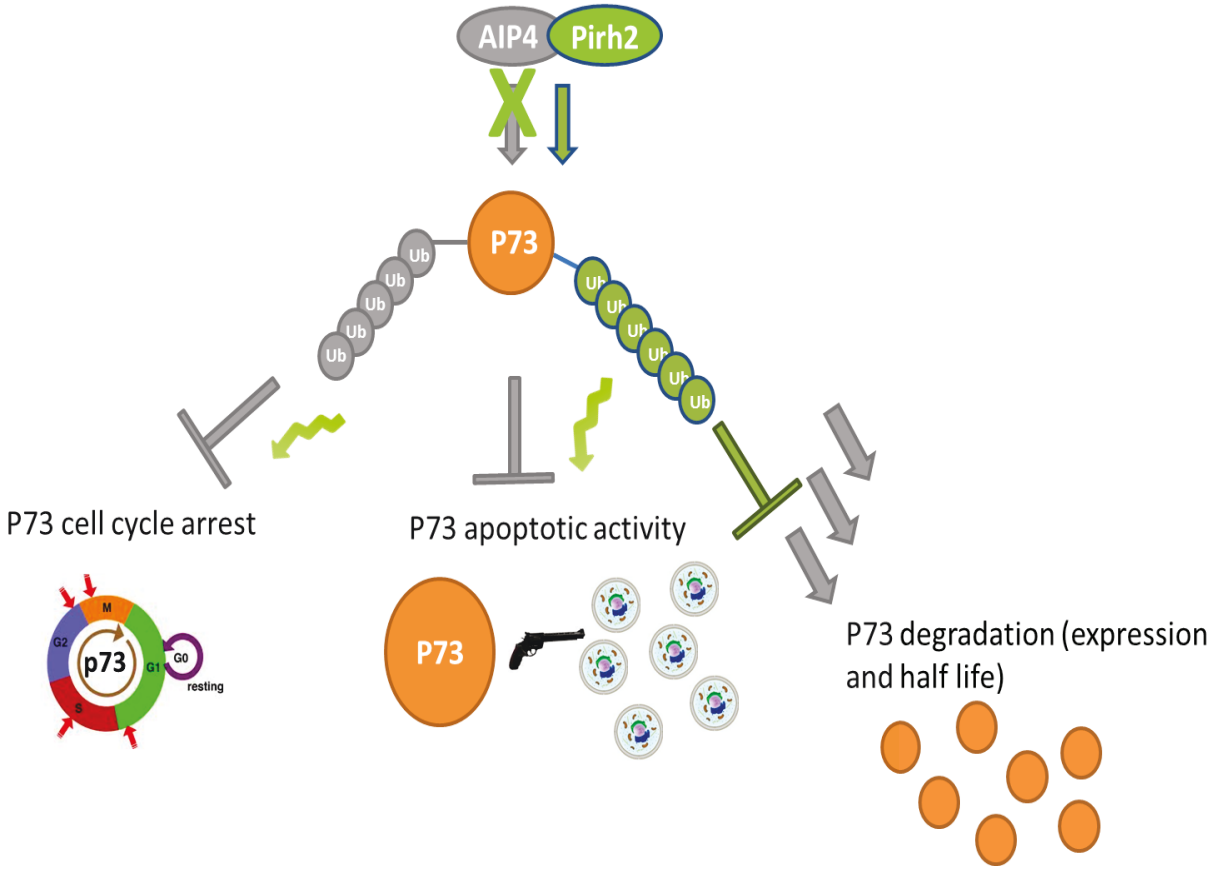

Figure 1.12. A schematic model representation of the proposed correlation between Pirh2 and AIP4 and their role in ubiquitinating and regulating p73.

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Chapter Two

RESIDUES 240-250 IN THE C-TERMINUS OF PIRH2 ARE ESSENTIAL FOR PIRH2 SELF-UBIQUITINATION

This chapter has been modified from a previous publication:

Abou Zeinab R, Wu H, Sergi C, & Leng R. (2013). Residues 240-250 in the C-terminus of the Pirh2 protein complement the function of the Ring domain in self-ubiquitination of the Pirh2 protein. *PlOS ONE* **8**, DOI: 10.1371/journal.pone.0082803.

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As first author, I designed and performed all the experiments described herein. I also wrote the manuscript.

2.1 INTRODUCTION

The function of the p53 tumor suppressor gene in maintaining genomic integrity (1) through its effects on cellular processes such as DNA repair, cell cycle arrest, and programmed cell death (2-4) is very significant. Thus, it is not surprising that 80% of human cancers are due to mutations in p53 or lack a functional wild type p53 allele (5). Clearly, tight regulation of the p53 protein is important. Although the precise mechanisms of p53 regulation are not fully understood, the involvement of many different proteins (6, 7) and many different processes, including sumoylation (8-10), neddylation (11-13), and acetylation or other post-translational modifications (14-16), have been proposed. Despite the apparent involvement of numerous proteins and processes, ubiquitination has been identified as the master regulatory mechanism (17). Three major enzymes are involved in the ubiquitination process: E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzyme), and E3 (ubiquitin ligating enzyme) (18). Ubiquitination is classified in three different types: mono, multi, and polyubiquitination. In definition, monoubiquitination indicates the attachment of one ubiquitin to the substrate. For multi ubiquitination more than one ubiquitin moiety at different residues are attached to the substrate; whereas in case of polyubiquitination multiple ubiquitin moieties are attached to the same substrate (18-19). Interestingly studies have shown that the same substrate could ubiquitinated differently by distinct E3 ligases, and the same E3 ligase can perform different types of ubiquitination on multiple substrates. In terms of p53, polyubiquitination is detected, which, in turn, promotes proteosomal degradation of p53 by 26S proteasome system (5, 19).

Many E3 ligases, including MDM2 (20, 21), Pirh2 (22), and COP1 (23), are tightly associated with the ubiquitination processes regulating p53 expression and activity. UBE4B was recently identified as an E3 and E4 ligases to promote p53 ubiquitination and degradation (24). Interestingly, of all the E3 ligases associated with p53, Pirh2 is the only one that is overexpressed in a wide range of human tumors, including primary breast cancer,

hepatocellular carcinoma, head and neck cancer, prostate cancer, and lung cancer (25-30). In normal unstressed cells, p53 is poly-ubiquitinated by Pirh2 and then degraded by 26S proteasome system. However, in response to cellular stress, Pirh2 is self-ubiquitinated leading to its degradation, which results in high levels of p53 (22). This phenomenon is important in cancer patients because constant Pirh2 expression leads to continuous p53 degradation, even in cases of cellular stress when the function of p53 is needed. It is noted that phosphorylation, a post translational modification of p53 that normally inhibits the binding of p53 to E3 ligases, such as MDM2, does not affect the binding of p53 to Pirh2, which has the ability to bind phosphorylated or non-phosphorylated p53 protein (31).

For these reasons, my main objective was to investigate Pirh2 self-ubiquitination by mapping each of the three main domains of Pirh2 and by identifying residues essential for its ubiquitination function. Domain mapping was accompanied by an analysis of ubiquitination conditions, such as the presence of E2 enzymes, ubiquitin mutations, and other factors that could affect self-ubiquitination. My investigations help explain the effects of Pirh2 overexpression, which can be associated either with a dysfunctional self-ubiquitination process or, in some cases, with a successful ubiquitination process that fails to lead to substrate degradation; hence resulting in altered regulation of end products (32). Studying the self-ubiquitination process of Pirh2 at the molecular level opens a new gateway to therapeutic techniques that can focus on counteracting the effects of Pirh2 overexpression in cancer patients by restoring self-ubiquitination activity, and thus releasing p53 continuous inhibition.

2.2 MATERIAL AND METHODS

2.2.1 Plasmids and antibodies

pcDNA3.1 served as the backbone mammalian expression vector for the p53 and Pirh2 used in the *in vivo* studies. WT and mutant Pirh2 constructs were generated by PCR-amplification

and subcloned into pGEX-5X-1 (22). Ubiquitin and ubiquitin mutants were PCR amplified and subcloned into pET-28-a (32). Similarly, His-p53 proteins were amplified by PCR and subcloned into pET-15-b (32). All PCR products were confirmed by sequencing. Pirh2 fusion proteins were detected using GST antibodies (B-14, Santa Cruz Biotechology), whereas mouse Pirh2 was detected using anti-Pirh2 antibodies. Mouse p53 was detected using the antibodies P421, P242, P1620, P122 and P248, whereas human p53 was detected using the antibodies DO-1, 1801, 1802, P421 and FL-393 (Santa Cruz biotechnology). Ubiquitin was detected using either anti-Myc (9E10, Roche, when Myc-Ub was used), anti HA (12CA5, Roche, when HA-Ub was used), or anti-Ubiquitin (BD) antibodies for the ubiquitin lysine mutant constructs. Anti-actin (Sigma) was also used as a loading control according to the manufacturer's description.

2.2.2 Cell culture and DNA transfection

H1299 cells were maintained in α-minimal essential medium supplemented with 10% fetal bovine serum. Calcium phosphate transfection methods were carried out for all in vivo ubiquitination reactions (22). 2M CaCl2 and 2XHEPES-buffered saline (pH: 7.05) were used. DNA was transfected to a maximum of 30µg per sample.

2.2.3 Expression and recombinant protein preparation

All GST- or His-tagged recombinant proteins were expressed in the *E. coli* strain BL21 (DE3, Novagene) that was treated with isopropyl-β-D-thiogalactoside (IPTG, 1 mM) for 4 hours at 30°C with shaking to induce fusion protein expression. Samples were centrifuged at 6000 rpm. Proteins were purified using glutathione Sepharose 4B (Amersham) for GST-fusion proteins or using Ni^{2+}-NTA agarose (Qiagene) for His-fusion proteins. All proteins were tested for purity prior to performance of ubiquitination reactions by separation on 10% SDS-PAGE gels, stained with Coomassie blue overnight, and destained for 8 hours the following day.

2.2.4 In vitro self-ubiquitination assay

In vitro self-ubiquitination assays were performed using purified GST-Pirh2 proteins. A total of 300, 600, or 900 ng of Pirh2 WT (wild-type) or mutant constructs was combined with E1 enzyme (40 ng, Calbiochem), E2 enzyme (H5B WT, 100 ng, Calbiochem, or other E2 variant), Myc-tagged ubiquitin WT or lysine mutant constructs (5 µg, Sigma), ubiquitination buffer (50 mM Tris-HCL pH 7.4, 2 mM ATP, 5 mM $MgCl_2$, and 2 mM DTT) and distilled water to a final volume of 40 µl. Reactions were incubated for 90 minutes in a 30°C water bath. The reactions were then stopped using SDS loading dye and heated at 95°C for 6 minutes. Proteins were separated on a 10% SDS-PAGE gel, transferred to PVDF membranes, and analyzed by Western blotting. Self-ubiquitination was visualized by immunoblotting with anti-ubiquitin antibodies. For p53 ubiquitination, purified His-p53 proteins (300 ng) were added to GST-Pirh2 WT. After the addition of all the ubiquitination factors mentioned above, reactions were also performed for 90 minutes in a 30°C water bath and then stopped using SDS loading dye and heated at 95°C for 6 minutes. Proteins were separated on a 10% SDS-PAGE gel, transferred to PVDF membranes, and analyzed by Western blotting. p53 ubiquitination was visualized using anti-p53 specific antibodies to exclude Pirh2 self-ubiquitination. In parallel, Pirh2 self-ubiquitination was tested using anti-ubiquitin antibodies to confirm the E3 activity of the Pirh2 proteins tested in the assay.

2.2.5 In vivo self-ubiquitination assay

H1299 cells were transfected with expression plasmids encoding Pirh2 (20 µg) and HA-tagged ubiquitin (5 µg). Plasmids ligated with the target genes were transformed to *Escherichia coli (E.coli)* DH5alpha strains and purified using Qiagene and Fermentous Max-Prep DNA extraction Kits. Cells were collected 30 hours post transfection and lysed in lysis buffer (50 mM Tris-HCL pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% NP40, 0.025% SDS, and 1 mM PMSF). Lysates were then sonicated and clarified by spinning at 4°C for 15 minutes to

remove cell debris. After boiling with SDS loading dye, 50 µg of total proteins was loaded and separated on 10% SDS-PAGE gels. After transferring to PVDF membranes, proteins were visualized using Western blotting. HA antibody was used to detect in vivo self-ubiquitination.

2.2.6 Ubiquitination integrated density

Ubiquitination levels were quantified using Adobe Photoshop CS4 to evaluate integrated band intensities. Tiff files of original films were uploaded, and inverted images were analyzed using measurement logs. Using the rectangular marque tool, a rectangle with an adjusted fixed size that included all ubiquitination smears was saved and applied to all the sample lanes, including the negative controls. All recorded integrated densities were compared to those of empty lanes or to the background of the original film. Data were saved as TXT files and analyzed.

2.3 RESULTS

2.3.1 Pirh2 in vitro self-ubiquitination optimal conditions

In previous studies, Pirh2 was shown to self-ubiquitinate (22, 33). Based on these studies, and because the self-ubiquitination process seems to be critical to Pirh2 expression and function as an E3 ligase, I designed three experiments to test the optimal conditions for Pirh2 E3 ligase activity. As shown in Figure 2.1a, Pirh2 self-ubiquitination was detected in 60 minutes. I found that there were no differences in Pirh2 self-ubiquitination after 60, 90, and 120 minutes, so I chose 90 minutes as optimal duration for later ubiquitination assays. Furthermore, I observed that 600 ng of Pirh2 is sufficient for Pirh2 self-ubiquitination (Figure 2.1b). Based on that, all ubiquitination reactions of Pirh2 WT and mutants were carried using 600ng. As shown in Figure 2.1c, four different E2 enzymes were tested; they were selected because they are among the 37 E2 human enzymes that are highly correlated

with RING-H2 E3 ligases (34, 35). I observed that H5B, H5A, and H5C, but not Cdc34, result in Pirh2 self-ubiquitination. As shown in Figure 2.1d, ubiquitination was not detected in the absence of E3, E2, E1, or ubiquitin, indicating that Pirh2-mediated ubiquitination is dependent on the presence of E1 and E2. Additionally, I found that proteins lose a portion of their enzymatic activity with time, despite being stored at -80°C with 10% glycerol. This is clear in lanes 5 and 6, in which Pirh2 WT a, which was purified two months earlier and stored at -80°C, showed significantly lower ubiquitination function than Pirh2 WT b, which was purified just two days prior to the performance of the ubiquitination reactions (Figure 2.1d).

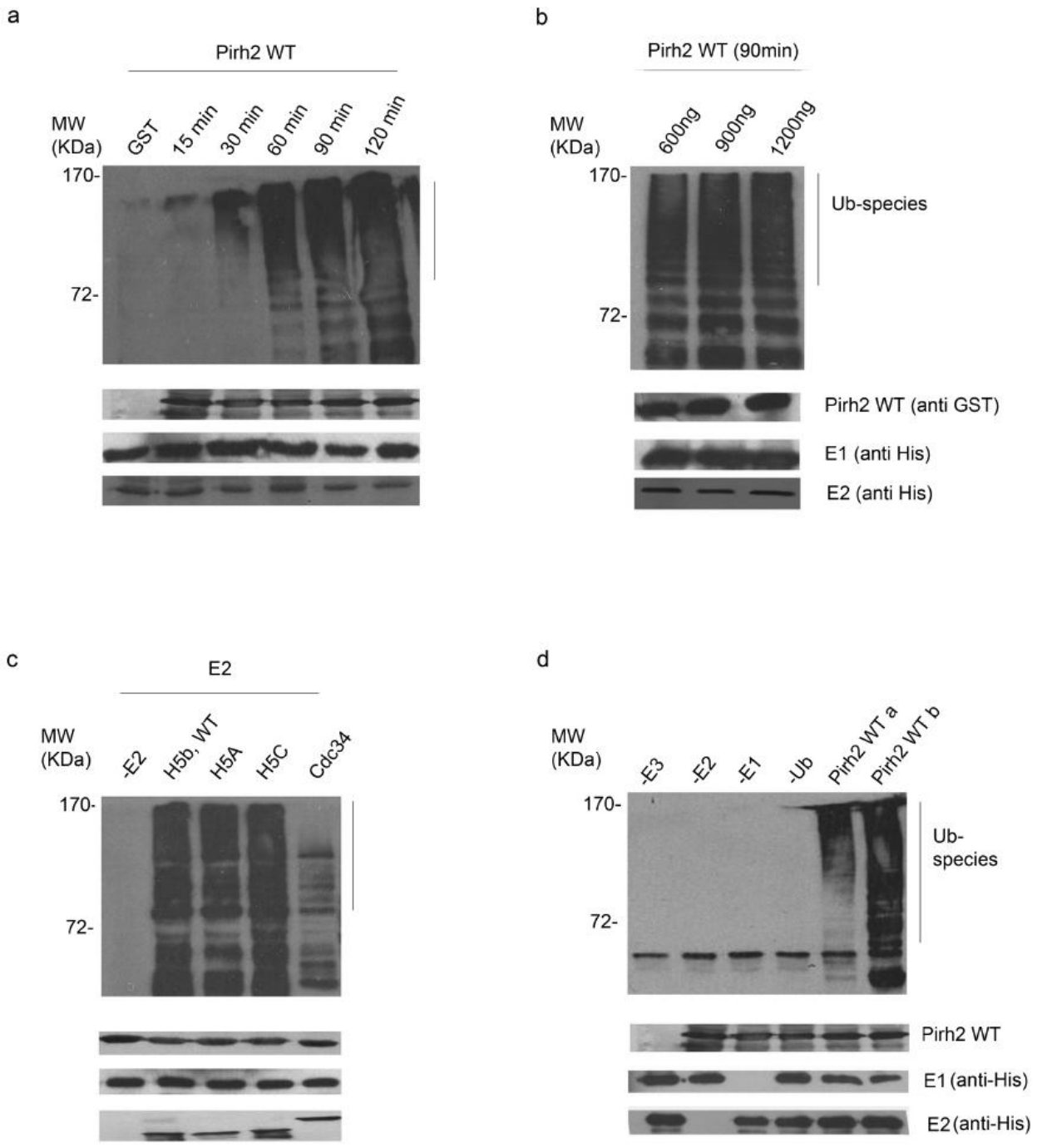


Figure 2.1. Pirh2 in vitro self-ubiquitination optimal conditions. (a) Self-ubiquitination time course of GST-Pirh2 WT protein. GST or GST-Pirh2 fusion proteins were affinity purified from *E.coli*. The self-ubiquitination of GST-Pirh2 was analyzed by immunoblotting with antibodies against ubiquitin (b) Similar to (a) except that different amounts of GST-Pirh2 were tested. (c) E2 dependent self-ubiquitination reaction. The E3

activity of Pirh2 was evaluated in the presence of E1, E2 (H5B, H5A, H5C, or Cdc34), and Myc-Ub. (d) In vitro self-ubiquitination in the absence of each enzyme (E1, E2, E3, and ubiquitin), with 600 ng of Pirh2 WT A or B that was purified 2 months or 2 days, respectively, prior to the performance of the reactions. For all ubiquitination, the reactions were run for 90 min; samples were subjected to SDS-PAGE and immunoblotting with anti-Myc antibodies to reveal ubiquitinated proteins. Anti-His antibody was used for all blots to confirm E1 and E2 presence, and anti-GST antibody was used for Pirh2 detection.

2.3.2 Mapping Pirh2 domains for self-ubiquitination in vitro

To reveal which residues in Pirh2 protein are essential for self-ubiquitination in vitro, three main domains of the Pirh2 protein (NTD, RING, and CTD) were mapped. In specific, three constructs that have residues deleted from the N-terminal domain were utilized: N40 has the first 40 residues of NTD deleted, N80 that has the first 80 residues of NTD deleted, and lastly N120 that has the first 120 residues of NTD deleted (22). Also a construct that lacked the RING domain (145-186) was utilized (22). The remaining three constructs were designed to test the function of the C-terminal domain: C60, C137, and C195. C60 has residues 70 to 261 deleted, C137 has residues 138-261 deleted, and C195 has residues 196 to 261 deleted (Figure 2.2a) (22). Also two extra constructs that had internal deletions were added. Δ120-137 which had residues 120-137, previously shown to be essential for p53 binding, deleted (22). Additionally, recent studies have related E3 ligase activity to the PHD domain that is present in some E3 ligases (36). Pirh2 protein has a PHD domain from residues 39-108 in its N-terminal domain. Therefore, a ΔPHD mutant construct was included in the analysis. Proteins were expressed and purified and run on a 10% SDS-PAGE gel to confirm their purity (Figure 2.2b). In vitro ubiquitination assays were performed using E1, E2 (H5B), and Myc tagged ubiquitin to test the potential E3 activity of Pirh2 WT and mutant constructs. As shown in Figure 2.2c, the NTD deletions had no effect on Pirh2 self-ubiquitination activity; rather, each of the three constructs N40, N80, and N120 showed successful ubiquitination despite lacking residues in their N-terminal domains. Additionally, Δ120-137 and ΔPHD, which have internal deletions in the N-terminal domain, showed positive ubiquitination, indicating that binding of Pirh2 to p53 is distinct from self-ubiquitination and that the PHD domain does not affect Pirh2 self-ubiquitination. I detected no ubiquitination with the ΔRING construct, indicating that the RING domain is involved in Pirh2 self-ubiquitination (39). Interestingly, constructs C60, C137 and C195, which have deletions in the CTD also showed no ubiquitination, indicating a role for the CTD in Pirh2

self-ubiquitination. I particularly noted that construct C195, which contains the whole RING domain but is missing residues 196 to 261 in the CTD, showed no ubiquitination activity. This surprising result indicates that residues within the CTD after the 195th residue are required for Pirh2 self-ubiquitination. Ubiquitination was further confirmed by measuring the integrated density of the ubiquitin smear detected on the original films (Figure 2.2d). All the deletions in the CTD clearly decreased ubiquitination, including the deletion in the C195 construct bearing the RING domain. This result explains my interest in mapping the C-terminal domain to investigate the role of its residues along with the RING domain in Pirh2 self-ubiquitination activity.

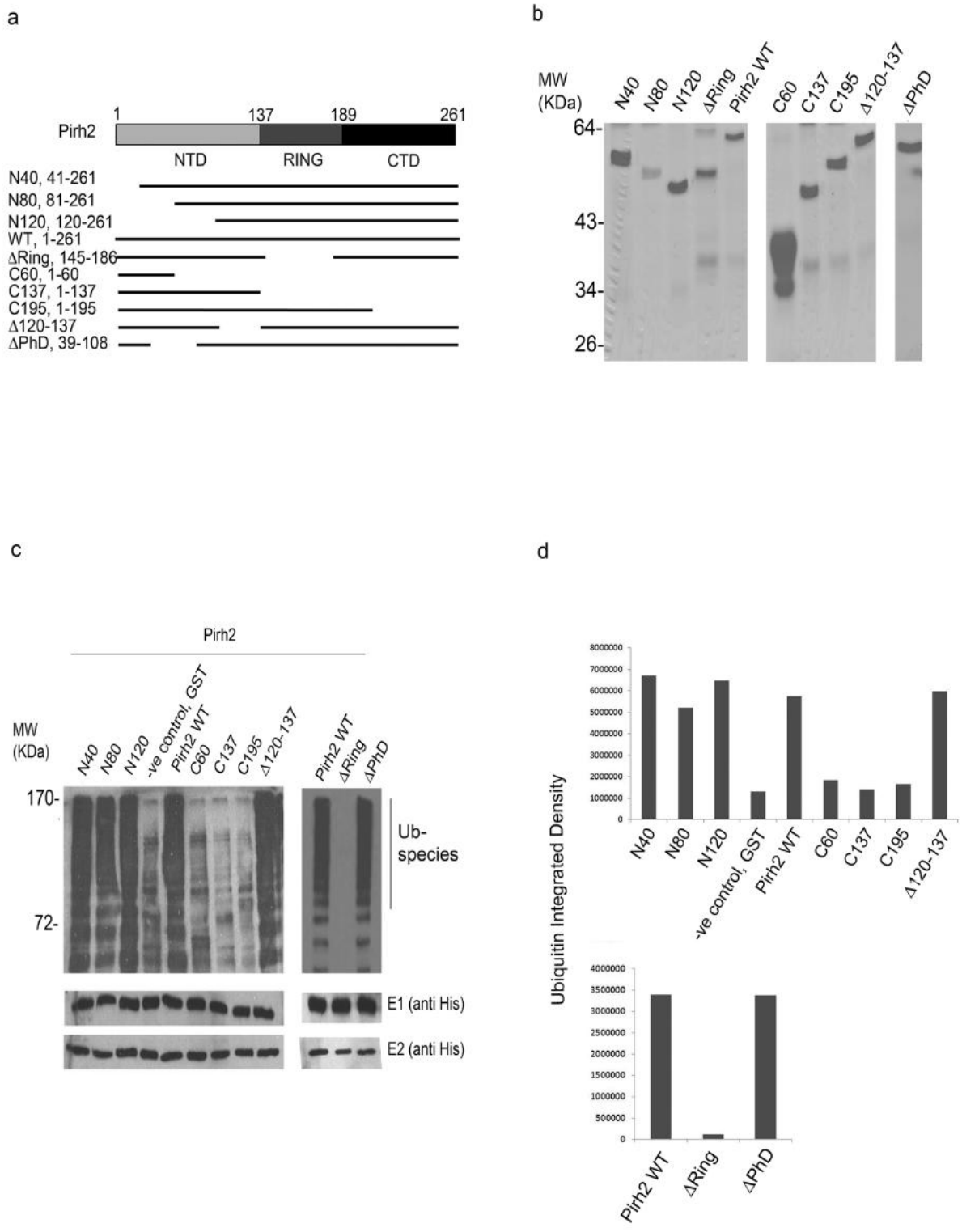


Figure 2.2. Mapping Pirh2 domain for in vitro self-ubiquitination. (a) Schematic representation of Pirh2 mutant constructs for the N-terminal domain, Ring domain, and C-terminal domain. (b) GST purified proteins run on a 10% SDS gel and stained with

Coomassie blue. (c) In vitro ubiquitination assays of Pirh2 and Pirh2 mutant constructs. Reactions were performed in the presence of E1, E2 (H5B), and Myc-Ub and analyzed as described in Figure 2.1. (d) The ubiquitin integrated density of the original films was evaluated using Photoshop density quantification to analyze the ubiquitination intensity of each band.

