STRUCTURAL AND FUNCTIONAL STUDIES OF HUMAN UBIQUITIN E3 LIGASE HUWE1

RAHIMA KHATUN

A DISSERTATION SUBMITTED TO
THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILLMENT OF THE REQUIRMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

GRADUATE PROGRAM IN BIOLOGY
YORK UNIVERSITY
TORONTO, ONTARIO

SEPTEMBER 2017

Abstract

Ubiquitin (Ub) acts as an intracellular signal once tagged covalently to the target proteins and regulates a myriad of cellular processes. Ubiquitin is attached to the target protein by the concerted action of E1, E2, and E3 Ub ligase enzymes, in the presence of ATP. During this process, E3 Ub ligases are responsible for the final step of protein ubiquitination and play crucial roles in substrate selectivity and specificity. Therefore, understanding the structure and function of E3 Ub ligases provides valuable mechanistic information regarding their specific roles in Ub-mediated regulatory processes. In this dissertation, we aimed to investigate the structure and function of the human E3 Ub ligase, HUWE1. HUWE1 regulates multiple cellular pathways, including DNA damage response, apoptosis, and transcriptional regulation through controlling the stability and fate of various proteins involved in these pathways. Using multidisciplinary approaches, we studied the functional domains, the regulation, and the new cellular substrates of HUWE1. We report that HUWE1 harbors a previously uncharacterized tandem ubiquitin-binding motif (UBM). It contains three independently folded unique UBMs, and as a tandem, it binds three different Ub chains. Most significantly, this tandem UBM enhances HUWE1-mediated ubiquitination. We also uncovered the mechanism of a X-linked mental retardation mutation, R2981H, of HUWE1. Our results show this mutation disrupts the structure of the UBM that attenuates HUWE1 function.

We also investigated the molecular mechanism of the HUWE1 inhibition by ARF. We identified an 8mer ARF peptide that binds HUWE1 and p53 *in vivo* to inhibit HUWE1-mediated p53 ubiquitination, which results in p53 transcriptional activation. Moreover, this peptide inhibited cancer cell growth in a p53-dependent manner.

Finally, we found that HUWE1 directly binds and ubiquitinates β-catenin, a master signal transducer in the canonical WNT signaling pathway. Overall, these findings identify a new HUWE1 substrate that provides new knowledge of HUWE1 function in cell regulation and development.

Acknowledgements

This dissertation would not have been completed without the help and guidance of the loving people surrounding me, who contributed their priceless assistance in completion of this project. I owe very much to them for extending their hand while I was struggling, and spending critical periods in the lab solving an unsolvable problem. First, I would like to express my sincere gratitude to my supervisor, Dr Yi Sheng for providing me with the opportunity to work in such an exciting, but slightly intimidating, structural biology project. A student like me, who had no clue how a protein crystal should look or how NMR works, began this project from scratch (only knowing there is a mutation in this protein, the valuable XLMR mutation, R2981H). However, Dr Sheng's endless support and devotion to research made me fearless in overcoming the obstacles I encountered at the beginning. I am deeply indebted to her for teaching me the critical thinking skills needed for the molecular research expertise I have gained. I wish to thank her profusely for making me work hard and touch new levels in a competitive research community.

I would also express utmost gratitude to my advisors Drs Chun Peng and Vivian Saridakis for providing all the valuable support and guidance during the PhD period. I appreciate the time and wonderful feedback I received regarding my progress every year, which helped me to resolve many difficult experiments. My sincere appreciation to my first research mentor, Dr Patricia Lakin-Thomas, with whom I worked while obtaining my M.Sc. In addition to research, without her humanitarian support and extra time, I would not have managed to survive in Toronto and finish my degree. Thank you very much Dr Pat for being such an incredible human being.