2.3.3 Residues 240-250 in the C-terminal domain are essential for Pirh2 self-ubiquitination

I designed a new set of mutant constructs (Pirh2A, B, C, D, and E) from Pirh2 WT (Figure 2.3a). Pirh2 WT and empty GST vectors were used as positive and negative control respectively. For a more accurate experimental design, additional constructs (Pirh2A and B) were used as a negative and positive control, respectively. Pirh2A lacks the NTD and was used to confirm that the NTD is not required for self-ubiquitination, and Pirh2B was used as a positive control to confirm that the RING domain along the CTD, without any residues from the CTD, is not sufficient for successful self-ubiquitination. Pirh2C, D, and E were designed on a 10 residue shift interval starting with the end terminal of the C domain (260-250; 250-240; 240-230, respectively). All proteins were expressed in GST vectors and purified using a GST purification protocol prior to performance of the ubiquitination reactions (Figure 2.3b). As expected, Pirh2A showed normal ubiquitination and Pirh2B showed no ubiquitination, consistent with my previous findings (Figure 2.3c). Interestingly, I was able to show that when residues 240-250 were deleted from the protein, Pirh2 self-ubiquitination activity was absent, despite the presence of the RING domain (Figure 2.3c, lane 6 and 7). The results were further analyzed by ubiquitination quantification (Figure 2.3d), which confirmed that ubiquitination was totally abolished after the deletion of residues 240-250.

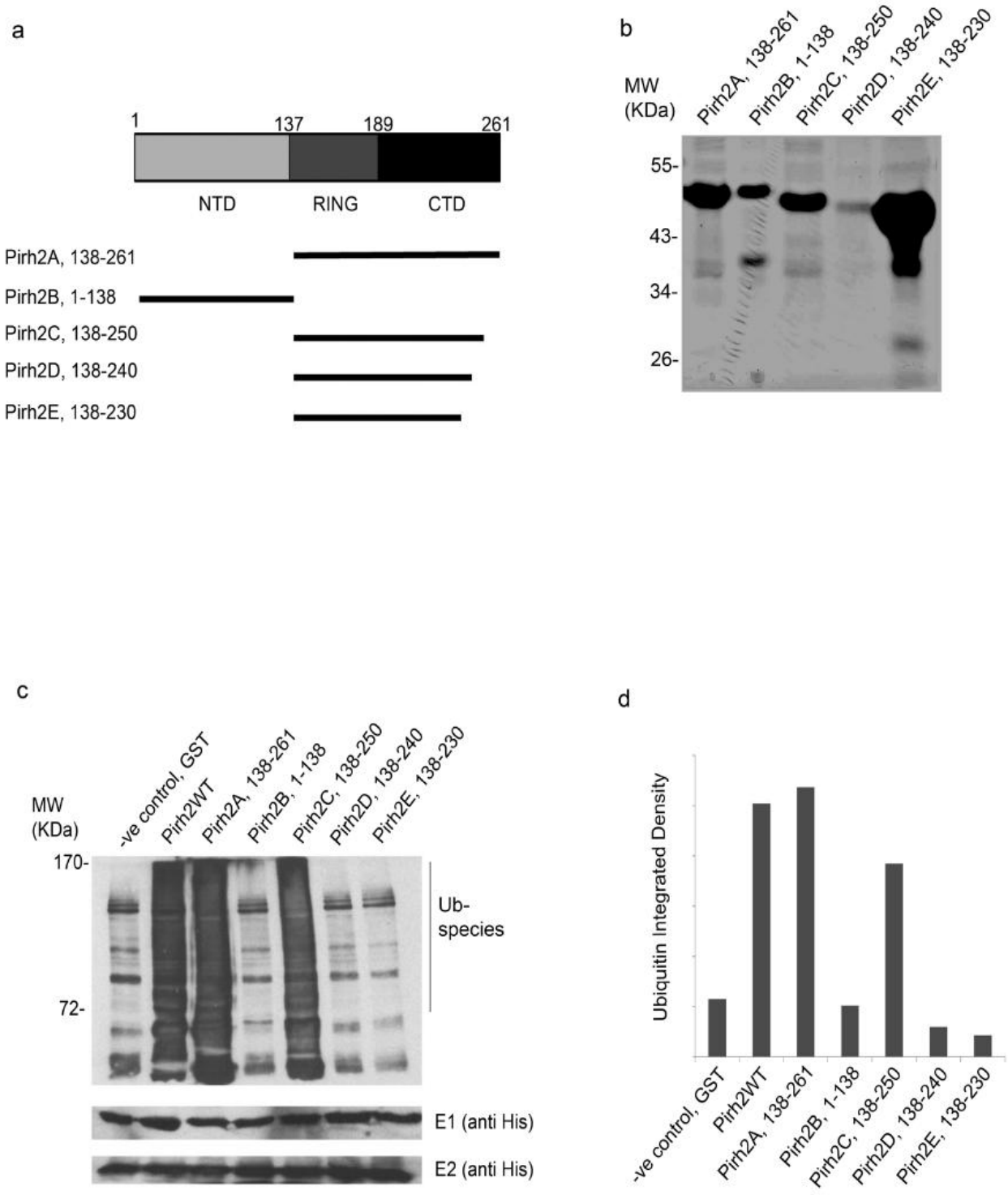


Figure 2.3. Residues 240-250 in the C-terminal domain are essential for Pirh2 self-ubiquitination. (a) Schematic representation of Pirh2 mutations in the C-terminal domain. (b) GST purified proteins run on a 10% SDS gel and stained with Coomassie blue. (c) In vitro ubiquitination assays of Pirh2 and Pirh2 mutant constructs. Reactions were performed

and analyzed as described in Figure 2.1. (d) The ubiquitin integrated density of the original films was evaluated using Photoshop density quantification to analyze the ubiquitination intensity of each band.

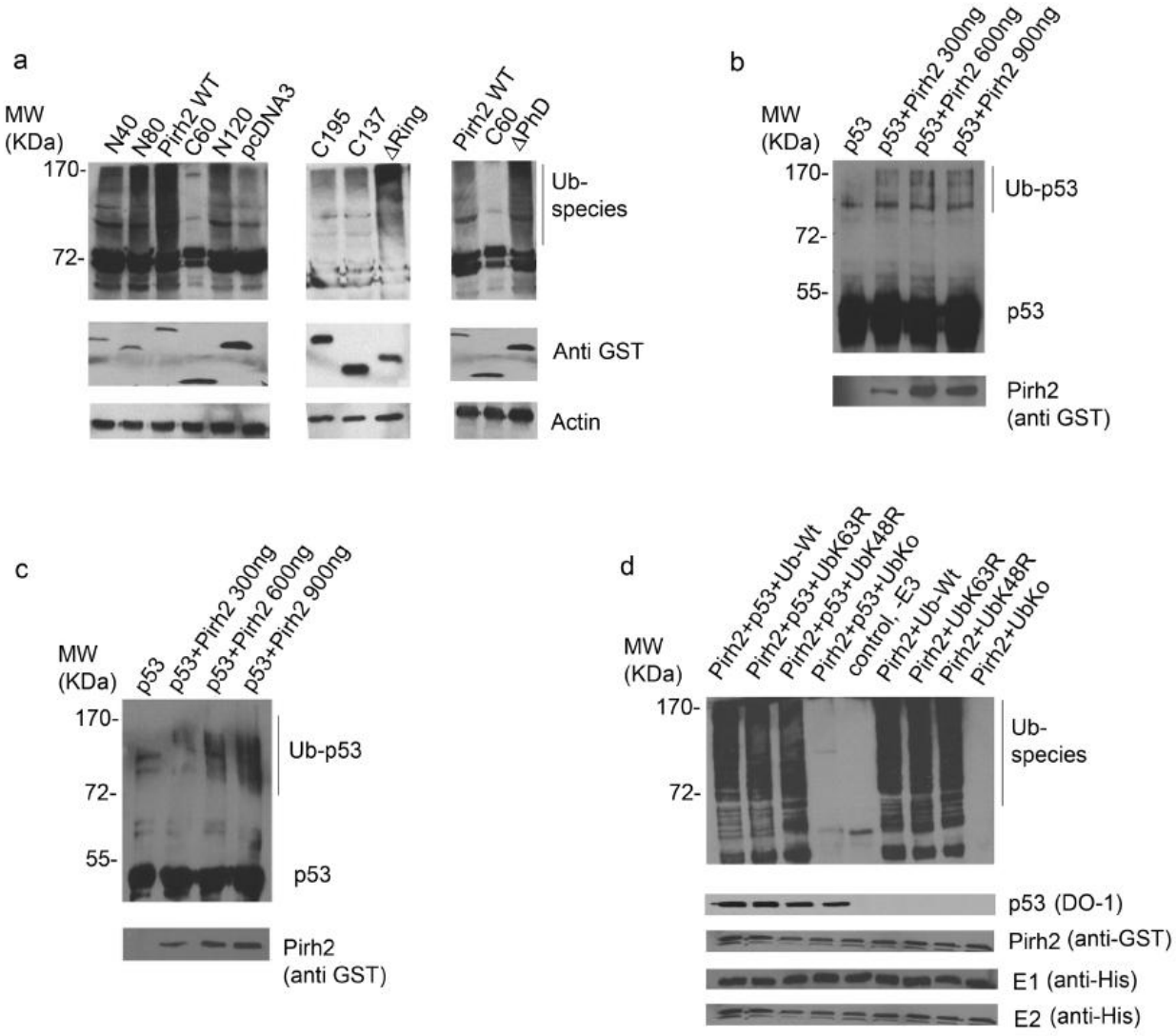
2.3.4 Pirh2 preferentially utilizes Lys-48 of Ub to mediate p53 ubiquitination in vitro

To confirm previous findings regarding in vitro ubiquitination, in vivo ubiquitination assays were performed. H1299 cells ($p53^{-/-}$) were transfected with plasmids expressing Pirh2 WT, various Pirh2 mutants, or vector pcDNA3, along with HA tagged ubiquitin (Figure 2.4a). Proteins were analyzed by Western blots with anti-HA antibodies to detect ubiquitinated species (Figure 2.4a: upper image) or with anti-GST antibodies to detect Pirh2 WT or Pirh2 mutants (Figure 2.4a: lower image). Consistently, ubiquitinated Pirh2 was detected in assays performed with Pirh2 WT and all the N-terminal domain mutants but not in assays performed with C-terminal domain mutants.

Because mouse and human p53 have commonly been used to study p53 regulation, I tested the ubiquitination of both human and mouse p53 by human and mouse Pirh2 proteins respectively. I observed that Pirh2 successfully ubiquitinates p53 highlighting that antibody DO-1 was more efficient in detecting human p53 ubiquitination (Figure 2.4b), while antibody P122 was more efficient at detecting mouse p53 ubiquitination (Figure 2.4c). Additionally, as indicated in Figure 2.4c, different concentrations of Pirh2 proteins were used. The level of p53 ubiquitination increased slightly with higher Pirh2 concentrations.

In the ubiquitination system, proteins are covalently tagged with an ubiquitin chain in which the terminal residue of one ubiquitin molecule is linked through an isopeptide bond to a lysine residue within another (37). Seven lysine residues are used for chain formation, and each may have a distinct effect on substrate fate. For example, K48 is known to cause substrate degradation, whereas K63 cannot (37, 38). Much less is known about the functions of chains with other topologies, which explains my interest in investigating the role of different lysine residues in the ubiquitination process. GST-Pirh2 and His-p53 were purified from *E.coli,* and reactions were carried out in the presence of wild-type ubiquitin (Ub-WT) or one of three ubiquitin mutants: Ub-KO, in which seven lysine residues -- K6,

K11, K27, K29, K33, K48, and K63 -- were replaced by arginine; Ub-K48R, in which only lysine K48 was replaced by arginine; or Ub-K63R, in which only lysine K63 was replaced by arginine (32). I tested Pirh2 self-ubiquitination activity in the presence or absence of p53 in vitro. As presented in Figure 2.4d, the self-ubiquitination of Ub-K63R and Ub-K48R were similar to the self-ubiquitination of Ub-WT; however, the self-ubiquitination of Ub-KO was significantly decreased. Interestingly, I found that the presence or absence of p53 protein did not affect Pirh2 self-ubiquitination (Figure 2.4d, lanes 1-4 compared to lanes 6-9). My results were further analyzed by ubiquitination quantification revealing the significance of the decrease in case of KO chains (Figure 2.4e). I further examined whether Pirh2 promotes p53 ubiquitination in vitro in the presence of Ub-WT, Ub-K48R, or Ub-K63R. As shown in Figure 2.4f, the presence of Ub-K48R significantly reduced the Pirh2-mediated ubiquitination of p53 compared to the presence of Ub-K63R or Ub-WT, suggesting that Pirh2 mediated p53 ubiquitination occurs through the K48 of ubiquitin in vitro. Together, these data demonstrate that Pirh2 preferentially utilizes ubiquitin K48 to ubiquitinate p53 in vitro.


a
MW
(KDa)
N40
N80
Pirh2 WT
C60
N120
pcDNA3
C195
C137
ΔRing
Pirh2 WT
C60
ΔPhD
170-
72-
Ub-
species
Anti GST
Actin
b
MW
(KDa)
p53
p53+Pirh2 300ng
p53+Pirh2 600ng
p53+Pirh2 900ng
170-
72-
55-
Ub-p53
p53
Pirh2
(anti GST)
c
MW
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p53
p53+Pirh2 300ng
p53+Pirh2 600ng
p53+Pirh2 900ng
170-
72-
55-
Ub-p53
p53
Pirh2
(anti GST)
d
MW
(KDa)
Pirh2+p53+Ub-Wt
Pirh2+p53+UbK63R
Pirh2+p53+UbK48R
Pirh2+p53+UbKo
control, -E3
Pirh2+Ub-Wt
Pirh2+UbK63R
Pirh2+UbK48R
Pirh2+UbKo
170-
72-
Ub-
species
p53 (DO-1)
Pirh2 (anti-GST)
E1 (anti-His)
E2 (anti-His)

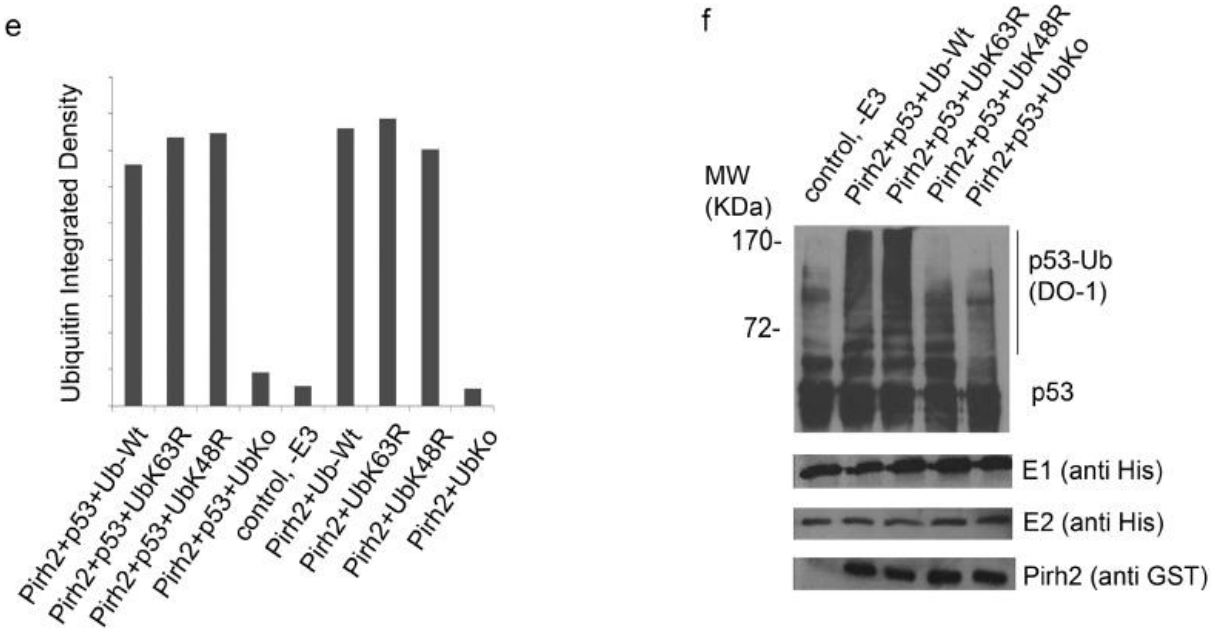

Figure 2.4. Pirh2 mediated p53 ubiquitination mainly through the K48 chain of ubiquitin in vitro. (a) In vivo self-ubiquitination reactions were performed in H1299 cells. Cells were transfected with plasmids expressing Pirh2 WT or the mutant constructs illustrated in Figure 2.2 and analyzed by Western blotting using anti-HA antibodies to reveal ubiquitinated proteins (top) and using anti-GST antibodies to detect Pirh2 WT or mutant proteins (bottom). Actin expression was used as a loading control. (b) and (c) Pirh2-p53 in vitro ubiquitination using human (b) and mouse (c) derived proteins. Ubiquitination reactions were performed in the presence of GST-Pirh2, His purified p53, E1, E2 (H5B), and Ub-WT. Pirh2 human and mouse derived proteins were used in three different concentrations (300, 600, and 900 ng). Following ubiquitination reactions (90 min), reactions were stopped by the addition of SDS loading dye and resolved on a 10% SDS-PAGE gel, and then, immunoblotting was performed using anti-p53 antibodies to reveal ubiquitinated p53 proteins. DO-1 was the most efficient in detecting human p53 ubiquitination (b). P122 was highly efficient in detecting mouse p53 ubiquitination (c). Anti-GST antibodies were used for Pirh2 detection as indicated. (d) Pirh2 ubiquitination using Ub-WT, Ub-K63R, Ub-K48R, or Ub-KO in the presence or absence of p53. Data was collected after immunoblotting with anti-ubiquitin antibodies to reveal ubiquitinated proteins, with DO-1 anti-p53 antibody to reveal p53, with anti-His antibody to reveal E1 and E2, and with anti-GST antibody to detect Pirh2. (e) The ubiquitin integrated density of the original films was evaluated using Photoshop density quantification to analyze the ubiquitination intensity of each reaction. (f) p53 in vitro ubiquitination by Pirh2 using the four ubiquitin constructs

described above. Immunoblotting was performed using anti-p53 antibody (Do-1) to reveal ubiquitinated p53 protein. Anti-His antibody was used to detect E1 and E2 and anti-GST antibody to detect Pirh2.

2.4 DISCUSSION

Because Pirh2 is an E3 ligase that regulates the p53 and p73 tumor suppressors (22, 32), Pirh2 ubiquitination has always been a focus of attention among researchers investigating the p53 system. Pirh2 has been shown to be involved in the ubiquitination of both proteins, independently of Mdm2 E3 ligases (31), as well as to be involved in negative feedback loop with its substrates (22, 24, 32). However, after Pirh2 was shown to be overexpressed in several human tumors, more attention was paid to its self-ubiquitinating activity, especially as it had been previously confirmed that Pirh2 levels are not affected by p53 WT in cancer cells but are elevated in p53 mutant (-/-) cells after DNA damage (39). Pirh2 is a RING E3 ligase, and its RING domain has always been a target of functional studies of Pirh2 proteins (40). The constructs shown in Figure 2.2a were first presented in a study by Leng et al. (2003) (22), and since then, have been used in many additional studies analyzing the activity of Pirh2 proteins. My study, however, is the first that analyzes the C-terminal domain of Pirh2. The loss of self-ubiquitination that I observed in a C195 mutant that lacks most of the C terminus and yet contains the RING domain is the first indication that residues outside of the RING domain are involved in self-ubiquitination. I specifically found that the absence of residues 240-250 inhibits Pirh2 self-ubiquitination despite the presence of the RING domain. I note that this role of the C-terminus in self-ubiquitination was first demonstrated in experiments using construct Pirh2C, which demonstrated a change in self-ubiquitination due to the lack of the last 10 residues (Figure 2.3a). These results were verified by ubiquitin integrated density quantification. In parallel to the experiments above, and because defects in ubiquitination end products might be due to the ubiquitin moieties involved in the process of ubiquitination, I investigated the role of lysine residues in the ubiquitination process. In other words, overexpression of Pirh2 might not be only due to the absence of self-ubiquitination but could also be due to a lack of substrate degradation despite successful ubiquitination due to lysine interference. At this point, I focused my

attention on specific lysine residues that had previously been shown to affect proteosomal degradation (32). Initially, the biological importance of K48-linked poly-Ub chains was demonstrated by the lethality of the Ub-K48R mutation in the yeast *Saccharomyces cerevisiae* (41). In contrast, in yeast Ub-K63R mutants, short-lived and abnormal proteins, the canonical substrates of the Ub-proteasome system, are still degraded at a normal rate (42). In my study, neither the Ub-K63R nor Ub-K48R mutant had a decreased level of Pirh2 self-ubiquitination, eliminating the possibility that either mutation might affect ubiquitination and/or degradation of Pirh2 in cancer patients. Interestingly, the mutations had different effects on p53 ubiquitination, with the Ub-K48R mutant unable to ubiquitinate p53. These data support a negative feedback loop between Pirh2 and p53 and highlight the role of lysine residues in a substrate's fate. In brief, this study confirmed the role of Pirh2 in promoting the cancer independently of p53 and provided additional insights into the process of self-regulation that might play a key role in the disruption of the Pirh2-p53 family system.

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Chapter Three

PIRH2 REPRESSES P73 TRANSCRIPTIONAL ACTIVITY BY PROMOTING ITS UBIQUITINATION

This chapter has been modified from a previous publication:

Wu H, **Abou Zeinab R**, Flores E, & Leng R. (2011). Pirh2 represses p73 transcriptional activity by promoting its ubiquitination. *Molecular Cancer Research* **9**(12):1780-1790.

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As second author, I performed the experiments regarding the ubiquitination assay presented in Figures 3.1 and 3.2.

3.1 INTRODUCTION

p73 was identified as a homologue of p53, and is capable of inducing apoptosis or cell cycle arrest (1-3). Unlike p53 that is commonly mutated in human tumors, p73 is rarely mutated (4). The tumor suppressor functions (apoptosis or cell cycle arrest) of p73 were at one point misleading because p73-deficient mice were not tumor prone or displayed an increase in tumor incidence. This was opposite to p53 mutations that triggered aggressive tumor formation (5, 6). However, a number of studies have shown that p73 expression is lost or reduced in certain human tumors (loss of heterozygosity, allele silencing), implicating that p73 and its tumor suppressive activities are correlated with tumor formation (7-9). Also p73 has been shown to respond to DNA damage, and the p73 responsive genes (such as bax, PUMA, Noxa, etc.) were also involved in apoptotic pathways and cell cycle arrest function (10-12). A study done by Flores et al. (2005) showed that $p73^{+/-}$ mice develop spontaneous tumors, and p73 loss can cooperate with the loss of p53 in tumor suppression. Also p73 mutations in combination with p53 mutations lead to a more aggressive tumor phenotype, indicating that p73 plays an important role in tumor suppression in the mouse model (13). The regulation of p73 apparently involved several signaling pathways. Please refer to Chapter one for a detailed explanation. In this chapter, I will limit my overview to E3 ligases and ubiquitination pathway with respect to p73 regulation.

MDM2, the major p53 regulator, is also correlated to p73 regulation. MDM2 can successfully bind and down regulate the transactivation activity of p73, yet does not lead to p73 degradation and does not shorten p73 half-life (14-16). On the other hand, a study done by Rossi et al. (2005), introduced the significant role of AIP4 ligases in regulating p73. In brief, they showed that AIP4 binds, poly-ubiquitinates, and proteosomaly degrades p73 in vivo and in vitro. Also in response to DNA damage stimuli, AIP4 is down regulated and p73 levels are consequently elevated. With respect to ubiquitination, AIP4 was shown to be required for p73 induced polyubiquitination followed by proteosomal degradation (17).

Similarly, the role of Pirh2 in regulating p53 hints for a possible similar pathway incorporating p73. Pirh2 is a RING finger E3 ligase that binds to p53 and promotes its degradation (18-21). Overexpression of Pirh2 in mouse tumors is accompanied by low p53 protein expression independent of Mdm2 levels (22, 23). Interestingly, a study done be Jung et al. (2011), confirmed that Pirh2 is a p73 inducible gene (24). Based on that, a new project done by Dr. Wu et al. (2011) aimed to investigate Pirh2-p73 interaction, ubiquitination, and p73 protein levels and transcriptional activity. Data confirmed that Pirh2 can interact with p73α and p73β in vivo. To determine whether endogenous p73 and Pirh2 could interact under more physiological conditions in the absence of overexpression, an IP/Western blotting experiment was performed using extracts prepared from human cells. Observations confirmed the presence of p73 in the anti-Pirh2 immunoprecipitates, and vice versa. In vitro pull down assays were also performed revealing the Pirh-p73 binding (25). To identify the regions of Pirh2 that interacts with p73, a number of Pirh2 deletion mutants were generated (18). All of the Pirh2 fragments, with the exception of those lacking residues 100-137, were capable of binding to p73. Thus residues 100-137 of Pirh2 are essential for p73 binding. Similarly, p73-binding site on the Pirh2 protein was mapped through generating four p73 constructs along the full length that served as the control. The DNA binding domain of p73, and in specific residues 123-313, was shown to be essential for Pirh2 interaction (25). Also this study analyzed the physiological consequence of the p73-Pirh2 interaction on p73 endogenous levels. Cells were transfected with increasing amounts of the Pirh2 expression plasmid. Interestingly overexpression of Pirh2 with dose dependency reaching 20μg of Pirh2 had no effect on p73 protein level. Investigations regarding p73 half-life confirmed the previous hypothesis which highlights the idea that Pirh2 does not regulate p73 stability since the half-life of p73 in the presence of Pirh2 was not significantly changed (25). It was expected to determine the lack of effect of Pirh2-p73 binding on p73 function as a tumor suppressor; however this was not the case. Luciferase assays utilizing p21-Luc (luciferase reporter construct) revealed a repression in p73 transcriptional activity in

response to Pirh2 overexpression. This was dependent on Pirh2 RING domain hinting for the role of ubiquitination in this process as the RING domain possesses the catalytic E3 ligase activity. AIP4 described above was used a positive control knowing that it binds, degrades, ubiquitinates, and inhibits p73 activity. Also inactive catalytic mutant construct of AIP4 (AIP4C830A), a direct site mutagenesis at the HECT domain responsible for AIP4 catalytic activity, was used as a negative control (25). Interestingly, reverse transcriptase PCR confirmed the decrease of p73 mRNA levels in response to active Pirh2 ligase overexpression. This was ceased when replacing Pirh2 WT with non-catalytic constructs (ΔRING) or when knocking-down Pirh2 by siRNAs. Pirh2 siRNAs alone showed an increase in p73 transcriptional activity (25). However in all experiments, p73 protein levels remained unaffected. Despite showing no differences at the protein level, the decrease of p73 transcriptional activity in response to Pirh2 overexpression was also tracked over the cell cycle arrest function of p73 which was decreased in response to Pirh2. For this reason knowing that the type of ubiquitination and the lysine chains utilized determine the substrate's fate, we aimed to investigate Pirh2-p73 ubiquitination and lysine chains utilized.

3.2 MATERIAL AND METHODS

3.2.1 Plasmids and antibodies

pcDNA3-Pirh2, GST-Pirh2, and Myc-Pirh2 were PCR amplified and generated as described earlier (18). Myc-AIP4 and Myc-AIP4 mutant (C830A) were provided by Dr. T. Pawson. His-p73α and His-p73β were PCR-amplified and subcloned into pET15b. Also, all ubiquitin or ubiquitin mutants were PCR-amplified and subcloned into pET-28-a (25). Flag-p73α or Flag-p73β or Flag-p73 mutants were generated by PCR and subcloned into pCMV-Tag1 (Stratagene) (25). pSUPER-RT (VEC-pRT-0002) vector served as the backbone for siRNAs generated. All PCR products have been confirmed by sequence. p73 specific antibody (ER-15, Abcam), anti-Myc specific antibody (9E10, Roche), anti-Flag (M5, M2, Sigma), anti-GST

(B-14, Santa Cruz Biotechology), anti-HA (12CA5, Roche), anti-Ubiquitin (BD), and anti-actin (Sigma) were used according to manufacturer's instructions.

3.2.2 Cell culture and DNA Transfection

All cells were maintained in α-minimal essential medium supplemented with 10% fetal bovine serum. H1299 cells, HEK293, HCT116 WT, and Saos-2 cells were transfected by the calcium phosphate method (18). 2M CaCl2 and 2XHEPES-buffered saline (pH: 7.05) were used. DNA was transfected to a maximum of 30μg per plate.

3.2.3 Expression and recombinant protein preparation

All GST- or His-tagged recombinant proteins were expressed in the *E. coli* strain BL21 (DE3, Novagene) that was treated with isopropyl-β-D-thiogalactoside (IPTG, 1 mM) for 4 hours at 30°C with shaking to induce fusion protein expression. Samples were centrifuged at 6000 rpm. Proteins were purified using glutathione Sepharose 4B (Amersham) for GST-fusion proteins or using Ni^{2+}-NTA agarose (Qiagene) for His-fusion proteins. All proteins were tested for purity prior to performance of ubiquitination reactions by separation on 10% SDS-PAGE gels, stained with Coomassie blue overnight and destained for 8 hours the following day.

3.2.4 Immunoprecipitation

After 30 hours from transfection, cells were lysed using lysis buffer (50 mM Tris-HCL (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.5% NP-40), with the addition of protease inhibitor table (Roche), and immunoprecipitated with the indicated antibodies. The immune complexes were collected with protein (A/G-agarose beads or A-agarose beads following the manufacturer recommendation) and washed three times with the lysis buffer (50 mM Tris-HCL (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.2% NP-40). The immunoprecipitates were

analyzed by SDS-PAGE, transferred to PVDF membranes, and analyzed by Western blotting and autoradiography.

3.2.5 In vitro Ubiquitination assay

The in vitro ubiquitination assay was performed as described previously in chapter 2. For Pirh2 mediated ubiquitination, reactions were carried out by adding E1 (40ng, Calbiochem), E2 (H5B WT, 100ng, Calbiochem), Ubiquitin or His-ubiquitin (3-5 μg, Sigma), His-p73 and GST-Pirh2 (0.2-0.5 μg) in ubiquitination buffer ubiquitination buffer (50mM Tris-HCL {pH 7.4}, 2mM ATP, 5mM $MgCl_2$, 2mM DTT) to a final volume of 30 μl. Reactions were incubated for 90 minutes in 30°C water bath. The reactions were then stopped using SDS loading dye and heated at 95°C for 6 minutes. Proteins were separated on a 10% SDS-PAGE, transferred to PVDF membranes, and analyzed by Western blotting. Self-ubiquitination was visualized by immunoblotting with ubiquitin tag antibodies. To eliminate the possible autoubiquitination of Pirh2, for the coupled in vitro ubiquitination/IP, the mixtures after 2 hr incubation were immunoprecipitated with the ER-15 (for p73) monoclonal antibody and protein A agarose beads, rotated at 4°C for 2-3 hr, washed with lysis buffer (50 mM Tris-HCL (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.2% NP-40), separated on 10% SDS-PAGE, transferred to PVDF membranes, and analyzed by Western blotting.

3.2.6 In vivo ubiquitination assay

Cells were transfected with expressing plasmids to total of 30μg. Plasmids ligated with the target genes were transformed to *E.coli* DH5alpha strains and purified using Qiagene and Fermentous Max-Prep DNA extraction Kits. The mix included expressing plasmids encoding: p73, Pirh2, AIP4, and HA-tagged ubiquitin or various HA-ubiquitin mutants either alone or in combination. Cells were collected 30 hours post transfection, lysed in lysis buffer (50mM Tris-HCL pH:7.4, 1mM EDTA, 150mM NaCl, 1% NP40, 0.025% SDS, and 1mM PMSF). Lysates were then sonicated and clarified by spinning at 4°C for 15 minutes to remove cell

debris, then immunoprecipitated with indicated antibodies. Immune complexes recovered with protein A-Sepharose were washed four times with lysis buffer (50 mM Tris-HCL (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.2% NP-40), separated on 10% SDS-PAGE, and analyzed by immunoblotting as described previously.

3.3 RESULTS

3.3.1 Pirh2 promotes the ubiquitination of p73 in vivo.

We investigated whether Pirh2 promotes p73 ubiquitination in vivo. Saos-2 cells were co-transfected with plasmids expressing Flag-p73α, Flag-p73β, and pcDNA3-Pirh2, along with HA-tagged ubiquitin. p73 was immunoprecipitated with specific monoclonal antibody for Flag (M5) and analyzed by immunoblotting with the HA antibody to detect ubiquitinated p73 (Figure 3.1a, upper image) or with the p73 specific monoclonal antibody (ER-15) to detect both p73 isoforms (Figure 3.1a, lower image). As shown in Figure 3.1a (upper image), immunoprecipitated p73α and p73β were ubiquitinated in the presence of Pirh2. Level of ubiquitination was assessed in comparison to our controls which were the empty vector (lane 1) or p73 (lane 2). Similar results were obtained when immunoprecipitating p73 using the ER-15 antibody (Figure 3.1b). It was also further found that Pirh2ΔRING (deleted RING domain in Pirh2) was unable to promote p73 ubiquitination, indicating that RING domain of Pirh2 is required to mediate p73 ubiquitination (25).

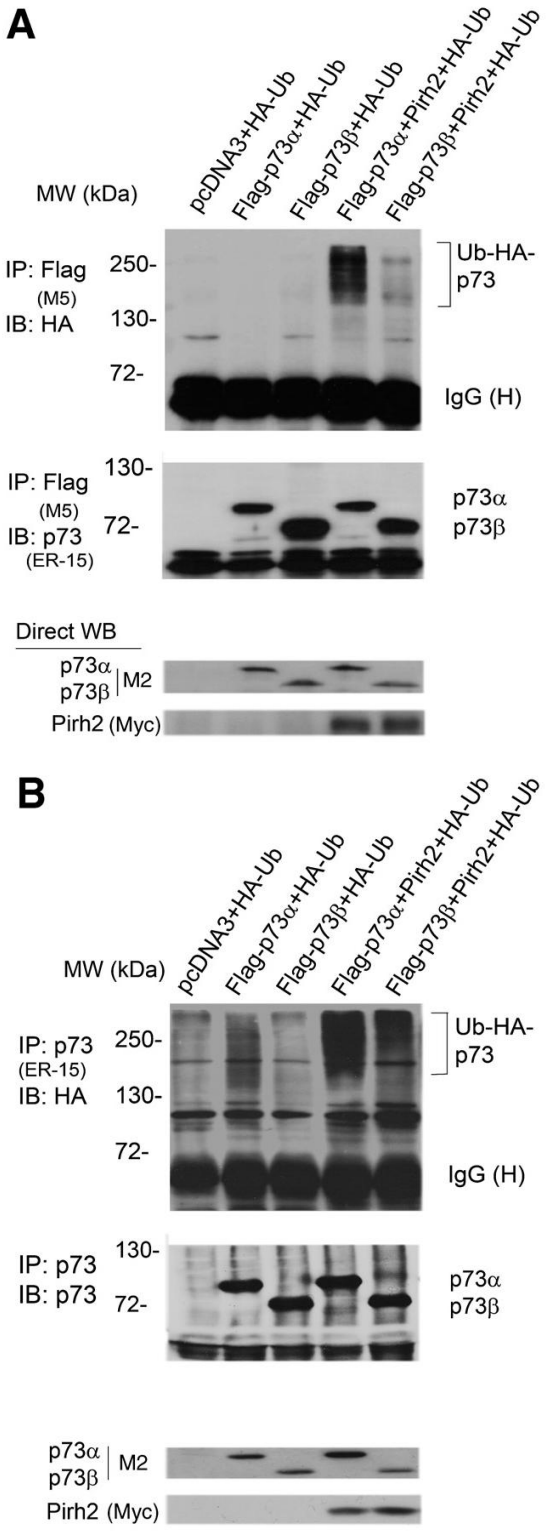

Figure 3.1. Pirh2 promotes p73 ubiquitination in vivo. (a) Saos-2 cells were co-transfected with plasmids expressing Flag-p73α, Flag-p73β, pcDNA3-Pirh2, and HA-tagged

ubiquitin. p73 was immunoprecipitated with anti-Flag (M5) monoclonal antibody and analyzed by immunoblotting with HA antibody (upper image) or with ER-15 for p73 (lower image). (b) Similar to (a) except that p73 was immunoprecipitated with anti-p73 (ER-15).

3.3.2 Pirh2 promotes the ubiquitination of p73 in vitro using K63 lysine chains.

To determine whether Pirh2 is required for p73 ubiquitination in vivo, HCT116 $p53^{-/-}$ cells were treated with the indicated siRNA constructs and, 40 hours later, further transfected with the HA-Ub expression plasmid and immunoprecipitated with a p73-specific monoclonal antibody (ER-15). The ubiquitinated p73 was significantly decreased upon Pirh2-siRNA treatment when compared to treatment with the control-siRNA. Stronger polyubiquitination of p73 was detected when cells were treated with MG132 (25). Notably, the levels of p73 were not significantly changed. Given that Pirh2 promotes p73 ubiquitination in vivo, we sought to determine whether p73 is a direct substrate for Pirh2 in vitro. An in vitro ubiquitination assay was performed (18, 26, 27). GST-Pirh2, His-p73α or His-p73β were purified from *E.coli,* and the reactions were added to E1, E2 (H5B), and ubiquitin or ubiquitin mutants (KO in which seven lysine residues K6, K11, K27, K29, K33, K48, and K63 were replaced by arginine; K48R in which only lysine K48 was replaced by arginine; K63R in which only lysine K63 was replaced by arginine). Ubiquitination of purified p73 was analyzed by Western blotting. Surprisingly, UbK63R mutant significantly reduced Pirh2-mediated p73α and p73β ubiquitination in comparison to UbK48R or Ub-WT, suggesting that Pirh2 mediated p73 ubiquitination mainly through K63 chain of ubiquitin in vitro (Figure 3.2a and 3.2b). This finding was further confirmed when repeated, as described above, however using a new set of ubiquitin mutants that contain one lysine with the remaining six lysine residues mutated to arginine (K6, K11, K29, K48, K63). Such measure reveals the direct dependency on the exact lysine position for ubiquitin tail formation. Constructs were purified from *E.coli,* and used for in vitro ubiquitination assay (25). In the presence of Pirh2 and p73, data revealed high levels of Ub-Lys-63, Ub-WT conjugation, moderate levels of Ub-Lys-11, 29 conjugation, and low levels of Ub-Lys-6, 48 conjugation (25). To eliminate the possible autoubiquitination of Pirh2, experiments were repeated however after 2hr reactions, the mixtures were immunoprecipitated with a specific monoclonal antibody for

p73 (ER-15), and analyzed by immunoblotted with the anti-Ub monoclonal antibody to detect ubiquitinated p73; data was similar.

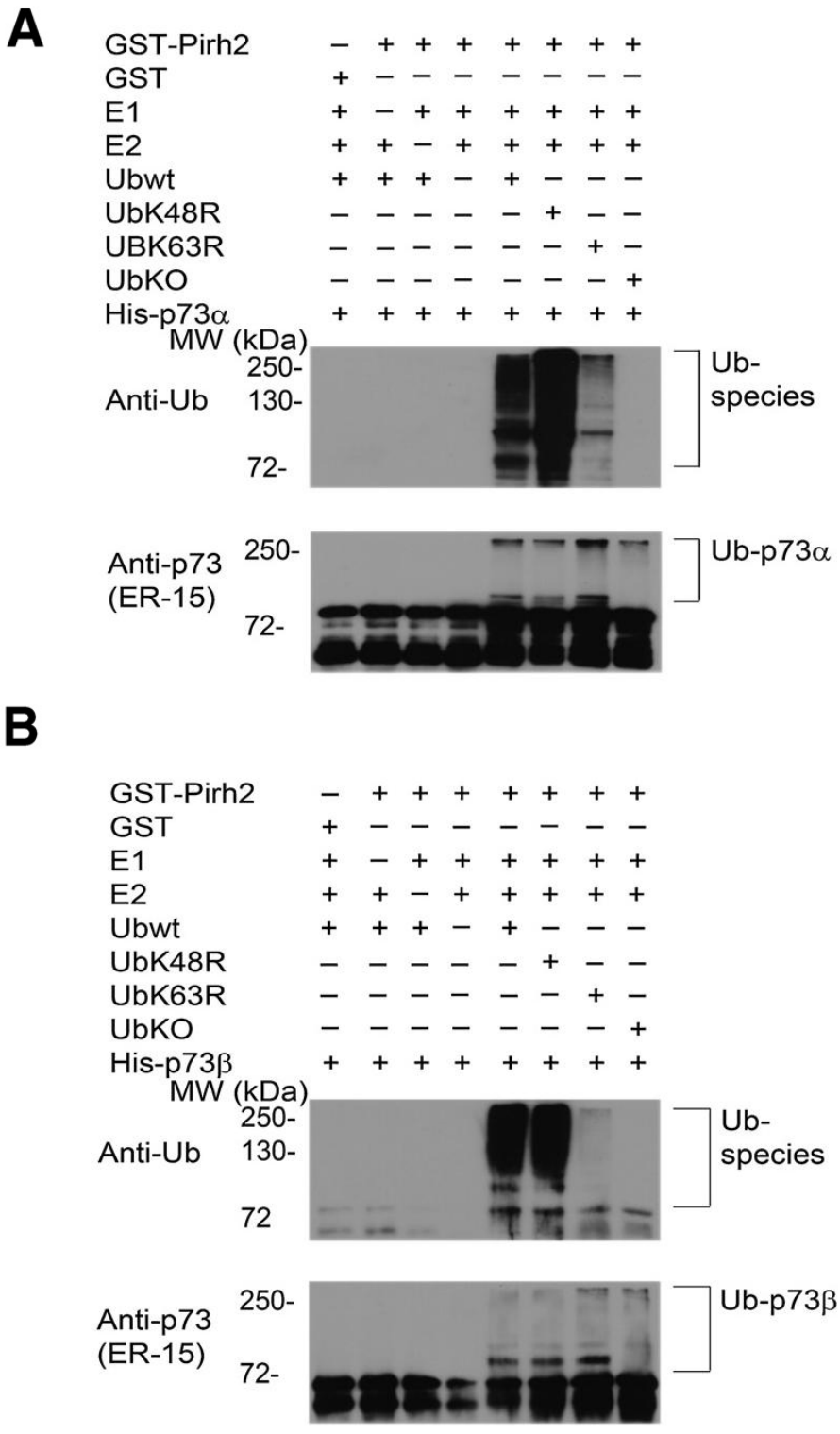

Figure 3.2. Pirh2 is an E3 ligase for p73 in vitro. GST-Pirh2 was evaluated for its capacity to ubiquitinate purified His-p73α (a) and His-p73β (b) utilizing Ub-WT or ubiquitin mutant constructs as indicated. Data was analyzed by immunoblotting with anti-Ub to

reveal ubiquitinated products (upper image) or with an antibody directed to p73 (ER-15) to reveal ubiquitinated p73 species. (c) Similar to (a) and (b), Pirh2 preferentially uses Ub with Lys residue at Lys-63, Lys-29, or Lys-11 for p73 ubiquitination in vitro. Affinity purified GST-Pirh2, His-p73aα, or His-p73β were added to bacterial extracts containing recombinant E1 and E2 (H5B) and wild-type Ub or ubiquitin mutants as indicated and analyzed by immunoblotting with anti-Ub to reveal ubiquitinated products (top) or anti-p73 (ER-15) to reveal ubiquitinated p73 species (bottom). D, similar to (C); samples after the ubiquitination reaction were immunoprecipitated with anti-p73 (ER-15) and analyzed by immunoblotting with anti-Ub (top) or anti-p73 (ER-15, top) as indicated. IB, immunoblotting.

3.4 DISCUSSION

Regarding p73 and as a Pirh2 substrate, more attention to its tumor suppressor function was drawn. On the other side, it makes it harder for us to understand how Pirh2 signals the regulation of p53 versus p73 especially that the fate and consequence of each regulation is distinct. In this project we reported the binding of Pirh2 and p73 with clear ubiquitination evidence. Notably the Pirh2 residues utilized in p53 or p73 binding are overlapping (18, 25). In other words under physiological conditions it is not known whether Pirh2 can bind both proteins or a selective mechanism signals the binding of one substrate. Further analysis using mass spectroscopy and NMR could answer these questions especially that protein folding and domain shielding can alter the binding process.

Also we observed that Pirh2 represses p73-dependent transcriptional activity by promoting p73 ubiquitination without targeting its degradation. Depletion of Pirh2 by siRNA can restore p73 transactivation function. Knowing that Pirh2 lacks the ability to degrade p73, the role of other cofactors in this pathway is highly possible and opens a new gateway in the same signaling/regulatory pathway. Since p73 deletion or point mutants that lack transactivation activity were stable compared to wild-type p73, Wu et al. (2004) proposed that p73 transcriptional activity is linked to its degradation (28). Besides, our findings indicate that p73-dependent transcriptional activity is regulated by ubiquitination. However, it is hard to justify that Pirh2 cannot degrade p73 with the current findings regarding the lysine chains utilized as the data collected here is insufficient. In chapter two, lysine at position 48 and 63 were used as hot spots for analyzing ubiquitination. Lys-63-linked chains appear to be used primarily for nonproteasome-dependent regulation of processes (such as DNA repair, endocytosis and chromatin remodeling) (29, 30). The biological importance of K48-linked poly-Ub chains is demonstrated by the lethality of the K48R mutation in the yeast *Saccharomyces cerevisiae* (30). In contrast, the K63R mutation of Ub in yeast still degrades short-lived proteins and abnormal proteins, the canonical substrates of the Ub-proteasome

system, at a normal rate (32). It is true that K48 lysine chains are the commonly known chains for being recognized by the 26 S proteasome system; yet also it has been reported that substrates with four or more Ub moieties, linked via Lys-29 or Lys-11, are targeted for degradation by the 26S proteasome as well (33, 34). Besides, recent studies suggested that K63-linked chains also support the proteosomal degradation (35-37). This was later correlated to E2 enzymes involved in the ubiquitination pathway. For example, it was reported that Rad6, as an E2, normally supports Lys-48 chain-dependent degradation in yeast and Rad6 also supports Lys-63 chain of Ub, suggesting that linkage specificity may depend on either the target proteins or the E3 for conjugation (38). In our ubiquitination analysis, Pirh2 catalyzes K11, K29, and K63-linked chains to ubiquitinate p73 protein in vitro, and markedly utilizes multiple lysine residue(s) (including Lys-48) to promote p73 ubiquitination in vivo. Based on that the current analysis is insufficient and requires further investigations regarding E2 enzymes involved in Pirh2-p73 ubiquitination and the 26S proteasome system post Pirh2 induced p73 ubiquitination. Interestingly, like Parkin et al. (2005), we weren't the first to notice that Pirh2 can act as a dual Ub E3 ligase, in which it utilizes K48-linked chain promoting p53 degradation and K63-linked chain repressing p73 transactivation (39). Again, many researches introduced the possibility that different E2s are used by Pirh2 through distinct mechanisms. This is not surprising knowing that Pirh2 belongs to the RING-H2 family ligases that do possess ability to bind ubiquitin but only transfer it from E2 to the substrate.