My graduate studies could have been endangered without the help of two amazing friends, Olga Egorova and Heyam Hyder, whom I met when I joined Dr Sheng's lab. I thank both of you very much for being such incredible lab mates and talented peers, with whom I could discuss many complicated experiments. Olga, your microscopic eyes and artistic computer skills so often shaped my writing and presentation meaningfully. Heyam, because of you I do not feel alone in Canada. Your considerable supports in all the ups and down since we met are still providing me with extra strength to survive in post-grad studies. I am also indebted to our former undergraduate research student Raghav Ramabadran, who worked with me in one of the projects presented in this dissertation. Your cheerful presence and support brought new ideas for our project. I would also like to thank Sahar Forhadi, a Masters student in our lab, for her hard work to solve the structure of UBM1 presented here. I also appreciate all the undergrads in our lab for their cooperation in running the lab smoothly.

Ira, Sara, Niharika, Anna, and Hossein in Dr Saridakis's lab, offered valuable criticisms and support during lab meetings. I am grateful to Marsha Kriss and Niharika for reading the draft and giving valuable feedback, and to Mohammed from Dr Peng's lab for the help he offered, sometimes at midnight.

My special thanks to Dr Pat and Dr Howard Hunter for editing the final draft.

Finally, I thank my Mother and siblings for their continuous support, so I could stay abroad and pursue science. At this very auspicious time in my life, I miss my dearly departed Father. Thank you Dad, for giving me the privilege of education in a society where, while I was growing up, the majority of girls were denied the opportunity to go to school.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

APC	Adenomatous Polyposis Coli
APC/C	Anaphase promoting complex/Cyclosome
APF-1	ATP-dependent protein factor 1
ARF	Alternative Reading Frame Protein
ARF-BP1	ARF Binding Protein 1
ATP	Adenosine Triphosphate
CD	Circular Dichroism
CDK	Cyclin-dependent kinase
DIUM	Double-sided Ubiquitin Interacting Motif
DUB	Deubiquitinating Enzyme
ERAD	Endoplasmic Reticulum Associated Degradation
GST	Glutathione-S Transferase
HDX	Hydrogen to deuterium exchange
HECT	Homologous to E6-AP C-terminus Domain
HSQC	Heteronuclear Single Quantum Coherence
HRS	Hepatocytes Growth factor-related Tyrosine Kinase Substrate
HUWE1	HECT, UBA and WWE Domain containing protein 1
IAP	Inhibitors of Apoptosis
LCMS	Liquid Chromatography and Mass Spectrometry
MDM2	Murine Double minute 2
MJD	Machado Josephin Domain
Mule	Mcl-1 Ubiquitin Ligase E3
NEDD4	Neural Precursor cell expressed, Developmentally Down-regulated 4
NEM	N-Ethylmaleimide
NF-kB	Nuclear Factor-kappa Light Chain Enhancer of activated B Cells

NMR	Nuclear Magnetic Resonance
OTU	Ovarian Tumor Domain
PCNA	Proliferating Cell Nuclear Antigen
PH	Pleckstrin Homology Domain
PKC	Protein Kinase C
PTM	Post Translational Modification
RAP80	Receptor Associated Protein 80
RING	Really Interesting New Gene Domain
SAXS	Small-Angle X-ray Scattering
SKP	Skip1-Cullin-F-box
TLS	Translesion Synthesis; a cell survival mechanism in eukaryote
Ub	Ubiquitin
UBA	Ubiquitin Associated Domain
UBC	Ubiquitin Conjugating Domain
UBD	Ubiquitin Binding Domain
UBM	Ubiquitin Binding Motif
UIM	Ubiquitin Interacting Motif
USP7	Ubiquitin Specific Protease 7
WWE	Tryptophan and Glutamate containing Protein-Protein Interaction Domain
YFP	Yellow fluorescent protein

Chapter 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

Ubiquitination is a form of protein modification that regulates almost every cellular process in eukaryotic cells. Ubiquitination involves covalent attachment of the carboxyl-terminal group of ubiquitin (Ub) to the Ɛ-amine group of substrate lysine in an ATP-dependent manner (Hershko and Ciechanover, 1998; Pickart and Eddins, 2004). This modification often alters the intracellular localization, modulates the enzymatic activity, or marks the protein for degradation through the 26S proteasome (Glickman and Ciechanover, 2002). This dissertation focuses on characterization of a large, multi-domain HECT Ub E3 ligase HUWE1 (HECT, UBA and WWE domain containing protein 1, also known as ARF-BP1, HUWE1, HectH9, Lasu1), an enzyme involved in protein ubiquitination pathways. This protein plays a crucial role in regulating diverse cellular functions, including cell cycle progression, DNA repair, and transcriptional regulation. Thus, the general introduction of this dissertation includes two major parts: (I) the mechanism and function of protein ubiquitination in the cell, and (II) the structure and function of the Ub E3 ligase, HUWE1.