Finally, our data also reveal that Pirh2 regulates the transactivation activity of p73 in terms of cell cycle arrest function and not apoptosis. This highlights the possibility that each pathway is controlled by different mechanisms. At this point since the p73 responsive genes with respect to cell cycle arrest and apoptosis are known, hence then monitoring their mRNA level and protein expression in response to Pirh2-p73 regulation can answer several questions.

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Chapter Four

PIRH2 E3 LIGASE INHBITS THE AIP4-P73 NEGATIVE REGULATORY PATHWAY BY MEDIATING AIP4 EXPRESSION AND UBIQUITINATION

This chapter has been modified from a manuscript in preparation:

Abou Zeinab R, Wu H, & Leng R. (2014). Pirh2 E3 ligase inhibits the AIP4-p73 negative regulatory pathway by mediating AIP4 expression and ubiquitination. In preparation.

As first author, I wrote the manuscript, and designed, and performed all the experiments as described herein.

4.1 INTRODUCTION

p73 is a tumor suppressor that belongs to the p53 family known for its role in apoptosis and cell cycle arrest function (1-3). At the beginning, less attention was drawn to p73 in comparison to p53 that showed stronger apoptotic activity and cycle arrest function despite the high structural homology between the two proteins (4-6). Also the knockout of p73 gene in mice showed no effect on tumor growth (7-8). Besides, in contrary to p53 that is mutated in more than 50% of human tumors (9, 10), p73 was rarely mutated (11-14). However, the role of p73 in cancer was highlighted when several studies focusing on mouse models later revealed that mutations in p73 when accompanied with p53 mutants lead to more aggressive tumors. Also, in mice $p73^{+/-}$ deletions developed spontaneous tumors indicating the role of p73 in tumor suppression (15). At the molecular level, it was shown that overexpression of p73 can activate p53 responsive genes and trigger apoptosis (16, 17). p73 has been shown to respond to DNA damage and induce cell cycle arrest through the transactivation of p21 gene (18, 19). Also, p73 expression has been shown to be irregular in several human tumors (20, 21). However, the tight regulation of p73 remains unclear. As a member of the p53 family, many E3 ligases that were previously shown to regulate p53 proteins such as: MDM2, Pirh2, COP1, UBE4B, etc., (22-26) were suspected to be associated with p73 as well. In 2011, a study done by Wu et al. (2011) showed that Pirh2, a RING-H2 E3 ligase initially discovered as negative regulator to p53, can bind, poly-ubiquitinate, and decrease p73 transcriptional activity; but lacks the degradation effect at the protein level (27). This was further explained with the utilization of K63 ubiquitin lysine chains that do not induce proteosomal degradation post-ubiquitination as it is the case with K48 lysine chains utilized by Pirh2 to regulate p53 (28-31). Despite the polyubiquitination of p73 by Pirh2, the absence of proteosomal degradation raises many questions regarding this regulatory mechanism. In parallel, a study done by Rossi et al. (2005) showed that p73 is regulated by an ubiquitin protein ligase: AIP4. AIP4 belongs to HECT (Homologous to the E6-AP Carboxyl Terminus) domain proteins and is

shown to bind and ubiquitinate p73. Also, AIP4 decreases p73 dependent transcriptional activity and protein level (32). Based on that, I suspected a possible Pirh2-AIP4 correlation and started my study with investigating: physical interaction, expression at the molecular level, and the effect of this mechanism on p73 ubiquitination, fate, and tumor suppressor activity.

In this study, I was able to detect that Pirh2 physically interacts with AIP4 in vivo and in-vitro. Also I was able to reveal the residues/domains (N terminal domain for Pirh2, and HECT domain for AIP4) are involved in binding. At the molecular level, I showed that Pirh2 overexpression down-regulates AIP4; whereas AIP4 enhances Pirh2 expression when overexpressed. Data was confirmed when utilizing knockdown constructs of AIP4 and Pirh2 that significantly depleted their endogenous expression. With respect to p73, the regulatory mechanism was further defined as Pirh2 ceased AIP4-p73 negative regulation. p73 expression at the endogenous level confirmed the proposed pathway. Moreover, my findings confirmed that AIP4 self ubiquitination is mediated by the HECT domain and that AIP4 ubiquitination induced by Pirh2 results in AIP4 down-regulation. Regarding p73 ubiquitination, in vivo and in vitro analyses confirmed the ubiquitination of p73 by AIP4 which is abolished when Pirh2 is introduced and restored when Pirh2 is knocked down. Regarding p73 functional activity in cell cycle arrest, it was only detected when p73 is overexpressed alone or in combination with Pirh2 that ceases AIP4 induced down-regulation.

4.2 MATERIAL AND METHODS

4.2.1 Plasmids and antibodies

As described earlier, pcDNA3 served as the backbone mammalian expression vector for p73, AIP4, and Pirh2 used in in vivo studies. pSUPER-RT (VEC-pRT-0002) vector served as the backbone for siRNAs generated for AIP4 and Pirh2 (27). Myc-Pirh2 Wild type (WT) and mutant constructs were generated by PCR amplify and subcloned into pcDNA3-Myc vector as

described earlier (24); similarly GST-Pirh2 WT was generated by PCR amplify and subcloned into pGEX-5X-1 vector as described earlier (24). Flag-p73α and Flag-p73β were generated by PCR and subcloned into pCMV-Tag1 (Stratagene) (27). Also, His-p73α and His-p73β were PCR-amplified and subcloned into pET15b; and GST-p73α and GST-p73β were PCR amplified and subcloned into pGEX-5X-1 vector (27). Myc-AIP4 and AIP4 mutant (C830A) were provided by Dr. T. Pawson. Mutant AIP4 constructs were provided by Dr. Atfi (35). HA-ubiquitin was PCR-amplified and subcloned into pET-28-a (24). All PCR products have been confirmed by sequence. Regarding antibodies, 3 different p73 specific antibodies were used: ER-15- Abcam and p73-BD and p73α-Santa Cruz Biotechnology. Also two AIP4 antibodies were used: anti-AIP4/ITCH-BD and anti-AIP4 (28367)-Sant Cruz Biotechnology. Pirh2 polyclonal antibody was used as described previously (27). p53 antibody, 1801, was used as described previously (34). Anti-Myc specific antibody (9E10, Roche), anti-Flag (M5, M2, Sigma), anti-GST (B-14, Santa Cruz Biotechology), anti-HA (12CA5, Roche), anti-His (Novagen) and anti-Actin (Sigma) were used according to manufacturer's instructions.

4.2.2 Cell culture and DNA transfection

All cell lines used (H1299, HEK293, HCT116 WT, HCT116 $p53^{-/-}$) were maintained in α-minimal essential medium supplemented with 10% fetal bovine serum. Calcium phosphate transfection methods were utilized as described earlier (24). 2M CaCl2 and 2XHEPES-buffered saline (pH: 7.05) were used. DNA was transfected to a maximum of 30μg per sample.

4.2.3 siRNA experiments

The Pirh2 target sequences used were: CCTTGCTGTGACAAGCTTT and GCTTTAAAGTGAAGGAAGT and GCTTTATACTTGCCGCTTG representing Pirh2 siRNA1, 2, and 3 respectively. The AIP4 target sequences used were: AAGTGCTTCTCAGAATGATGA and AACCACAACACACGAATTACA representing AIP4 siRNA 1 and 2 respectively.

4.2.4 Expression and recombinant protein preparation

All GST or His-tagged recombinant proteins were expressed in *E. coli* strain BL21 (DE3, Novagene), treated with isopropyl-β-D-thiogalactoside (IPTG, 1mM) for 4 hours at 30°C while shaking to induce fusion protein expression. Samples were centrifuged at 6000 rpm for 10 minutes. Proteins were purified using the glutathione Sepharose 4B (Amersham) for GST-fusion proteins (Pirh2, AIP4 WT and mutants, GST p73 α orβ) or using Ni^{2+}-NTA agarose (Qiagene) for His-fusion proteins (His p73 α orβ). All proteins were tested for purity prior to ubiquitination reactions through separating them on 10% SDS-PAGE gels and staining with Coomasie blue overnight; and then destained for 12 hours the following day.

4.2.5 Immunoprecipitation

After 30 hours from transfection, cells were lysed using lysis buffer (50 mM Tris-HCL (pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40), with the addition of protease inhibitor table (Roche), and immunoprecipitated with the indicated antibodies. The immune complexes were collected with protein (A/G-agarose beads or A-agarose beads following the manufacturer recommendation) and washed three times with the lysis buffer (50 mM Tris-HCL (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.2% NP-40). The immunoprecipitates were analyzed by SDS-PAGE, transferred to PVDF membranes, and analyzed by Western blotting and autoradiography.

4.2.6 In vitro ubiquitination assay

The in vitro ubiquitination assay was performed as described previously (34). Reactions were performed using purified GST-AIP4 WT or mutant constructs. GST-Pirh2 alone or in combination with AIP4 WT was used as well. Along the E3 enzyme, E1 (40ng, Calbiochem), E2 (H5B WT, 100ng, Calbiochem), Myc or HA-tagged ubiquitin WT (5 µg, Sigma), and ubiquitination buffer (50mM Tris-HCL {pH 7.4}, 2mM ATP, 5mM $MgCl_2$, 2mM DTT) were added and using distilled water the final volume was adjusted to 40µl. Reactions were

incubated for 90 minutes in 30°C water bath. The reactions were then stopped using SDS loading dye and heated at 95°C for 6 minutes. Proteins were separated on a 10% SDS-PAGE, transferred to PVDF membranes, and analyzed by Western blotting. Self-ubiquitination was visualized by immunoblotting with ubiquitin tag antibodies. For p73 ubiquitination, purified p73 proteins (300ng) were added to the mix. Reactions were performed and analysed as described above. However, ubiquitination through Western blotting was also visualized using p73 specific antibodies to exclude Pirh2/AIP4 self-ubiquitination. To avoid false negative data when analyzing p73 ubiquitination due to Pirh2 and AIP4 self ubiquitination, CO-IP experiments were performed as well. The lysate were incubated with p73 antibody (ER15) for one hour on ice. Afterward samples were rocked for 3 hours with protein agarose beads "A". Afterwards, beads are washed with lysis buffer (same as described above) and separated on 10% SDS-PAGE. Analysis for p73 ubiquitination was determined by WB using p73-BD/antibody and not ER15. Also similar experiments were done using AIP4 antibody for immunoprecipitation known that AIP4 and p73 are shown to successfully bind.

4.2.7 In vivo ubiquitination assay

Cells were transfected with expressing plasmids to total of 30µg. Plasmids ligated with the target genes were transformed to *E.coli* DH5alpha strains and purified using Qiagene and Fermentous Max-Prep DNA extraction Kits. The mix included: p73 (5ug), HA-tagged ubiquitin (5µg), in addition to AIP4 and Pirh2 alone or in combination and at a different concentrations depending on the experiment. Cells were collected 30 hours post transfection, lysed in lysis buffer (50mM Tris-HCL pH:7.4, 1mM EDTA, 150mM NaCl, 1% NP40, 0.025% SDS, and 1mM PMSF). Lysates were then sonicated and clarified by spinning at 4°C for 15 minutes to remove cell debris. 60µg total proteins were loaded on a 10% SDS-PAGE after heating the samples at 95°C for 6 minutes with SDS loading dye. HA antibody is used to detect in vivo ubiquitination.

4.2.8 Cell Cycle Analysis

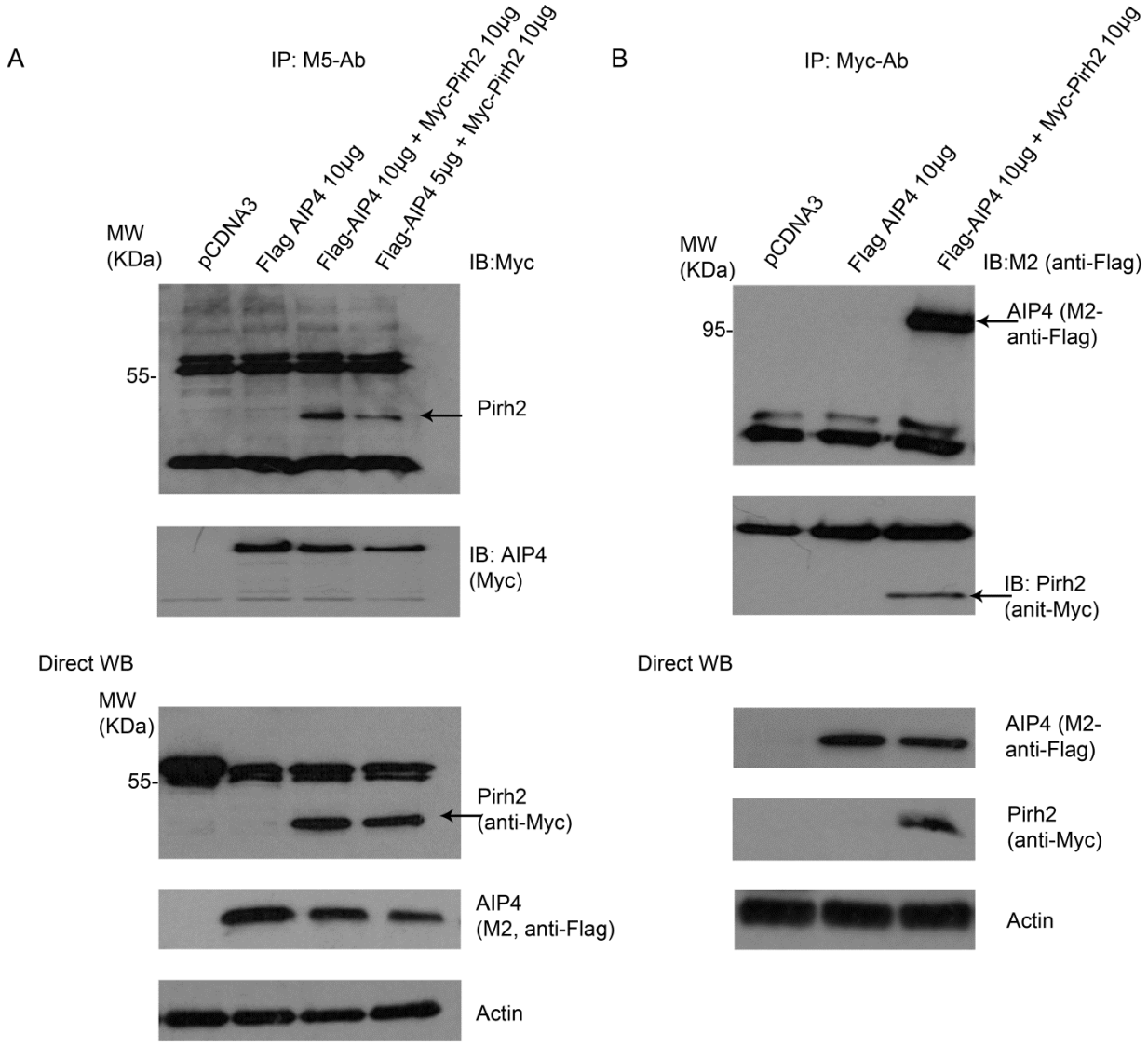
H1299 cells were transfected to a total of 10 µg with p73α and β in combination with a different mix of E3 ligases and knockdowns. 30 hours post transfection; cells were washed twice with 1XPBS and fixed overnight in -20^{o}C with 70% cold ethanol to a total volume of 3ml. Pelleted cells are collected and dissolved in FCS buffer and washed to a total of two times. Cells were then dissolved in Sodium Citrate (3.8M) and 10mg/ml RNAse and 50 mg/ml Propidium iodide to stain the nuclei. Cell cycle arrest (G0/G1; S; G2/M) were examined by flow cytometer using FACScan Flow cytometer and Cellquest software. The relative proportion of cells in each phase will be determined using the automated ModFit program. In parallel Western blot analysis were carried to determine the transfection efficiency of p73 and the used ligase mix.

4.3 RESULTS

4.3.1 In vivo and in vitro binding of Pirh2 and AIP4 utilizing the N-terminal domain of Pirh2 and HECT domain of AIP4

It has been previously shown that Pirh2 binds p53 and p73 proteins (24, 27), whereas AIP4 binds p73 exclusively (32) despite the homology (~80%) between p73 and p53 at DNA binding sequence (19). For this reason I aimed to investigate Pirh2 and AIP4 binding and map their major domains because such findings may later aid in understanding how Pirh2 signals binding to p53 or p73. For example, AIP4 binding to Pirh2 may interfere with the same regions of Pirh2 that are utilized in p53 binding. This could also act as a gateway to understand how the binding of AIP4 to other effector proteins could be affected due to Pirh2 interference. Accordingly, co-immunoprecipitation (Co-IP) experiments followed by Western blot analysis in H1299 and HEK293 cells were performed (Figure 4.1). Cells were co-transfected with Flag-AIP4 and Myc-Pirh2. In parallel, transfection with Flag-AIP4 or Myc-Pirh2 was used as controls. Data showed that Pirh2 co-immunoprecipitated with AIP4 when

using AIP4 antibodies (Figure 4.1a) and similarly AIP4 co-immunoprecipitated when using Pirh2 antibodies (Figure 4.1b). To confirm in vivo binding, GST-Pirh2 and GST AIP4 proteins were purified from BL21 *E.coli*. Proteins were purified and analyzed for purity and molecular size prior to Co-IP experiments. Pull down assay was performed and analysis using AIP4 or Pirh2 antibodies through WB revealed the in vitro binding between both ligases. Also, an AIP4 construct bearing a point mutation in the HECT domain (C830A) known for ceasing the HECT catalytic activity (35) was added to my samples. Data still showed successful binding with Pirh2 confirming that catalytic activity of the HECT is not related to its role in binding (Figure 4.1c).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IP: M5-Ab
MW (KDa)
pCDNA3
Flag AIP4 10μg
Flag-AIP4 10μg + Myc-Pirh2 10μg
Flag-AIP4 5μg + Myc-Pirh2 10μg
IB:Myc
55-
Pirh2
IB: AIP4 (Myc)
Direct WB
MW (KDa)
55-
Pirh2 (anti-Myc)
AIP4 (M2, anti-Flag)
Actin
B
IP: Myc-Ab
MW (KDa)
pCDNA3
Flag AIP4 10μg
Flag-AIP4 10μg + Myc-Pirh2 10μg
IB:M2 (anti-Flag)
95-
AIP4 (M2-anti-Flag)
IB: Pirh2 (anit-Myc)
Direct WB
AIP4 (M2-anti-Flag)
Pirh2 (anti-Myc)
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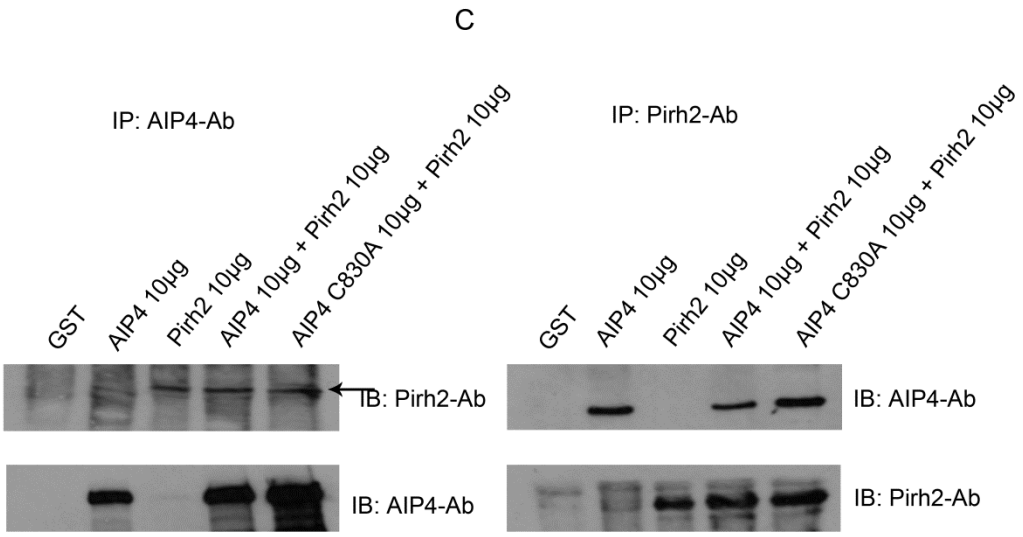


Figure 4.1. In vivo and in vitro binding of Pirh2 and AIP4. (a) In vivo binding of Pirh2 and AIP4 was assesseed where H1299 cells were co-transfected with plasmids expressing Myc-Pirh2 and Flag-AIP4 and, immunoprecipitated with M5 antibody for Flag-AIP4, and analyzed by Western blots with anti-Myc for Pirh2 and M2 (anti- Flag) for AIP4 as indicated. Also portions of the cell lysate were isolated for direct Western blot analysis (bottom panel). Similarly anti-Myc was used to detect Pirh2 and M2 (anti- Flag) to detect AIP4, and Actin antibody was used as loading control. (b) opposite to (a), Co-IP experiment was the same except that cell extracts were immunoprecipitated with anti-Myc and immunoblotted with M2 (anti-Flag) as indicated. Also portions of the cell lysate were isolated for direct Western blot analysis (top panel). Anti-Myc was used to detect Pirh2 and M2 (anti- Flag) to detect AIP4, and Actin antibody was used as loading control (bottom panel). (c) In vitro interaction of Pirh2 and AIP4 was evaluated using GST-Pirh2 and GST-AIP4 fusion proteins. The ability of GST-Pirh2 to bind GST-AIP4 was analyzed by immunoprecipitating for AIP4 using AIP4 antibody and immunoblotting for Pirh2 using Pirh2 antibody (left panel); or immunoprecipitating for Pirh2 using Pirh2 antibody and immunoblotting AIP4 using AIP4 antibody (right panel).

Second, the major domains of AIP4 and Pirh2 were mapped to reveal what residues in specific are essential for a successful binding. Accordingly, seven AIP4 mutant constructs (35) were utilized focusing on the 3 major domains of AIP4: C2, multiple WW domains (WW1/2, WW3/4), and HECT domain (Figure 4.2a). Two terminal mutant constructs, one lacking only the C2 domain and the other lacking only the HECT domain, were used as well. In addition to that, five WW constructs: C2-WW1/2, WW3/4-HECT, WW1/2, WW3/4, and WW1/2-WW3/4 were included in the analysis. Prior to Co-IP experiments, all constructs were transfected at the same concentration to detect their efficient expression in cells. Similar to the experiment described above, Co-IP was performed. AIP4 WT was used as a positive control for Pirh2 binding. All constructs showed a decrease in binding affinity to Pirh2 except construct 1 and 6, both of which do not lack HECT domain (Figure 4.2b). Hence, the deletion of the HECT domain and not the WW domains, commonly known for their role in binding (33), inhibited AIP4-Pirh2 binding confirming its role in the binding mechanism. Also Pirh2 domains: N-Terminal, RING, and C-Terminal domain were mapped for AIP4 binding. Based on that, Pirh2 constructs described in chapter 2 were utilized for mapping the residues involved in AIP4 binding (Figure 4.2c). Of the seven constructs, three have deleted residues in the N-terminal: N40 that has the first 40 residues of NTD deleted, N80 that has the first 80 residues of NTD deleted, and N120 that has the first 120 residues of NTD deleted. Also one construct lacked the RING domain (145-186 residues). The remaining 3 constructs focused more on the C-terminal where C60 has residues 70 to 261 deleted, C137 has residues 138-261 deleted, and C195 has residues 196 to 261 deleted. Co-IP experiments as described above revealed the role of NTD of Pirh2 in binding AIP4. Deletion of the RING domain, known for its catalytic activity, did not affect the AIP4 binding and similarly for CTD deletions (Figure 4.2d). To conclude, the binding of both ligases, Pirh2 and AIP4, at the in vivo and in vitro level was confirmed and the domains, HECT for AIP4 and NTD for Pirh2, are required for interaction.

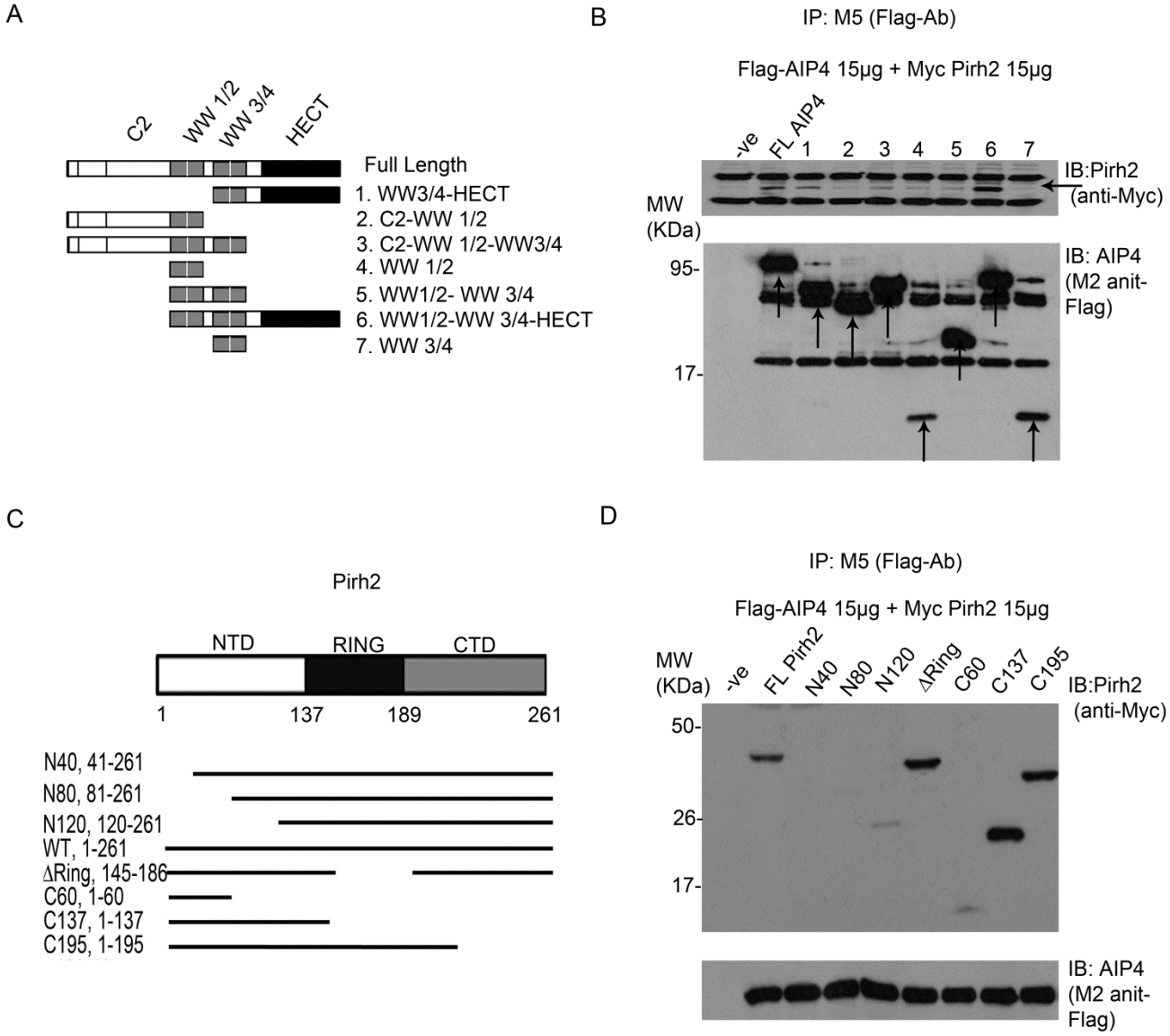


Figure 4.2. Mapping the major domains of Pirh2 and AIP4. (a) Schematic representation of AIP4 7 different constructs designed for domain mapping (C2, WW, and HECT domains) along the full length. All constructs were expressed in Flag-tagged vectors. (b) Cell extracts prepared from H1299 cells ectopically expressing Myc-Pirh2 and Flag-AIP4 (full length and truncated constructs) were analyzed by Co-IP. Cell lysates were immunoprecipitated using M5 antibody to pull down AIP4 constructs. Pirh2 binding was analyzed through immunoblotting using Myc antibody for Myc-Pirh2. Also M2 antibody was used for immunoblotting to detect the transfection efficiency of AIP4 constructs. (c) Schematic representation of Pirh2 (N-terminal, RING, and C-terminal domain) 7 different constructs designed for domain mapping along the full length. All constructs were expressed

in Myc tagged vectors. (d) Cell extracts prepared from H1299 cells ectopically expressing Flag-AIP4 and Myc-Pirh2 (full length and truncated constructs) were analyzed by Co-IP. Cell lysates were immunoprecipitated using M5 antibody to pull down AIP4. Pirh2 binding was analyzed through immunoblotting using Myc antibody for Myc-Pirh2 (full length and truncated constructs). Also M2 antibody was used for immunoblotting to detect the transfection efficiency of AIP4.

4.3.2 Pirh2 down-regulates AIP4 protein expression whereas AIP4 up-regulates Pirh2 expression

After confirming the binding between the two ligases, I aimed to investigate their correlation at the expression level. Based on this, I studied the expression of each ligase in response to overexpression or knockdown of the other ligase. As a first step, exogenously AIP4 expression was monitored in response to Pirh2 overexpression in a dose dependent experiment (Figure 4.3a left panel). Data revealed the decrease of AIP4 expression by increasing Pirh2 doses. The down-regulation of AIP4 by Pirh2 is highly significant where AIP4 expression was almost completely lost at the highest Pirh2 dose (20μg) (Figure 4.3a left panel lane 4). On the contrary, when Pirh2 was transfected at a fixed concentration and dose dependent AIP4 experiment was performed, Pirh2 expression was remarkably elevated (Figure 4.3a right panel). This may be explained by a possible existence of a feedback loop between AIP4 and Pirh2. To further investigate and confirm the exogenous findings, the expression levels of each ligase, at the endogenous level, were determined in response to overexpression of the other ligase. siRNA knockdowns of both proteins were generated. Two AIP4 knockdowns and three Pirh2 knockdowns were generated and tested for knockdown efficiency prior to investigation in comparison to WT (data not shown). Similar to the experiments described above; co-transfection was performed with the overexpression of siRNAs in comparison to WTs. Endogenously, AIP4 expression was successfully ceased in response to Pirh2 overexpression as detected earlier; and interestingly this mechanism is abolished when replacing Pirh2 WT with knockdowns (Pirh2 siRNAs 1, 2 and 3) (Figure 4.3b left panel, lanes: 4-6). Regarding Pirh2, when overexpressing AIP4, Pirh2 was endogenously elevated and again this mechanism was abolished when replacing AIP4 WT with knockdowns (AIP4 siRNAs) (Figure 4.3b right panel). In both experiments, empty vector (pcDNA3) was used as a negative control. To sum up, I propose that Pirh2 may fall upstream of AIP4 and act as the rate limiting factor in AIP4-Pirh2 regulatory mechanism.

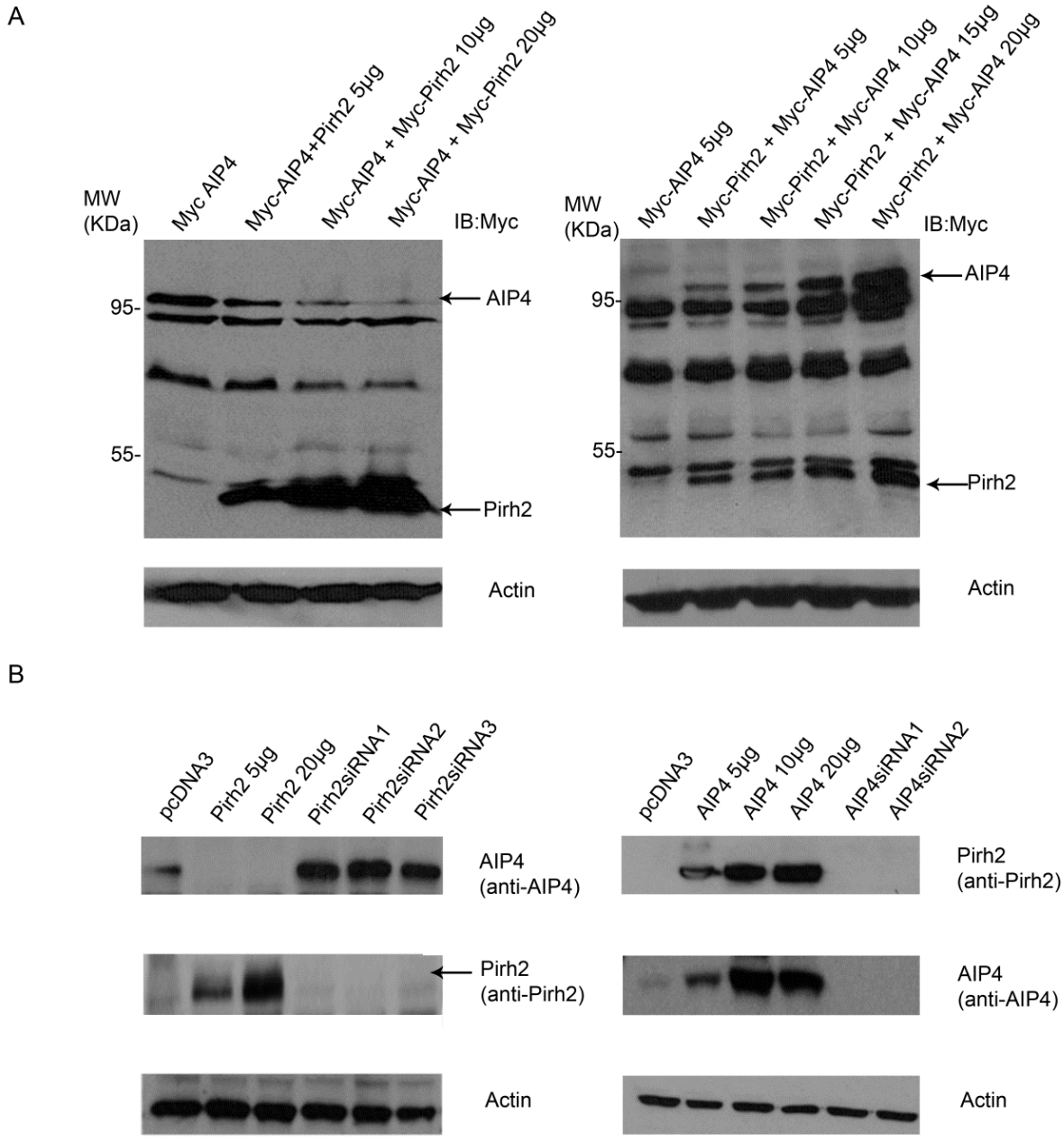


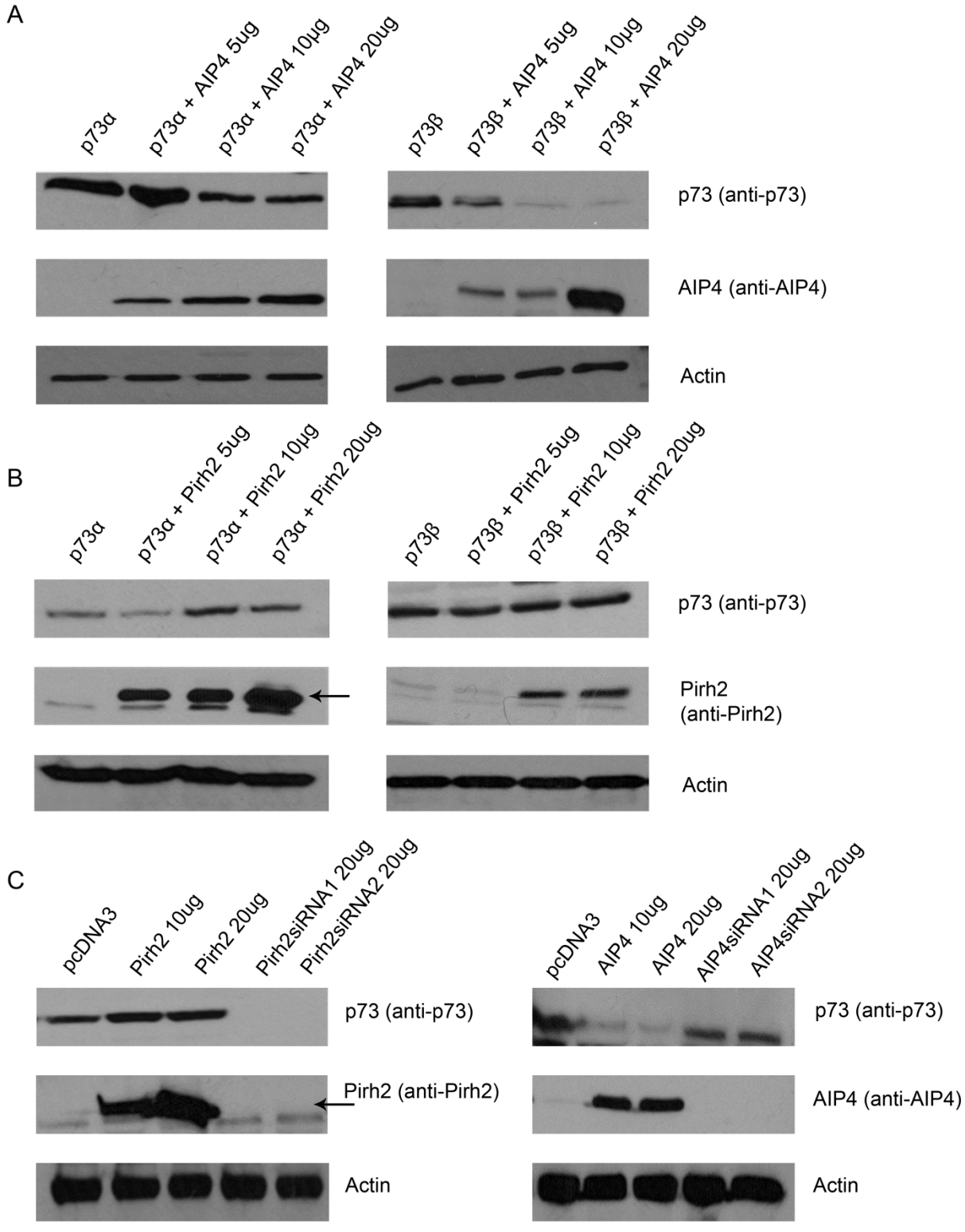
Figure 4.3. Pirh2-AIP4 regulatory pathway. (a) Exogenously, H1299 cells were co-transfected with plasmids expressing Myc-AIP4 at a constant concentration (5µg) along a dose dependent concentration of Myc-Pirh2 (5, 10 and 20 µg). Western blot analysis was performed using Myc antibody to detect both AIP4 and Pirh2 (left panel). To the right, cells were co-transfected with plasmids expressing Myc Pirh2 at a constant concentration (5µg) along a dose dependent concentration of Myc-AIP4 (5, 10 and 20 µg). Western blot analysis was performed using Myc antibody to detect both AIP4 and Pirh2. (b) Similarly, except at the endogenous level, H1299 cells were transfected with plasmids expressing Myc-Pirh2 at

two concentration (5μg and 20μg) along three Pirh2 knock-downs (Pirh2 siRNA1,2, and 3). Western blot analysis to detect AIP4 endogenous levels was performed using AIP4 antibody (left panel); whereas Pirh2 antibody blotting and actin, as indicated, served as controls for transfection efficiency and protein loading. To the right, cells were transfected with plasmids expressing Myc-AIP4 at three concentration (5, 10, and 20μg) along two AIP4 knock-downs (AIP4 siRNA1 and 2). Western blot analysis to detect Pirh2 endogenous levels was performed using Pirh2 antibody; whereas AIP4 antibody blotting and actin, as indicated, served as controls for transfection efficiency and protein loading.

4.3.3 p73 expression in response to AIP4 and/or Pirh2 overexpression/ knockdown at the endogenous and exogenous level.

To get insight over the effect of Pirh2-AIP4 regulatory mechanism on p73 proteins, knowing that p73 is a direct substrate for both ligases, I investigated the consequence of overexpression, silencing, and co-transfection of Pirh2 and AIP4 on p73 endogenous and exogenous expression. First, data confirmed the previous findings of Rossi et al. (2005) that showed the decrease of exogenous p73 expression in response to AIP4 transfection (Figure 4.4a). Isoforms, alpha (Figure 4.4a left panel) and beta (Figure 4a right panel), known for their tumor suppressor role showed a decrease at the protein level. Furthermore, exogenous expression of p73 was elevated when overexpressing Pirh2 (Figure 4.4b). This can be explained by inhibiting endogenous AIP4 which down-regulates p73 proteins. Data was consistent regarding both alpha (Figure 4.4b left panel) and beta (Figure 4.4b right panel) isoforms. At this point, endogenous p73 analyses were of huge importance to confirm the previous findings. All experiments described above were repeated without transfecting p73 and the analysis was performed on p73 endogenous levels in response to Pirh2 overexpression or knockdown (Figure 4.4c left panel); or in response to AIP4 overexpression or knockdown (Figure 4.4c right panel). Results at the endogenous levels supported all the exogenous findings and confirmed the proposed signaling pathway. Interestingly, co-transfection of a combination of AIP4 and/or Pirh2 WT and Pirh2 siRNAs revealed the down-regulation of exogenous p73 by AIP4 that was ceased when combined with Pirh2 WT and restored when combined with Pirh2 siRNAs (Figure 4.4d left panel). At the endogenous level, data reinforced the exogenous findings proposing the down-regulation of p73 in response to AIP4 overexpression. This inhibition is ended when transfecting Pirh2 that down-regulates AIP4 ceasing its negative regulatory effect on p73 (Figure 4.4d right panel). Results above were also performed on HEK293 cells showing similar findings; hence indicating that the mechanism is not cell type specific. Furthermore,

I aimed to investigate the regulatory pathway in normal cell lines such as HCT116 cell lines (Figure 4.4e). Duplicate experiments were performed on HCT116 cell lines expressing p53 WT (Figure 4.4e left panel) and HCT116 $p53^{-/-}$ (Figure 4.4e right panel). Interestingly, p73-AIP4-Pirh2 correlation was only detected in HCT116 $p53^{-/-}$ cell line matching the previous findings that were performed in $p53^{-/-}$ cell lines such as H1299 and HEK293. The presence of p53 affected the pathway and Pirh2-AIP4 correlation was lost, and similarly their translational effect on p73 was lost as well. Further investigations are required to reveal the role of p53 in disrupting Pirh2-AIP4-p73 signaling pathway.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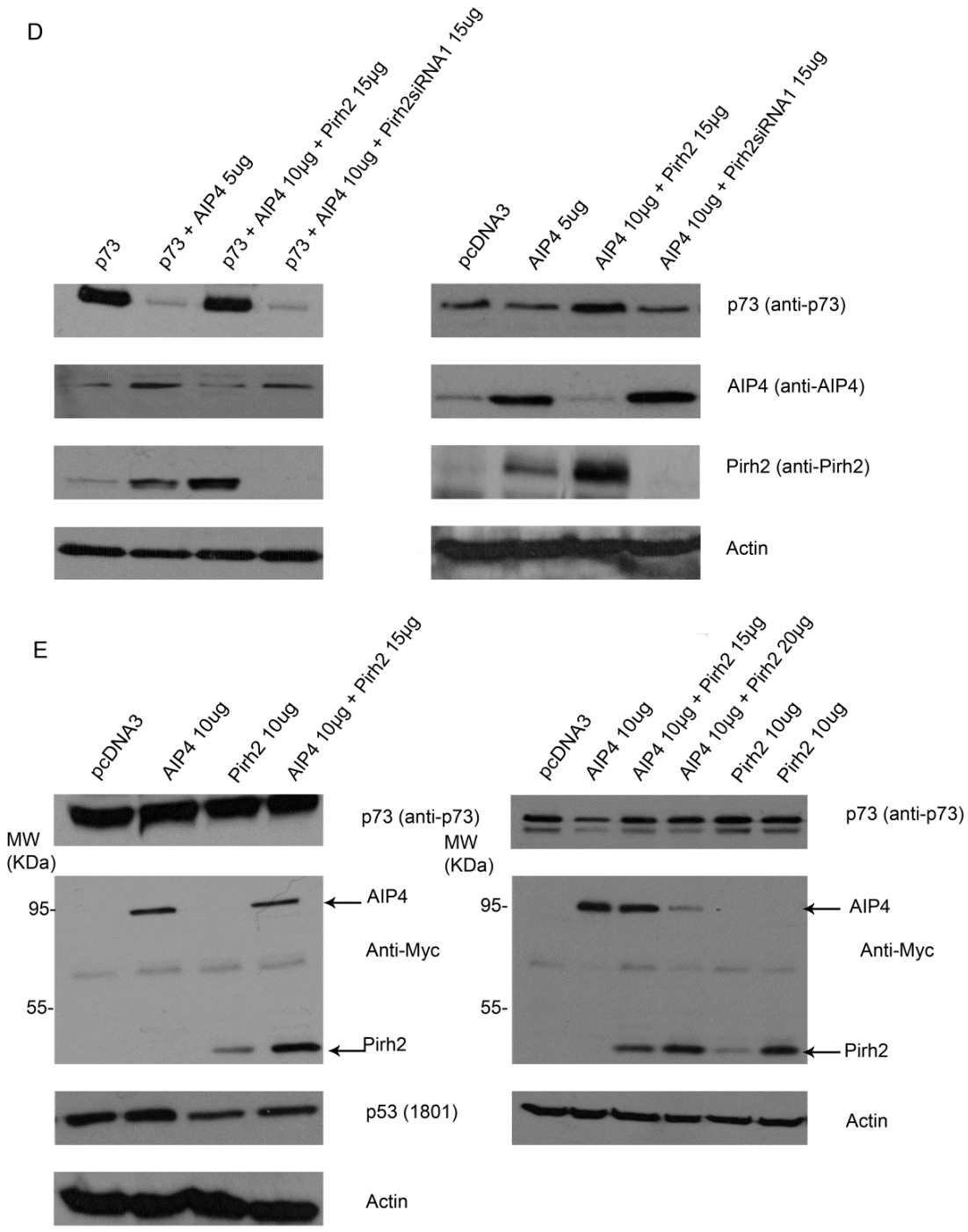