Part I. The mechanism and function of protein ubiquitination in the cell

1.1 Protein ubiquitination

Ub is a 76-amino acid protein, first isolated in the bovine thymus by Goldstein *et al.*, in 1975 (Goldstein et al., 1975). Later studies showed that Ub is expressed ubiquitously from Yeast to human. The ubiquitination process was first observed and reported by Goldkeopf and Busch, (1977) with the observation that histone proteins could be modified by lysine-linked isopeptide bonds. This study expanded the forms of protein post-translational modification (PTM) beyond phosphorylation, acetylation, and methylation, and was the first discovered PTM by a small

protein (Goldknopf and Busch, 1977). Later, Ub-mediated protein degradation, *i.e*. ATP-dependent protein factor 1 (APF-1) mediated intracellular proteolysis, was reported by Ciechanover *et al.*, through seminal biochemical studies using reticulocyte lysate in the early 1980s (Ciechanover et al., 1980; Ciechanover et al., 2012; Hershko et al., 1980). Since then, ubiquitination has been recognized as a key PTM signal in controlling nearly every aspect of eukaryotic biology.

Ub structurally adopts a conserved globular fold, termed the β-grasp fold. Its structure contains a central β-sheet with a helix packed on one side and an exposed canonical hydrophobic surface on the other side of the β-sheet. Ub has a flexible C-terminal tail composed of a di-glycine motif (Dye and Schulman, 2007; Husnjak and Dikic, 2012). The C-terminal carboxyl group of the residue Gly76 in the Ub di-glycine motif is covalently attached to the Ɛ-amine-group of a lysine residue of the substrate protein via an isopeptide bond, through an enzymatic process named ubiquitination. This reaction is accomplished by the concerted actions of three classes of enzymes: E1- Ub activating enzyme, E2- Ub conjugating enzyme, and E3 Ub ligase enzyme. The covalent attachment of Ub to the substrate lysine serves as a cellular signal, which often alters the function and fate of the substrate protein, resulting in changes in the intracellular localization, enzymatic activity, or protein degradation through the 26S proteasome (Glickman and Ciechanover, 2002; Pickart, 2001). A schematic diagram of Ub and the sequential steps of a typical ubiquitination reaction are provided in Figure 1.1. The human proteome contains one confirmed E1 enzyme, approximately 36 E2 enzymes, and more than 600 E3 ligases (Grabbe et al., 2011; Husnjak and Dikic, 2012). E3 Ub ligases directly bind to their cognate substrate proteins, thus conferring the substrate specificity and diversity.