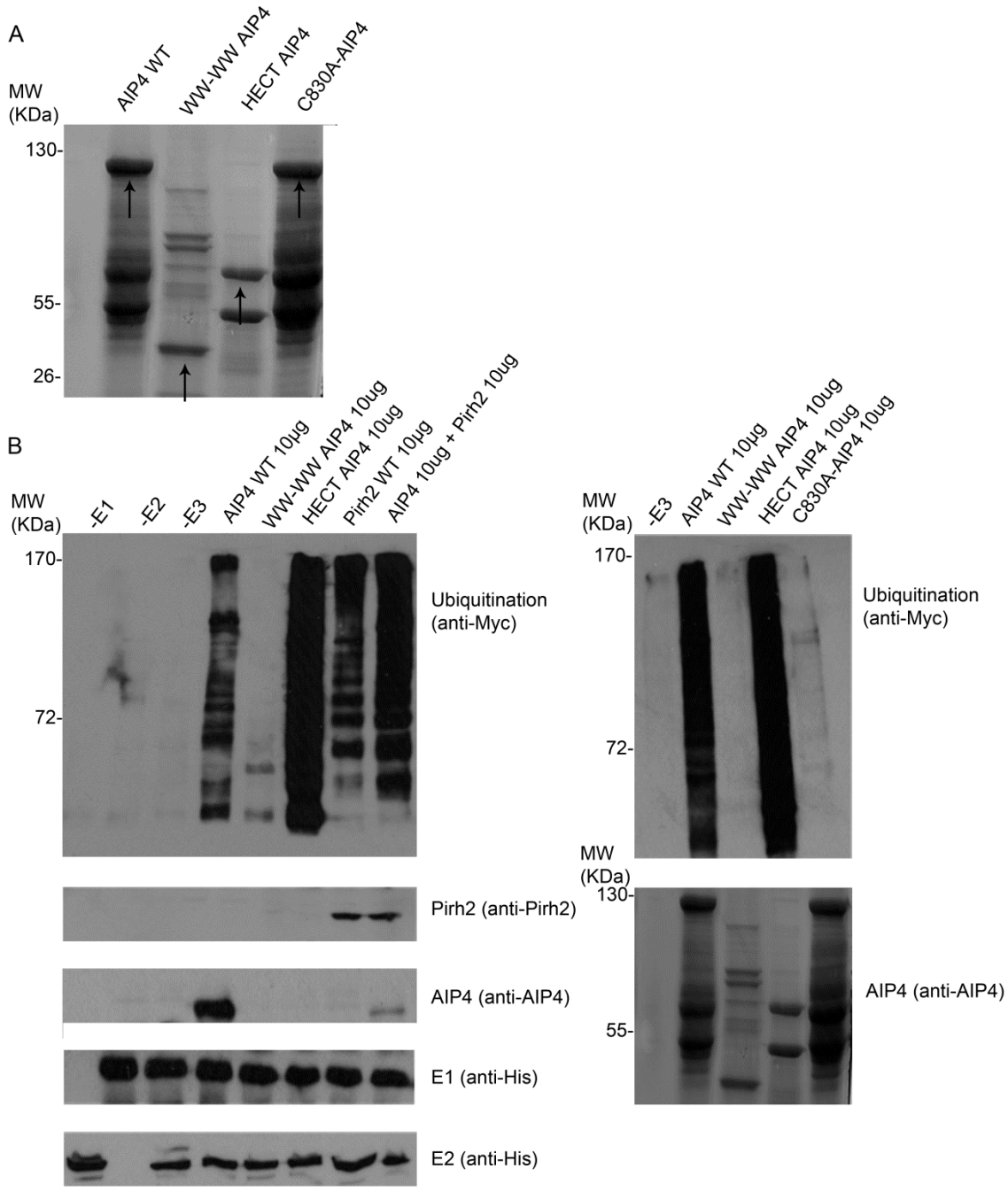
Figure 4.4. p73 expression in response to Pirh2-AIP4 correlation. (a) Exogenous expression of p73 isofroms α and β (transfected at 5µg) was detected in H1299 cells co-transfected with dose dependent concentrations of Myc-AIP4 (5, 10 and 20 µg). Western blot analysis was performed using p73 antibody to detect both p73 isoforms (α to the left and β to the right). Myc antibody blotting and actin, as indicated, served as controls for

Myc-AIP4 transfection efficiency and protein loading. (b) Similarly cells were transfected with dose dependent concentrations of Myc-Pirh2 (5, 10 and 20 μg). Western blot analysis was performed using p73 antibody to detect both p73 isoforms (α to the left and β to the right). Myc antibody blotting and actin, as indicated, served as controls for Myc-Pirh2 transfection efficiency and protein loading. (c) Endogenously, p73 expression was detected by Western blot using p73 antibody in response to Pirh2 transfection (10 or 20μg) or Pirh2 siRNA1 and 2 (left panel). Also p73 endogenous expression was detected by Western blot using p73 antibody in response to AIP4 transfection (10 or 20μg) or AIP4 siRNA1 and 2 (right panel). Pirh2 and AIP4 antibodies in addition to actin, as indicated, served as controls for transfection efficiency and protein loading. (d) Exogenous (left panel) and endogenous (right panel) levels of p73 in response to co-transfection of AIP4 alone or in combination with Pirh2 WT or siRNAs were detected. Pirh2 and AIP4 antibodies in addition to actin, as indicated, served respectively as controls for Pirh2 and AIP4 transfection efficiency and protein loading. (e) Similar to (d), p73 endogenous expression in HCT116 WT (left panel) and HCT116 $p53^{-/-}$ cells (right panel) was detected in response to overexpression of AIP4 or Pirh2 and when both ligases are combined together. Myc antibody and actin, as indicated, served respectively as controls for Myc-Pirh2 and Myc-AIP4 transfection efficiency and protein loading.

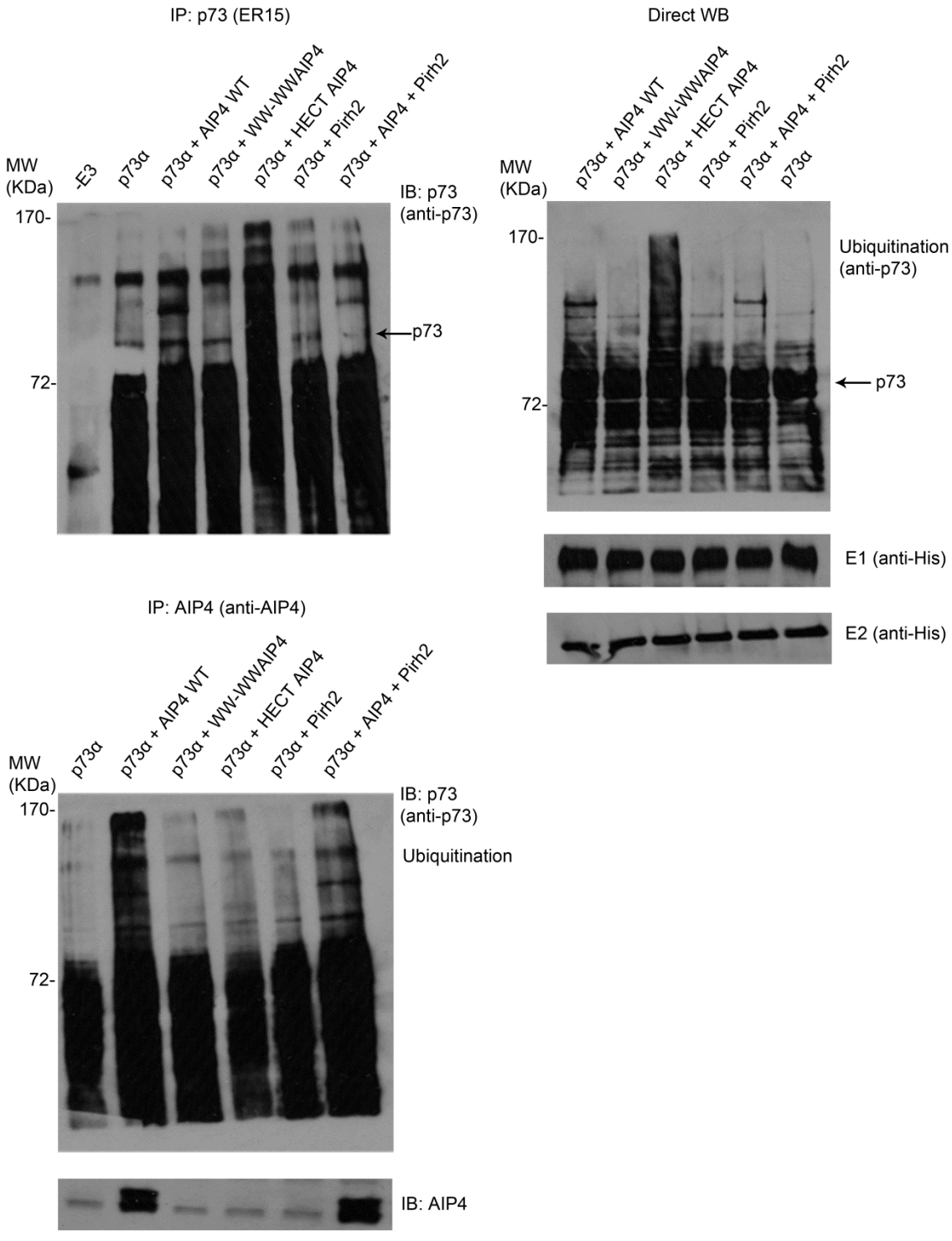
4.3.4 Pirh2 induces AIP4 ubiquitination and ceases AIP4-p73 ubiquitination in vivo and in vitro

Pirh2 and AIP4 are both shown to ubiquitinate p73 proteins despite having different substrate fate post ubiquitination. Also both are shown to be regulated by self ubiquitination mechanism (24, 27, 32). However, the consequence of Pirh2-AIP4 correlation remains unknown with respect to its effect on AIP4 self ubiquitination and p73 ubiquitination. First, I aimed to evaluate AIP4 self ubiquitination; and then I wanted to compare p73 ubiquitination induced by Pirh2 or AIP4 or in combination knowing that an antagonistic relation is revealed between the two ligases. To start with, to perform in vitro ubiquitination analysis I utilized purified GST AIP4 and GST Pirh2, and His p73 alpha and beta proteins. Also additional three mutant constructs of AIP4 were included in the analysis: one consist only of the HECT domain known for its catalytic activity, the second constitutes only of the WW domains, and the third was a full length that has a point mutation (C830A) in the HECT domain which has been reported to cease the catalytic activity of the ligase (35). All proteins were run on SDS-PAGE gels and stained with Coomasie blue staining to confirm their purity and correct molecular size (Figure 4.5a). AIP4 in vitro ubiquitination assays following the same conditions as reported earlier (34) were performed (Figure 4.5b). Western blots analysis using Myc antibody targeting Myc-Ub revealed that AIP4 WT showed positive ubiquitination (Figure 4.5b left panel); and surprisingly the HECT AIP4 showed more efficient ubiquitination compared to the Wild type (Figure 4.5b right panel). Interestingly, the mutant construct C830A totally abolished AIP4 self ubiquitination emphasizing the role of the HECT domain in the ubiquitination process. When accompanied with Pirh2, AIP4 ubiquitination level was increased endorsing the regulation of Pirh2-AIP4 through ubiquitination (Figure 4.5b left panel). In terms of p73 in vitro ubiquitination, ubiquitination assays were run as described above with the addition of p73 α or β. Regarding p73α, direct Western blots using p73 antibody showed positive p73 ubiquitination

in the presence of AIP4 which was decreased in the presence of Pirh2 (Figure 4.5c right panel). However, the limitation in this experiment for detecting ubiquitination using Western blot (Ub tagged antibody) is the false positive effect of Pirh2 and AIP4 self-ubiquitination. To eliminate the E3 ligases self ubiquitination factor, Co-IP experiments were performed immunoblotting for p73 using ER15 antibodies (Figure 4.5c left top panel) or AIP4 antibodies (Figure 4.5c left bottom panel). Data collected confirmed the decrease in p73 ubiquitination by AIP4 when Pirh2 is introduced. Regarding p73β, direct Western blots using p73 antibody showed positive p73 ubiquitination in the presence of AIP4 which was decreased in the presence of Pirh2 (Figure 4.5d right panel). For the same reason described above, Co-IP experiments were performed immunoblotting for p73 using ER15 antibodies (Figure 4.5d left panel). Data confirmed the previous findings. To draw better conclusion regarding the catalytic role of AIP4 domains, Co-IP experiments for p73α (Figure 4.5e left panel) and p73β (Figure 4.5e right panel) ubiquitination were done. Samples were immunoprecipitated for p73 using ER15 antibodies and ubiquitination was determined using p73 antibody. As expected, HECT domain showed the highest efficiency in p73 ubiquitination and the C830A construct lost the catalytic activity. Also I confirmed that WW domains are not essential for ubiquitination.


A
AIP4 WT
WW-WW AIP4
HECT AIP4
C830A-AIP4
MW (KDa)
130-
55-
26-
B
MW (KDa)
-E1
-E2
-E3
AIP4 WT 10µg
WW-WW AIP4 10ug
HECT AIP4 10ug
Pirh2 WT 10µg
AIP4 10ug + Pirh2 10ug
170-
72-
Ubiquitination (anti-Myc)
Pirh2 (anti-Pirh2)
AIP4 (anti-AIP4)
E1 (anti-His)
E2 (anti-His)
MW (KDa)
-E3
AIP4 WT 10µg
WW-WW AIP4 10ug
HECT AIP4 10ug
C830A-AIP4 10ug
170-
72-
Ubiquitination (anti-Myc)
MW (KDa)
130-
55-
AIP4 (anti-AIP4)

C


IP: p73 (ER15)
MW (KDa)
-E3
p73α
p73α + AIP4 WT
p73α + WW-WWAIP4
p73α + HECT AIP4
p73α + Pirh2
p73α + AIP4 + Pirh2
170-
72-
IB: p73 (anti-p73)
p73
Direct WB
MW (KDa)
p73α + AIP4 WT
p73α + WW-WWAIP4
p73α + HECT AIP4
p73α + Pirh2
p73α + AIP4 + Pirh2
p73α
170-
72-
Ubiquitination (anti-p73)
p73
E1 (anti-His)
E2 (anti-His)
IP: AIP4 (anti-AIP4)
MW (KDa)
p73α
p73α + AIP4 WT
p73α + WW-WWAIP4
p73α + HECT AIP4
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p73α + AIP4 + Pirh2
170-
72-
IB: p73 (anti-p73)
Ubiquitination
IB: AIP4

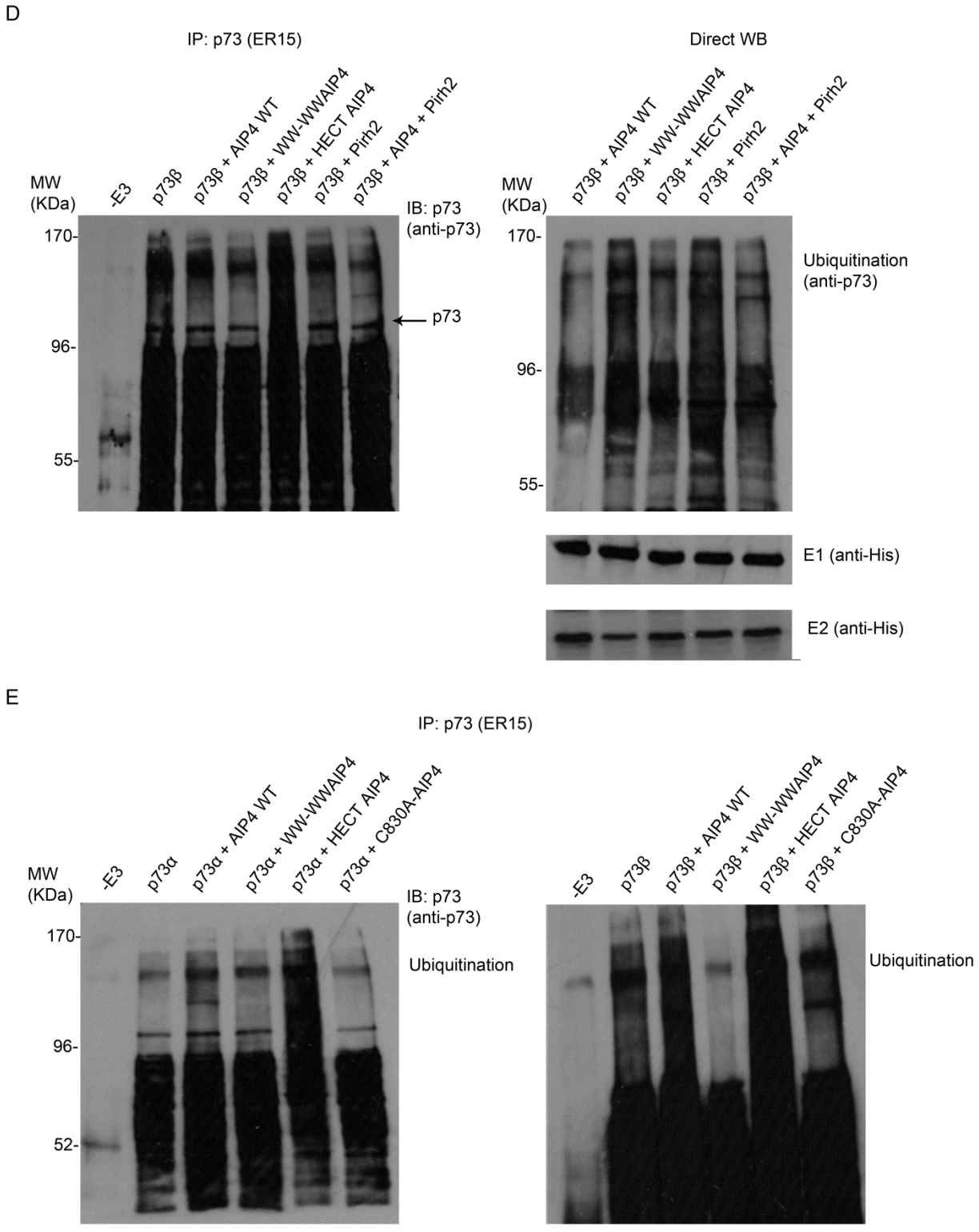
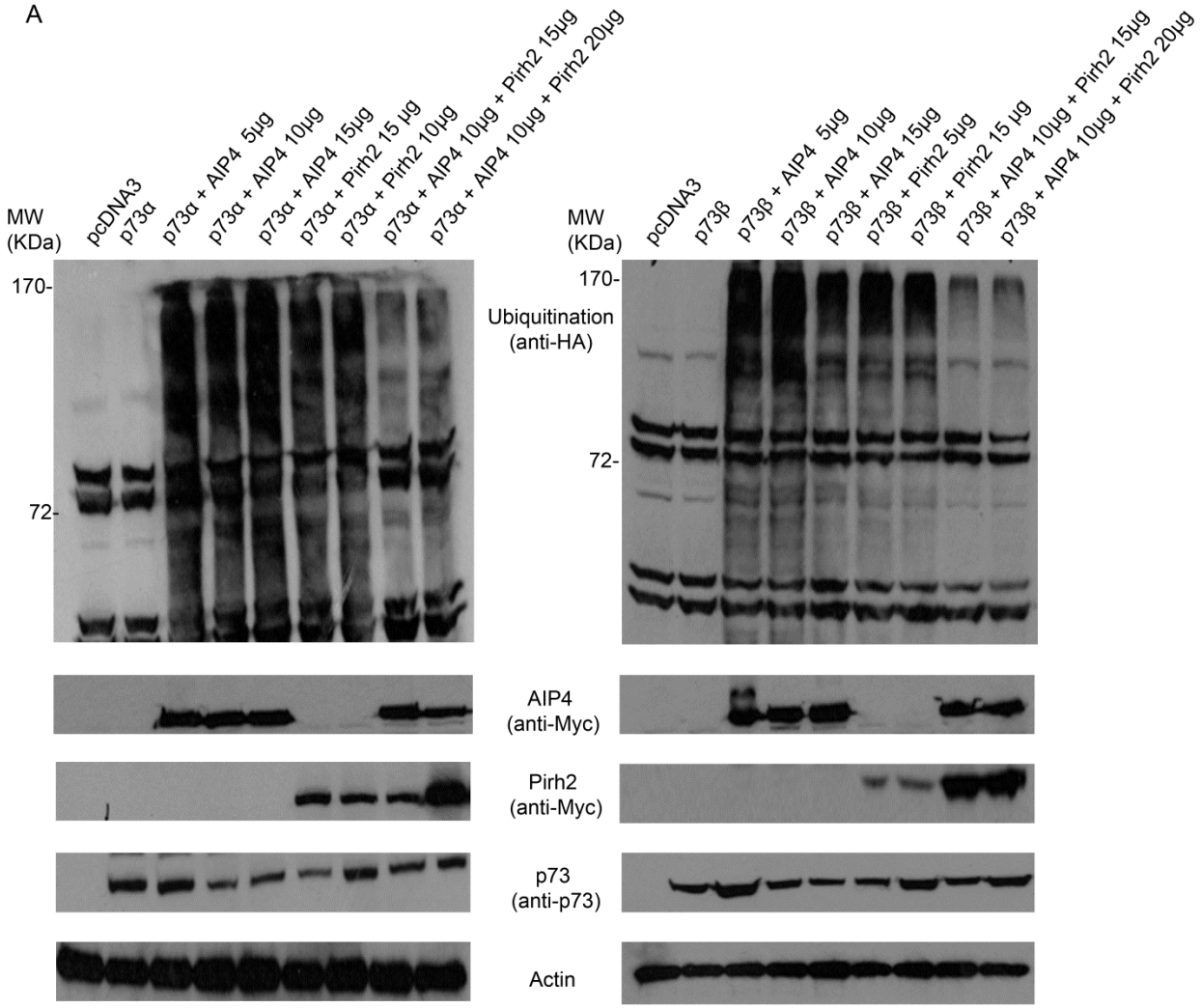


Figure 4.5. In vitro AIP4 and p73 ubiquitination. (a) GST-AIP4 mutant constructs were designed and purified from *E.coli*. Prior to ubiquitination assays, constructs were run on

SDS-PAGE gel and stained with Coomasie blue. (b) In vitro self ubiquitination reactions for AIP4 mutant constructs were performed using E1, E2 (H5B), and Myc-Ub (left panel). Similarly AIP4 self ubiquitination was compared to Pirh2 and also when the two ligases are combined (right panel). Following ubiquitination reactions (90min) data was collected after immunoblotting with Myc antibody to reveal ubiquitinated proteins and His antibody to reveal E1 and E2 presence as indicated. (c) p73 α ubiquitination, after purifying GST-p73 α from *E.coli*, was assessed using CO-IP (left panel) and direct Western blot (right panel) analysis when p73 α was combined with AIP4 alone (WT or mutants), Pirh2 alone, or in combination. p73 α ubiquitination was analyzed after immuncoprecipitating p73 using ER15 antibody (left top panel) or AIP4 antibody (left bottom panel) and immunoblotting for p73 using p73 antibody. (d) As described above, the same was applied for p73 β. To the right, p73 β ubiquitination was analyzed after immunoprecipitating p73 using ER15 antibody (left top panel) and immunoblotting for p73 using p73 antibody. Also portions were isolated for direct Western blot analysis (right panel). Anti-p73 was used to detect ubiquitinated p73 and His to confirm the presence of E1 and E2 (right). (e) As a replica to experiments described in (c) and (d), ubiquitination of p73 was analyzed with respect to AIP4 constructs including AIP4C830A, which lacks the HECT catalytic activity. p73 α ubiquitination (left panel) and β ubiquitination (right panel) were collected post CO-IP experiment, where immunoprecipitation was performed using ER15 and ubiquitination was deduced from p73 antibody immunoblotting.

At the in vivo level, H1299 cells were co-transfected with a combination of p73, Pirh2, and AIP4 expressing plasmids. Figure 4.6a represents p73α ubiquitination (left panel) and p73β ubiquitination (right panel). First, both ligases ubiquitinate p73 (α and β); however Myc-AIP4 showed stronger ubiquitination ability compared to Myc-Pirh2 with respect to p73. Ubiquitination was detected using HA antibody that targets HA-Ub utilized. Interestingly, when analyzing the ubiquitination level of p73 in the presence of both Pirh2 and AIP4, ubiquitination levels were lower; hence confirming the decrease of p73 ubiquitination due to down-regulation of AIP4 by Pirh2. Also, when replacing Pirh2 WT with Pirh2 siRNA co-transfected with AIP4 WT, the level of p73 ubiquitination was restored (Figure 4.6b). For the same reason stated above and to eliminate the E3 ligases self ubiquitination factor, the Co-IP experiments were performed using M5 antibody to pull down Flag-p73 (α left panel and β right panel) and ubiquitination was detected using tagged ubiquitin; HA in my case. Results from Co-IP experiments confirmed the previous findings and supported the drawn conclusions.


A
MW (KDa)
pcDNA3
p73α
p73α + AIP4 5μg
p73α + AIP4 10μg
p73α + AIP4 15μg
p73α + Pirh2 15 μg
p73α + Pirh2 10μg
p73α + AIP4 10μg + Pirh2 15μg
p73α + AIP4 10μg + Pirh2 20μg
MW (KDa)
pcDNA3
p73β
p73β + AIP4 5μg
p73β + AIP4 10μg
p73β + AIP4 15μg
p73β + Pirh2 5μg
p73β + Pirh2 15 μg
p73β + AIP4 10μg + Pirh2 15μg
p73β + AIP4 10μg + Pirh2 20μg
170-
72-
Ubiquitination (anti-HA)
170-
72-
AIP4 (anti-Myc)
Pirh2 (anti-Myc)
p73 (anti-p73)
Actin

B

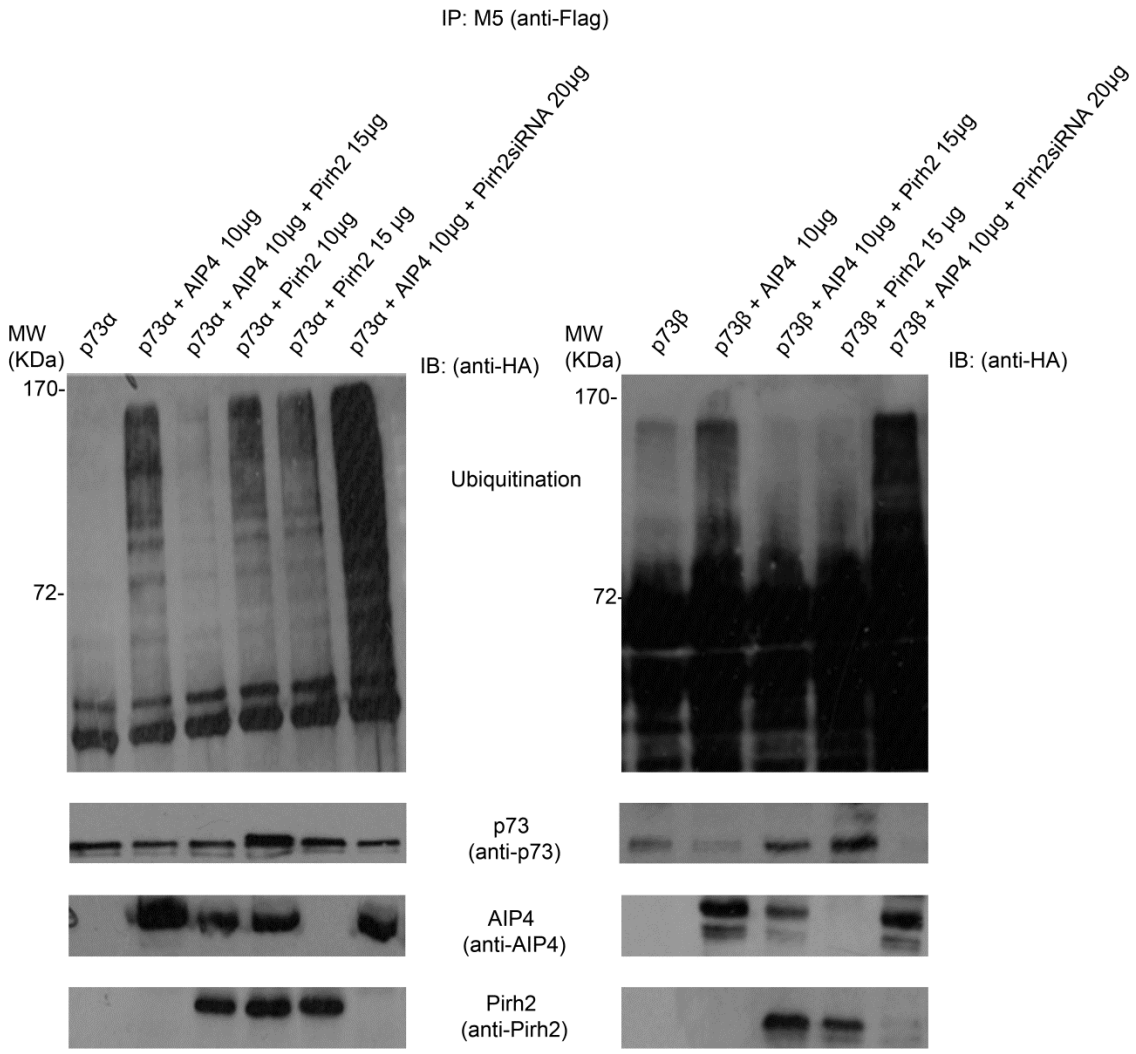


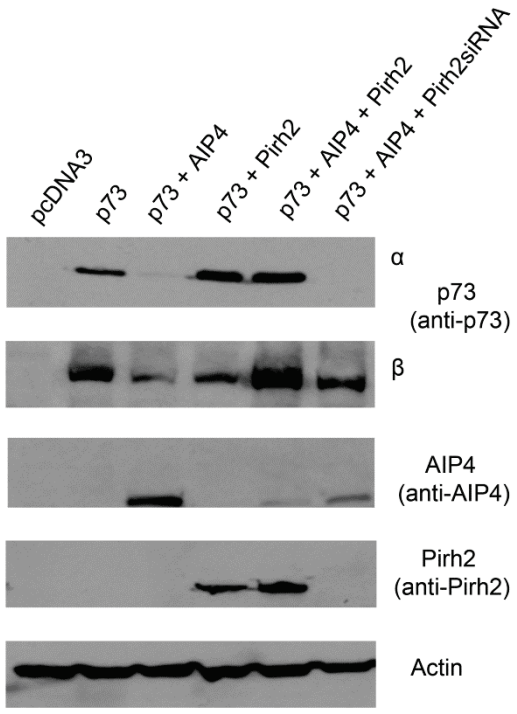
Figure 4.6. p73 α and β in vivo ubiquitination. (a) H1299 cells were co-transfected with plasmids expressing Flag-p73α or Flag-p73β, Myc-Pirh2 and Myc AIP4 (alone or in combination), in addition to HA-tagged ubiquitin. Forty hours later, cells were further transfected with the plasmid expressing HA-Ub. Lysates were analyzed by Western blot with anti-HA to detect ubiquitination. Myc antibody was used to detect AIP4 and Pirh2 transfection, p73 antibody to detect p73, and actin as a loading control. p73 α ubiquitination is detected in left panel and p73 β in right panel. (b) Similar to (a) except that ubiquitination is detected after immunoprecipitating the cell lysates with M5 antibody to pull down Flag-p73 and immunoblotted with HA antibody. Also, Pirh2 siRNA was introduced

along the Pirh2 WT. AIP4 and Pirh2 antibodies are used to reflect transfection efficiency, p73 antibody to detect p73, and actin as a loading control. p73 α ubiquitination is detected in left panel and p73 β in right panel.

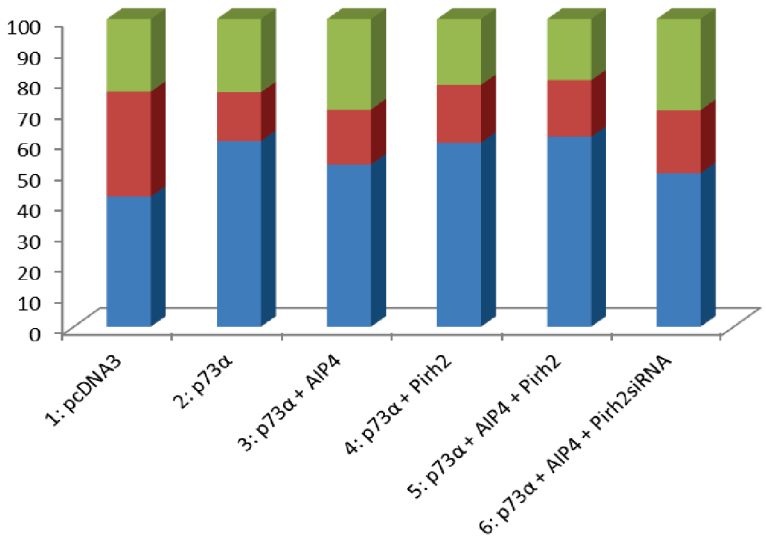
4.3.5 Pirh2 restores p73 cell cycle arrest function

Given that Pirh2 down-regulates AIP4 and ceases AIP4 negative regulation over p73 proteins, we aimed to investigate the physiological function of p73 proteins in terms of G1 cell cycle arrest (Figure 4.7). The G1 cell cycle arrest function of both isoforms alpha and beta were analyzed post overexpression of Myc-Pirh2 or Myc-AIP4 exclusively or in combination. Also knockdown of Pirh2 to restore AIP4 expression and function was added to the analysis. As a positive control, overexpression of p73 was used to assess the differences in the arrest function after regulation; and as a negative control pcDNA3 was used in transfection. Prior to flow cytometry analyses, direct Western blots of isolated portions from the cell lysates were run to confirm the transfection efficiency and the expression of the target proteins (Figure 4.7a). AIP4 overexpression decreased the arrest function which was restored when Pirh2 was introduced. G1/S ratios were calculated and presented in accompanying tables to confirm the G1 cycle arrest and give a clear indication on the arrest function differences. Interestingly the decrease was restored when Pirh2 WT was replaced with Pirh2 siRNA (Figure 4.7b). Data was consistent between the two isoforms (α: figure 4.7b top panel, and β; figure 4.7b bottom panel); yet p73β showed a stronger cell cycle arrest function where the percentage of cells at G1Go arrest was close to 80%. To confirm the G1 cell cycle arrest, G1/S ratios were provided as indicated in figue 4.7b.

A



B



G1/S ratio of pcDNA3= 1.22 ± 0.03
G1/S ratio of p73α= 3.59 ± 0.14
G1/S ratio of p73α + AIP4= 3.04± 0.12
G1/S ratio of p73α + Pirh2= 3.14± 0.06
G1/S ratio p73α + AIP4 + Pirh2= 3.42± 0.08
G1/S ratiop73α + AIP4 + Pirh2siRNA= 2.38± 0.18

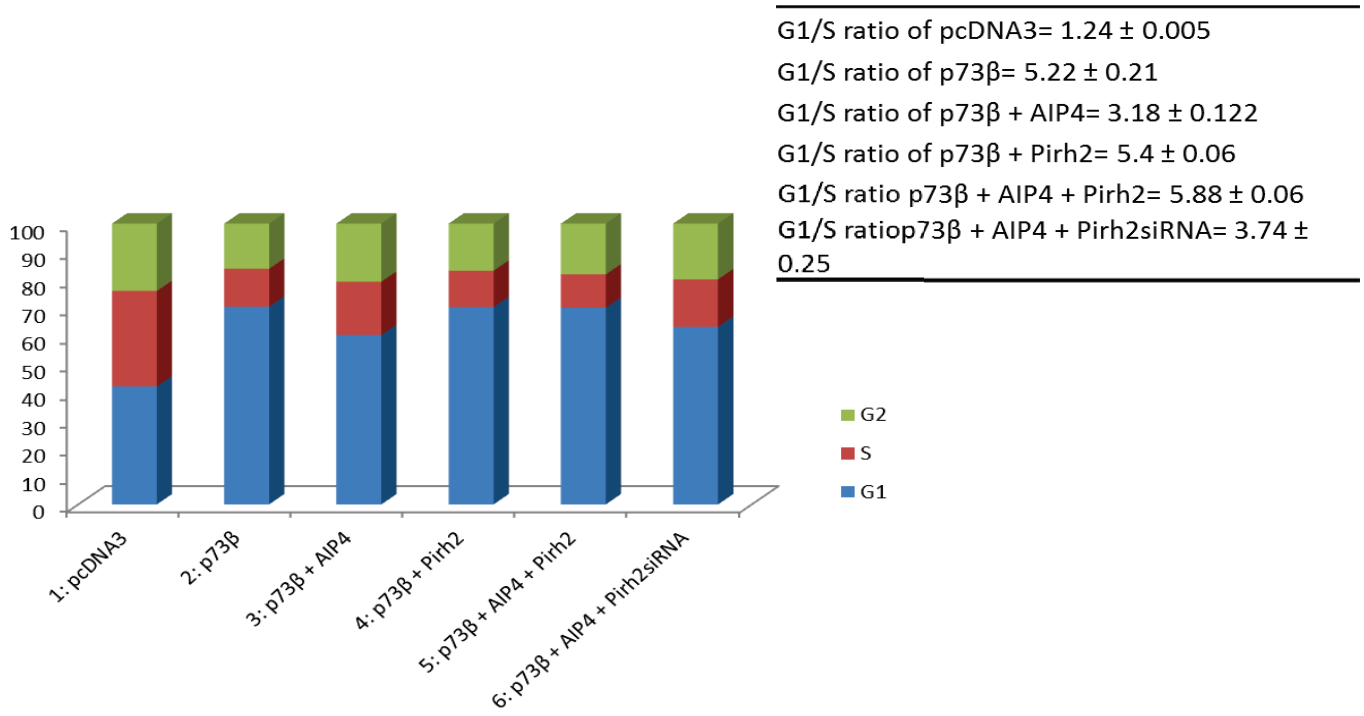


Figure 4.7. p73 cell cycle arrest function in response to AIP4/Pirh2 regulation. (a) H1299 cells were co-transfected with plasmids expressing p73 α or β, AIP4 alone or in combination with Pirh2 WT or Pirh2 siRNA as indicated. Empty vector served as the negative control and p73 alone served as the positive control. Prior to cell cycle analysis; portions of the cells were analyzed with Western blot using p73, AIP4, Pirh2, and Actin antibody to confirm successful transfection as indicated. (b) Cell cycle profile was determined for α (top panel) and β (bottom panel) by propidium iodide staining and flow cytometry. Results presented are the average of triplicate experiment ± SED. Also G1/S ratios are presented on the figure.

4.4 DISCUSSION

This study proposed a novel correlation between two E3 ligases that share a common regulatory substrate, p73 in my case. Previously it has never been demonstrated to have two E3 ligases that interact and have an antagonistic relationship within the same signaling pathway. The implication resides behind several aspects; but to start with, the binding between Pirh2 and AIP4 opens many gateways. Initially Pirh2 was shown to utilize residues 120-137 to bind p53 proteins (27). Similarly, same residues were essential for p73 binding highlighting the role of the NTD domain in protein-protein interaction (32). When I mapped Pirh2 domains for AIP4 binding, the NTD was also revealed to be important. Thus, Pirh2-AIP4 binding may actually affect the binding of Pirh2 to their substrates. In the near future, we aim to repeat Pirh2 and p53/p73 binding in the presence or absence of AIP4. Regarding AIP4, I reported in this study for the first time the role of HECT and not the WW domain, as it has been always shown (33), in binding. However, the same domain (HECT) was investigated earlier and shown not to have an effect on AIP4-p73 binding. Hence, there is a high possibility that Pirh-AIP4 form a complex which may interfere in the substrate fate post ubiquitination. For example the recognition of the complex by the 26S proteasome complex could be affected. For such reasons, structural analysis regarding Pirh2/AIP4 conformational change post binding and the complex formation may reveal whether certain domains are shielded and not exposed for further substrate binding or ubiquitination.

The down-regulation of AIP4 by Pirh2 overexpression and the up-regulation of Pirh2 by AIP4 expression with the consistency in the finding when monitoring the ligase levels endogenously propose a possible feedback loop between the two ligases. Also confirming the data with the ablation of the endogenous expression of each ligase utilizing siRNas while monitoring the endogenous expression of the other suggest that Pirh2 falls upstream of AIP4. Further investigation is required to confirm the loop. For example, stable cell lines with silenced Pirh2 or AIP4 could be a perfect model to run our investigations. At this point

it is worth mentioning that tumor tissues including: breast, head and neck, prostates, etc. showed elevated Pirh2 expression (35-42). I suspect that AIP4 levels in these tumor tissues should be significantly decreased yet it still needs to be proven. Also the exact mechanism by which Pirh2 induces AIP4 down-regulation is not clear. Proteosomal degradation or translocation of AIP4 alone or in complex with Pirh2 could be possible. For this reason, it is important to investigate the nuclear/cytoplasm translocation of AIP4 in response to Pirh2 regulation; and also it would be a key factor in this mechanism to determine the half-life of AIP4 and whether it is shortened in the presence of Pirh2 to further define their correlation at the molecular level.

Regarding p73, my study showed, at the endogenous and exogenous level, the degradation of p73 by AIP4 ligases only happens when alone or co-expressed with knockdowns Pirh2. Pirh2 WT restored p73 expression by terminating AIP4 negative inhibition. Such finding introduces a new insight for therapeutic approaches that may utilize the antagonistic relationship between two ligases to restore p73 expression. Also, we confirmed that this relationship is moderated through ubiquitination. A clear decrease in AIP4 induced ubiquitination when Pirh2 is introduced was detected at the p73 level. The ubiquitination was not totally disappeared as I showed that Pirh2 ubiquitinates p73 but not as efficiently as AIP4 does. However, the lysine chains utilized by both ligases may be different. Hence we couldn't eliminate the fact that both ligases will ubiquitinate p73 yet proteosomal degradation is blocked. Such questions can be answered by repeating our ubiquitination experiments but using K48R and K63R ubiquitin mutant constructs as described in chapter 2 (27, 34) instead of Ub-WT. p73 protein level can also be assed to determine how the utilization of the lysine chains affected the substrate fate knowing that Ub moieties, linked via Lys-48 or 29 or 11, are targeted 26S proteosomal degradation while Lys-63-linked chains appear to be used primarily for nonproteasome-dependent regulation of processes such as: DNA repair, endocytosis, and chromatin remodeling (28-31). Since p73 expression

and ubiquitination were affected, I anticipated changes in p73 cell cycle arrest function. Data showed that the translational role of p73 tumors suppressors in inducing G1 cell cycle arrest was only restored when AIP4 is accompanied by Pirh2, hence ceasing AIP4 negative regulation. Further analysis regarding p73 apoptotic function will complement the stated findings. Colony formation assays to monitor the apoptotic role of p73 or fluorescence-activated cell sorting using Annexin V staining could be used to assess p73 apoptotic activity.

To sum up, my results confirmed that Pirh2 binds and down-regulates AIP4, hence inhibiting AIP4 mediated proteosomal degradation of p73 post ubiquitination and consequently restores p73 tumor suppressor activity. The relationship between the two ligases, the domain involved in binding, and p73 ubiquitination, expression, and activity all add valuable knowledge to our understanding regarding the p73 regulatory pathway.

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Chapter Five

GENERAL DISCUSSION

5.1 Thesis overview

In this thesis, I have studied the role and regulatory pathways involving Pirh2 and other substrates. Regarding Pirh2 and with the absence of any substrate, studying the self ubiquitination pathway was extremely important because it is the major regulatory pathway that modulates Pirh2 levels in human (1, 2). Little is known on how this mechanism is initiated with respect to the post translational modifications such as phosphorylation, or the role of other cofactors. Also the type of external stimuli is significant knowing that differences in DNA damages might lead to distinct biological responses. Such aspects need to be answered especially that Pirh2 overexpression in human tumors is a common event and was the gateway that drew more attention to this ligase compared to other ligases involved in ubiquitination and regulation of tumor suppressors or oncogenes (1). In this thesis, we revealed the domains involved in Pirh2 self ubiquitination and added more knowledge to the fate of Pirh2 post ubiquitination.

Regarding Pirh2 substrates, many researchers are studying Pirh2 correlation with tumor suppressors. This thesis focuses on p53 family proteins and particularly p73 as a tumor suppressor only because Pirh2-p53 correlation has been widely studied and many evaluated their interaction, regulation, and function (3). The delay in revealing p73 role in cancer was an advantage for our research as p73 was less studied in terms of how it is controlled and kept under tight regulation (4-6). Pirh2 can regulate both p53 and p73, yet it was surprising to know that the type of regulation is distinct. In this thesis we further characterized Pirh2-p73 correlation and provided a novel explanation regarding the distinct effect in terms of p73 expression and function.

Lastly and most importantly, it has always been a challenge in different signaling pathways relative to cancer or other regulatory mechanisms in metabolism, autoimmune diseases, etc. to reveal whether multiple regulators are present for redundant effect, or to guarantee a

better regulation in response to different physiological conditions. For example, MDM2 Pirh2, AIP4, CHIP and many other ligases were all involved in p73 regulation (7, 8). Many researchers proposed hypotheses correlating these pathways to each other, yet nothing was physiologically confirmed and the big picture remains unclear. In this thesis a novel antagonistic correlation between two E3 ligases previously thought to be unrelated is proposed. Each ligase regulates p73 in a different manner. What was known about AIP4 in down regulating p73 through ubiquitination is now revealed to be under Pirh2 regulation. Accordingly, Pirh2 fine tunes p73 expression and its tumor suppressor effect. The pathway by which AIP4 binds and down regulates p73 at the transcription and protein level through ubiquitination is ceased when binding to Pirh2. Pirh2 negatively regulates AIP4 through ubiquitination. Figure 5.1 schematically presents the Pirh2-AIP4-p73 story.

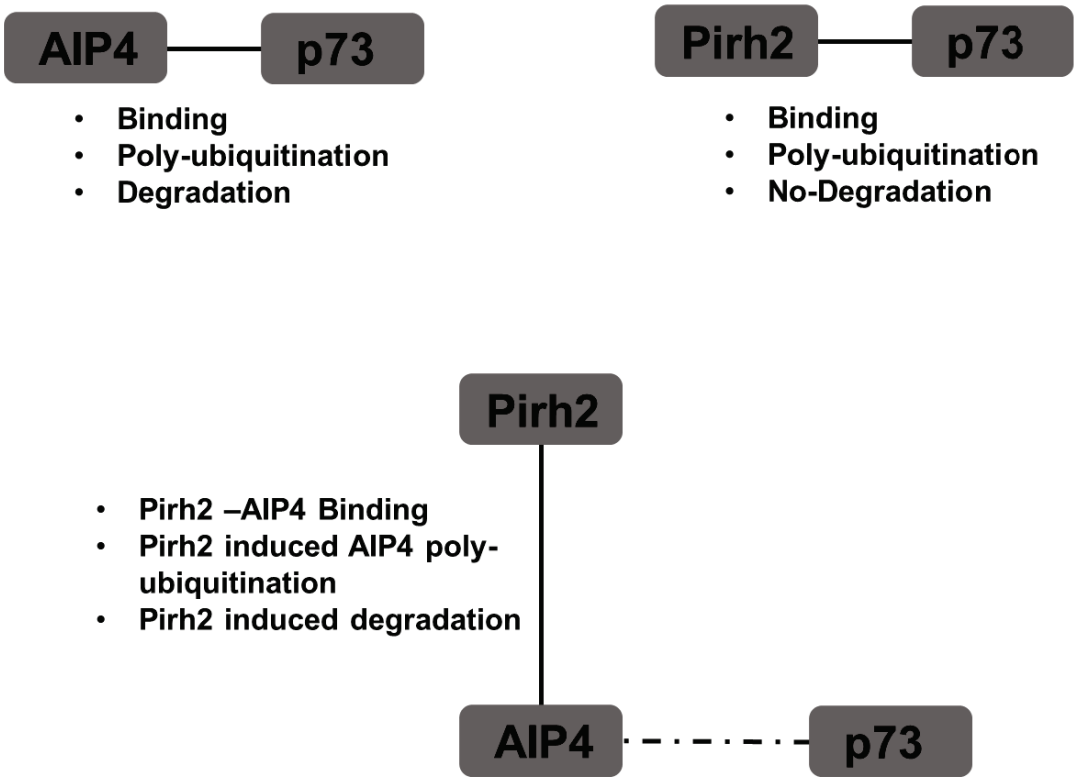

Figure 5.1. Schematic representation of Pirh2-p73; AIP4-p73; Pirh2-AIP4-p73 correlation.

5.2 Pirh2 self ubiquitination and p53 family association

The overexpression and mutations of tumor suppressors and oncogenes were always detected in human tumors. At the beginning the focus was limited to the effector proteins such as the p53 family. It was revealed from early investigations that these proteins are mutated in more than 50% of human tumors. Also their role is disrupted even when bearing a wilt type copy (9, 10). As more research was carried out, the regulatory molecules involved in these pathways became of high importance as well. For example, MDM2, the first E3 ligase highly correlated with p53 family, mutations and overexpression was monitored. Interestingly, it was minimal in the majority of human tumours (11-14). Of all the E3 ligases associated with p53, Pirh2 is the only one that is overexpressed in a wide range of human tumors. Pirh2 is overexpressed in 84% of 32 human lung neoplasms in a study evaluating Pirh2 expression in lung cancer. Complementary, p53 ubiquitination was detected at high levels in the same tumor tissues that highly expressed Pirh2 (15). Likewise, Pirh2 is overexpressed in prostate cancer (82%), hepatocellular carcinoma (78%), head and neck cancer (35%), and breast cancer (51%) (16-20). This highlights the fact that Pirh2 overexpression in human tumors might either be due to unsuccessful ubiquitination or disrupted degradation mechanism post ubiquitination. To further understand Pirh2 regulation, I will use the Pirh2-p53 model as an example. It has been shown that Pirh2 are p53 inducible proteins that bind to p53 and act as E3 ligases inducing substrate ubiquitination. Consequently, the substrate expression and activity is altered. Under unstressed conditions when the p53 tumor suppressor activity is not needed, Pirh2 binds to p53, polyubiquitinates it, and causes its proteosomal degradation. A negative feedback loop between Pirh2 and p53 proteins exists. Under stressed conditions, Pirh2 will be self-ubiquitinated and degraded. Therefore, the continuous repression of p53 will be released. As a result of the de-repression, p53 will be transcriptionally active (3, 21). The overexpression of Pirh2 in human tumors indicates a disruption in ubiquitination mechanism where the function of p53 is abrogated. Based on that, we investigated Pirh2 self

ubiquitination mechanism. We mapped the domains of Pirh2 involved in the self-regulatory pathway. The implication of domain mapping is sometimes unclear or lacks a strong significance. We argue that in terms of cell signalling pathways, when the same molecule is correlated in binding and function of several regulators, domain mapping becomes very important. The overlapping of domains explains how the molecule signals one pathway over the other. For example regarding Pirh2, the residues utilized for p53 and p73 binding happen to exist within the same domain region (3, 22). Structural analysis has also shown the protein confirmation post binding when the Pirh2 C-terminal recognizes the TET domain of p53 proteins (23). This was only carried for p53-Pirh2 binding. We anticipate a similar interaction with respect to Pirh2 and p73 proteins. Regarding the domain activity, the RING domain of Pirh2 is responsible for the ubiquitin catalytic activity. At this point it is worth mentioning that RING-H2 E3 ligases family lacks the ability to bind ubiquitin. Ubiquitin will only be transfered to the substrate after the E2-E3-substrate complex is formed (24). It remains unclear how the same E3 ligase that only transferred ubiquitin when binding the substrates becomes the host of ubiquitin moieties in the absence of the substrate. It was hypothesized earlier that this could be triggered by another E3 ligase that causes Pirh2 ubiquitination. The reason resides behind the evidence of self ubiquitination in catalytically inactive (Cys145or Cys146 RING mutations) Pirh2 proteins (1, 25, 26). Another possible explanation could be the shielding of the domains involved in self ubiquitination when binding the substrate. This applies only if the same residues required for substrate binding are the ubiquitin molecules host. Also the role of E2 enzymes could not be neglected (27, 28). In our data we confirmed that the RING domain and not the N-terminal domain are essential for Pirh2 self ubiquitination. However revealing the role of C-terminal domain along the RING was novel. Interestingly, the 240-250 residues in C-terminal domain are needed as well for Pirh2 self ubiquitination. Such a finding gives more value to future structural analysis as we suspect the formation of a loop, which possibly brings the RING domain in close proximity with C-terminal tail. For this reason both regions are required for self ubiquitination. Besides,

we investigated the role of E2 enzymes and lysine chains utilized in Pirh2 self ubiquitination. We confirmed the successful ubiquitination of Pirh2 using H5B, C, and A E2 enzymes but not Cdc34 E2 enzymes. Since the fate of the ubiquitination might also involve the role of E2, it is beneficial to analyze whether our E2 enzymes can lead to Pirh2 degradation post ubiquitination (29-32). This degradation can be investigated in vitro and in vivo to assess the importance of physiological conditions. Also, a wider screening of other E2 enzymes in Pirh2 self ubiquitination is advantageous. When it comes to the substrate's fate and as discussed earlier, the role of the lysine chains and their recognition by the 26S proteasome system is very important. Seven different lysine residues (6, 11, 27, 29, 33 48, 63) can be used for chain formation. Each chain may have a distinct effect on the substrate's fate (33, 34). Much less is known about the function of chains with other topologies. Poly-ubiquitinated substrates are mostly targeted for proteosomal degradation (35, 36). K48 lysine chains are recognized by the 26S proteasome system in vivo and induce substrate degradation post ubiquitination. Also, in some cases K11 and 29 were reported to induce the proteosomal degradation of the ubiquitinated substrate; however the mechanism remains under investigations. Alternatively, K63 chains, commonly utilized by many E3 ligases, are known to function in signal transduction, DNA repair, inclusion formation, endocytosis, lysosomal degradation, etc. Interestingly, recent studies did not eliminate the possibility that K63 may be recognized by the 26S proteasome system (37-45). This may highlight again the significance of E2 enzymes in the lysine chain analysis and substrate's fate. In this thesis, I detected no differences in terms of Pirh2 self ubiquitination when analysing K48 or K63 chains complemented by E2 H5B. However, we did confirm that self ubiquitination is done through lysine residues because when utilizing KO mutants (all lysine are mutated to arginine), ubiquitination was completely lost. So the questions that remain unanswered are: Do lysine chains determine the fate of Pirh2 post ubiquitination? Would the same lysine chain have a different fate on Pirh2 post ubiquitination when utilizing different E2 enzymes?

Lastly our in vitro ubiquitination analysis showed similar levels of Pirh2 self ubiquitination in the presence or absence of p53. This indicates that only under physiological conditions, Pirh2 can signal self or p53 ubiquitination. In other words, the catalytic function of the E3 ligases does not differentiate between p53 and self ubiquitination. This complies with the hypothesis stated above where E2-E3-substrate complex is the rate limiting step for differentiating between substrate/self ubiquitination mechanisms.

In terms of p73 regulation, a study done by Rossi et al. (2005) clearly shows the binding, polyubiquitination, and negative regulation of p73 at the transcriptional and protein level (46). The study did not investigate the lysine chains utilized. Since degradation is detected, the activation of the 26S proteasome system is anticipated to be activated (47, 48). This can be simply tested using protease inhibitors when analyzing the lysine chains utilized. As for Pirh2, in this project we reported the binding of Pirh2 and p73 with clear ubiquitination evidence. Evidently, the Pirh2 residues utilized in p53 or p73 binding are overlapping. In other words, under physiological conditions it is not known whether Pirh2 can bind both or a selective mechanism signals the binding of one substrate. Further analysis using mass spectroscopy and NMR could answer these questions especially that protein folding and domain shielding can affect the binding process. Also we observed that Pirh2 represses p73-dependent transcriptional activity by promoting p73 ubiquitination without targeting its degradation. Knowing that Pirh2 lacks the ability to degrade p73 (22), the role of other cofactors in this pathway is anticipated. In our ubiquitination analysis, Pirh2 catalyzes K11, K29, and K63-linked chains to ubiquitinate p73 protein in vitro, and markedly utilizes multiple lysine residue(s) (including Lys-48) to promote p73 ubiquitination in vivo. For this reason we can objectively state that the data collected is not all in favor of the lack of degradation. Since K48 is only utilized in vivo and the protein levels of p73 remain unaffected, cofactors determine the characteristic of this regulation rather than the E3 ligase function itself. We are not the first to propose this idea as it has been discussed earlier regarding p53 regulation.

MDM2 was the major E3 ligase regulating p53 function. Surprisingly polyubiquitination of p53 by MDM2 was only noticed when MDM2 is overexpressed and not under normal physiological conditions (11, 12, 49). In parallel, Pirh2-p53 polyubiquitination in response to DNA damage was detected in vivo and in an MDM2 independent manner (3). It was shown that in response to DNA damage, p53 is phosphorylated at Ser15. p53 Ser15 phosphorylation is sufficient to prevent its degradation by MDM2. The same condition of p53 phosphorylation is shown to be not effective in ceasing Pirh2-p53 regulation (50). This was further supported with in vivo data where Pirh2 knockout mice displayed high levels of phosphorylated p53. Furthermore, mouse models clearly showed that p53 phosphorylation at Ser18 (human serine 15) is required for p53 mediated apoptosis (51, 52). Based on that, it was proposed that MDM2 regulates p53 under unstressed conditions whereas Pirh2 fine tunes the DNA damage response (Figure 5.2).

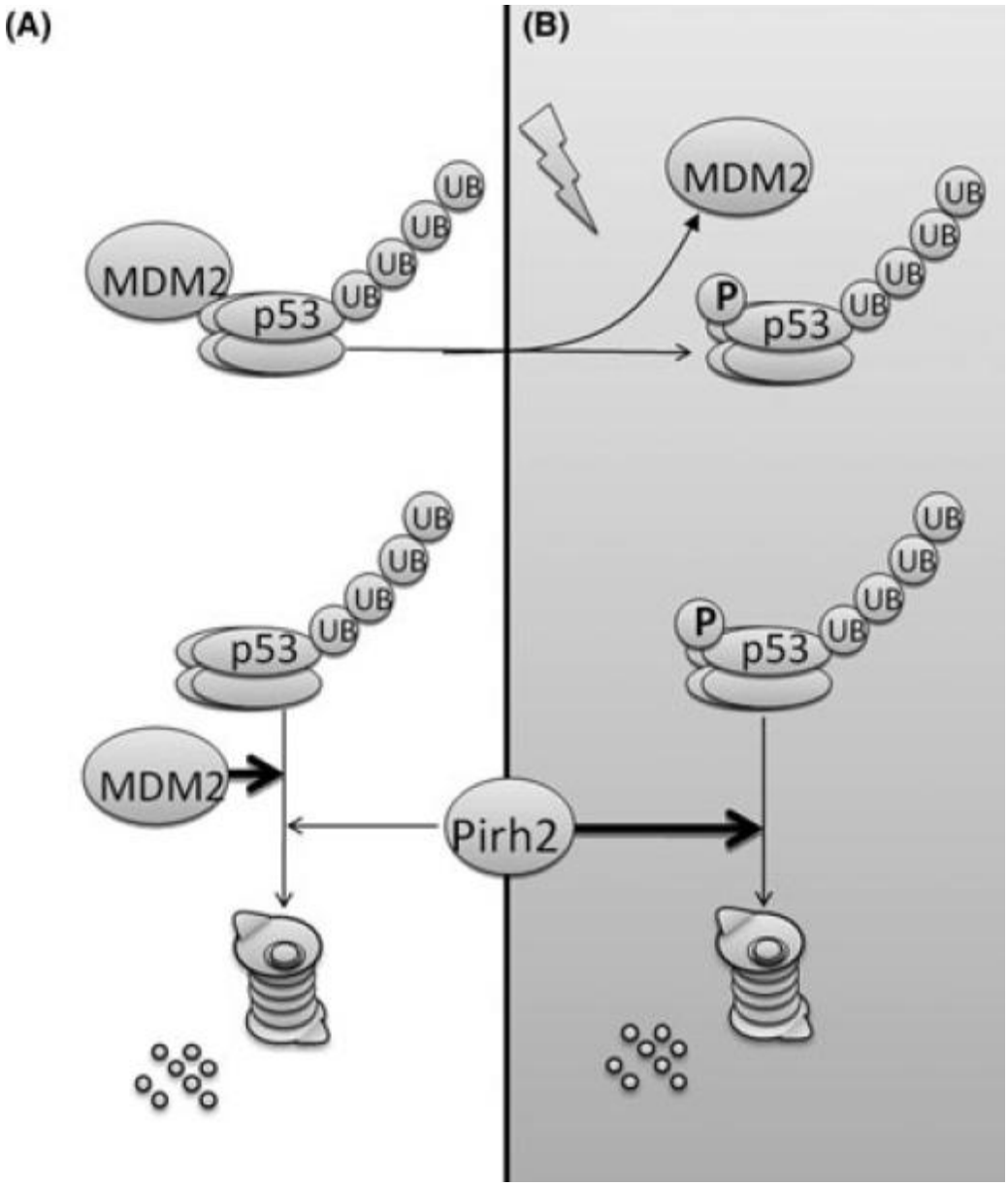

Figure 5.2. Schematic diagram illustrating the regulation of p53 by murine double minute 2 protein (MDM2) and Pirh2. (A) Under unstressed conditions, MDM2 is the main regulator of p53. (B) MDM2 and p53 are phosphorylated after DNA damage. MDM2 dissociates from p53, and Pirh2 becomes a main regulator of active p53. UB, ubiquitin; p, phosphorylation.

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Many scientists supported the proposed hypothesis while others did not. I am not introducing this dilemma to understand p53 regulation but to explain that this was a gateway for me to suspect whether Pirh2-p73 pathway and AIP4-p73 pathway are correlated. Also the preliminary data in our lab demonstrating Pirh2 and AIP4 binding using yeast two hybrid screening was the first step into this project.

5.3 Pirh2-AIP4-p73 story

In this thesis a novel correlation between two E3 ligases that share a common regulatory substrate (p73 in our case) is proposed. Pirh2 and AIP4 successfully bind in vitro and in vivo. When mapping the Pirh2 domains utilized, the N-terminal residues were shown to be involved in binding. This finding confirms that the N-terminal of Pirh2 is always involved in binding similarly to p53 and p73 binding. In this objective we did not reveal the exact residues as it is done with respect to p53 (residues: 120-137) and p73 (residues: 100-137) binding. For this reason, we cannot eliminate the fact that the same residues are utilized for AIP4 binding. Also, we do not know whether this mechanism is carried out in a competitive manner. For example, would p73 overexpression affect Pirh2-AIP4 especially that our in vivo data regarding Pirh2-AIP4 binding were collected from p73 bearing cell lines? In response to that, we aim to confirm the binding in $p73^{-/-}$ cells, and also we aim to repeat Pirh2 and p73 binding in the presence or absence of AIP4. The situation is more defined regarding AIP4 domain mapping, where the domain involved in p73 binding is distinct from that involved in Pirh2 binding. We confirmed that the HECT domain and not the WW domains, commonly known for their role in binding (53, 54), modulate AIP4-Pirh2 binding. The case is reversed with respect to p73, where WW domains modulate AIP4-p73 binding (46). In conclusion, AIP4 domain involved in Pirh2 binding is still vacant despite p73 binding. Based on that, two models could be proposed (Figure 5.3). Structural analysis such as mass spectroscopy and NMR can confirm the binding story.

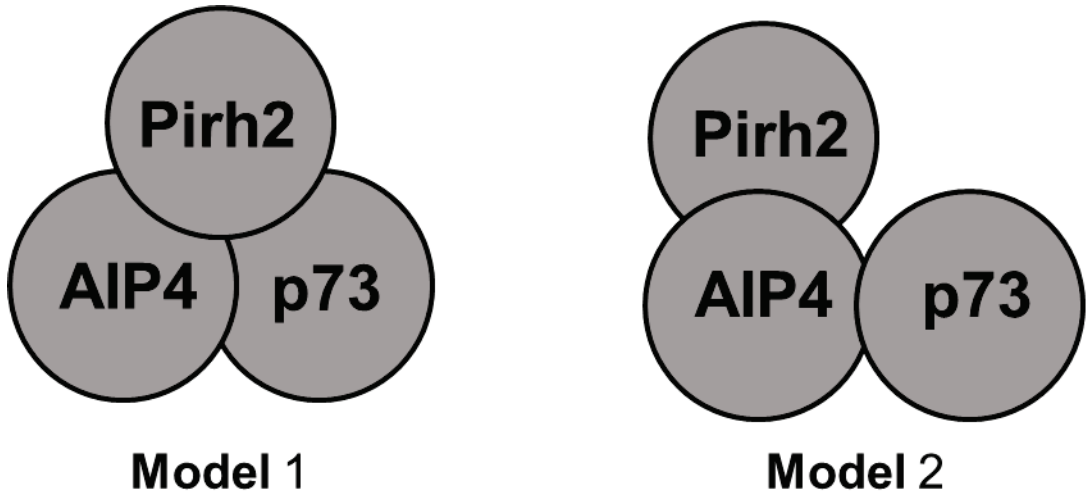

Figure 5.3. Schematic illustration of Pirh2-AIP4-p73 proposed binding models. Model 1 demonstrates the interaction of the 3 proteins (p73, AIP4, and Pirh2) whereas Model 2 demonstrates the binding of Pirh2 to AIP4 which in turn is bound to p73.

The exogenous and endogenous findings confirmed the down regulation of AIP4 by Pirh2 while stabilizing Pirh2 expression when AIP4 is present at high levels. This introduces the possibility of a negative feedback loop where Pirh2 falls upstream of AIP4. Further investigation is required to confirm the loop. For example, stable cell lines with knocked out Pirh2 or AIP4 can be models to run our investigations. Also we demonstrated that Pirh2 induced AIP4 degradation post ubiquitination. However, the lysine chains utilized are not known and the activation of the 26S proteasome system is to be determined. Nevertheless, we cannot eliminate the translocation of AIP4 and its lysosomal degradation in response to ubiquitination. To further elucidate the AIP4 degradation, we should monitor its half-life that we expect to be extended in the absence of Pirh2.

Interestingly when trying to replicate the data in HCT $p53^{+/+}$ cells, Pirh2 and AIP4 correlation disappeared. Thus we conclude that the presence of WT p53 ceased Pirh2-AIP4 correlation. When utilizing HCT $p53^{-/-}$ cells the correlation is restored similar to the p53 null cells (H1299 and HEK293) where we generated the data presented in chapter 4. We anticipate that the presence of p53 and its binding to Pirh2 affects Pirh2-AIP4 binding and regulation. As a result we need to investigate AIP4-Pirh2 when accompanied by p53 overexpression. Also data collected from HCT$p53^{+/+}$ cells should be repeated in other p53 bearing cells (tumor derived cell lines or normal cell lines) to confirm whether this regulation is cell type specific or a direct correlation with p53 presence.

Regarding p73, data collected at the endogenous and exogenous levels regarding its expression in response to AIP4-Pirh2 regulation is consistent with our previous findings. Pirh2 successfully ceased AIP4 down regulation and restored p73 expression. This introduces a new insight for therapeutic approaches that may utilize the antagonistic relationship between the two ligases to restore p73 expression. Our data collected at the ubiquitination level is in some cases misleading; for example, the ability of Pirh2 to ubiquitinate itself and ubiquitinate p73 while regulating AIP4 is hard to differentiate at the molecular level. Our measures to avoid

false negative data regarding p73 ubiquitination due to Pirh2 and AIP4 self ubiquitination mechanism were extremely helpful. In many western blots it is hard to define the ubiquitinated substrate. This was much enhanced when immunoprecipitating p73 and then detecting p73 ubiquitination using p73 rather than ubiquitin antibodies. However, since p73 succesfuly bind both AIP4 and Pirh2 then even after immunoprecipitation the ligases might be pulled down and self ubiquitination might still interfere. In our data, the level of p73 ubiquitination was decreased but not abolished when Pirh2 is introduced. This was expected since we previously confirmed in chapter 3 the Pirh2-p73 induced ubiquitination. However, we do not know the role of this Pirh2-p73 induced ubiquitination. Pirh2 as a negative regulator significantly inhibits AIP4 with respect to p73 but why does it lead to p73 ubiquitination? More investigations are necessary to answer and reveal what is unknown. Monitoring p73 half-life can be used as a preliminary step. Lastly, cell cycle analysis was the only objective that translationally investigated the role of Pirh2-AIP4 on p73 tumor suppressor function. Data was consistent yet Pirh2 was not effective in restoring p73 G1 arrest function at the same strength as restoring p73 protein expression level. It is too early to judge before further assessing p73 tumor suppressor role and specifically its apoptotic function. Colony formation assays to monitor the apoptotic role of p73 or fluorescence-activated cell sorting using Annexin V staining could be used to assess p73 apoptotic activity in response to Pirh2-AIP4 regulation.

5.4 Limitations and future directions

The major findings presented in this thesis each have its limitations, and accordingly future work for the short term has been proposed above when discussing the data. On the long term, many future directions could be suggested to support our hypotheses and confirm our anticipated outcomes. For example NMR analysis could confirm which of the proposed models (Figure 5.3) is accurate. The challenge in this work is the involvement of three proteins within the same signalling pathway. Most of the findings were confirmed at the exogenous and

endogenous levels, but this doesn't eliminate the possibility that the correlation is indirect. For example, Pirh2 and AIP4 correlation in terms of expression was studied in $p53^{-/-}$ cell lines where p73 is endogenously expressed. Using cell lines that lack endogenous p73 can reveal whether Pirh2-AIP4 correlation is p73 dependent. The same applies to Pirh2 and AIP4 since we only studied their expression through knockdowns or overexpression. Generating stable cell lines that are $Pirh2^{-/-}$ or $AIP4^{-/-}$ can be used as a control for our experiments. Regarding the role of p73 as a tumor suppressor, further work can be suggested. We have previously proposed to study the apoptotic function of p73 in response to Pirh2-AIP4 regulation. It was explained in chapter one that many p73 responsive genes are common with p53 (55-57). Therefore, analyzing the responsive genes in terms of expression and function is very important. Also in chapter 4, we showed that Pirh2-AIP4 correlation is dependent on the absence of p53. Based on that, studying the role of p73 responsive genes, in response to Pirh2–AIP4 regulation, in the presence or absence of p53 can open new gateways. Likewise we showed that the AIP4-Pirh2 correlation was lost in HCT WT $p53^{+/+}$ cells, yet data was not confirmed at the p73 expression level. This along with analyzing p73 tumor suppressor function (cell cycle arrest and apoptosis) can confirm whether p73 regulation is ceased just when p53 is introduced.

Knowing that Pirh2 activation depends on DNA damage regardless of p53 status, Pirh2-AIP4-p73 story can be investigated in response to different DNA damaging stimuli. Interestingly, it is known that Pirh2 phosphorylation triggers the self ubiquitination mechanism. This is indirectly triggered by cytoplasmic translocation of Pirh2. Accordingly Pirh2 cannot interact with the tumor suppressors present in the nucleus. In tumor cells, Pirh2 is shown to dominantly exist in unphosphorylated form (58, 59). This could be a gateway to investigate whether the same Pirh2 phosphorylation modulated by CaMKII kinases affects Pirh2-AIP4 binding. At the clinical level and since our ultimate goal is to provide insights into more effective therapeutic approaches, it is worth using tumor tissues as models for further

investigations. As a first step I suggest monitoring the AIP4 expression levels in breast, head and neck, prostates tumor tissues, all of which previously shown to possess elevated Pirh2 expression (16-20). Similar analysis should be carried on the same tissues regarding p73 expression levels and p73 responsive genes. Lastly, unlike MDM2 knockout mice, Pirh2 knockout mice are viable and develop normally without showing differences in p53 basal levels (1, 50). Thus, the AIP4-p73 regulation can be analyzed under normal conditions and in response to DNA damage. Pirh2 bearing mice will be used as controls.

Aside from Pirh2-AIP4-p73 signaling pathway, we provided insight towards possible correlation between other E3 ligases in regulating the same substrate. For example, it was previously shown that overexpression of Pirh2 leads to a substantial increase in MDM2 and MDMX levels and a milder increase in COP1 levels (60). The implications of this increase were partially investigated regarding p53 activity but the big picture remains unclear.

5.5 Closing remarks

In summary, my thesis provides a novel perspective of Pirh2 E3 ligases. Pirh2 overexpression in a wide range of cancer tumors may affect the regulation of p73 tumor suppressors. Discussion regarding Pirh2 dual function in regulating tumor suppressors and oncogenes at the same time has been proposed earlier. We showed that, within the same family of proteins (p53 family), Pirh2 has a dual role. Pirh2 binds and negatively regulates p53, while stabilizes p73 through ceasing the AIP4 down regulation and enhancing its expression. It remains unknown what signals Pirh2 to function as a tumor suppressor or an oncogene, and whether DNA damaging stimuli or cellular physiological conditions is a driving factor in these pathways. In conclusion, Pirh2 is a promising target for cancer therapy, and can be utilized as a novel prognostic marker and a major target molecule for restoring the disrupted activity of tumor suppressors in our human body system.

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