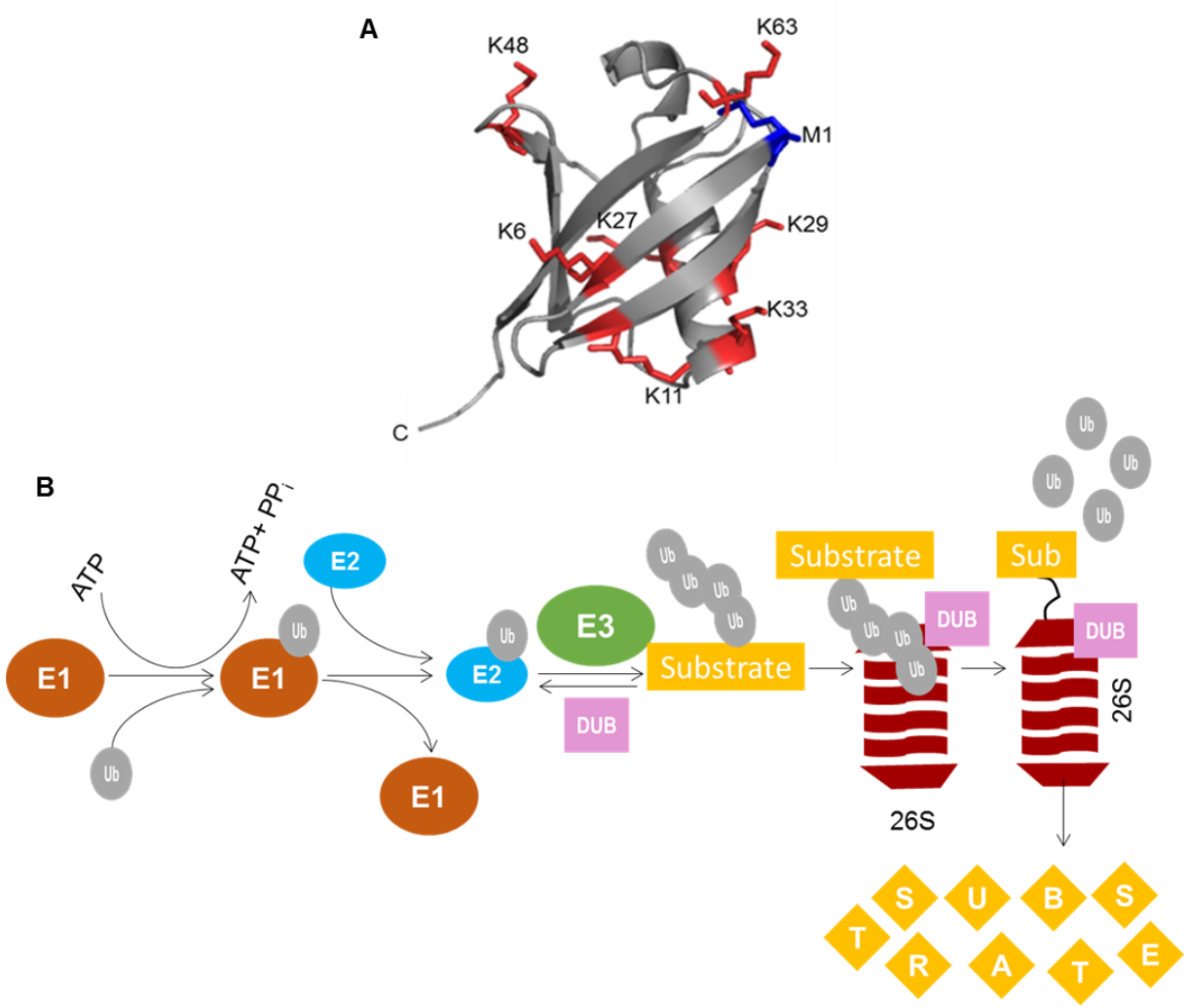

Figure 1.1: A schematic diagram of the ubiquitination pathway. (A) Cartoon representation of ubiquitin (Ub). Seven lysine residues are shown as red sticks. The N-terminal first residue methionine (M1) is shown as a blue stick. The C-terminus of Ub is indicated as "C". (B) Cartoon representation of the ubiquitination pathway. Three specialized enzymes E1, E2 & E3 work sequentially to attach Ub to the Ɛ-amine group of the substrate lysine through an isopeptide bond formation. Initially, Ub is activated by an E1 Ub-activating enzyme in an ATP-dependent manner, and forms an E1~Ub thioester at the catalytic cysteine residue of E1. Ub is then transferred to the catalytic cysteine of E2 in a transthiolation reaction and forms an E2~Ub thioester. The E3 ligases finally transfer Ub from the E2~Ub thioester to the substrate lysines. Similar to other PTMs, protein ubiquitination is reversible. The specialized enzymes, deubiquitinases (DUBs) remove Ub from the substrate for proper regulation of this pathway. Figure A is generated by RK from the Ub structure using PyMol (PBD code: 1UBI) and B is adapted and modified from (Chrysis et al., 2011).

Ub contains seven lysine residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63). All seven Ub lysine residues and also the amino terminus of the first residue methionine (Met1) can serve as the acceptor site for Ub conjugation, permitting assembly of polyUb chains (polyUb chains, Ub-Ub chain). Thus, the substrate protein can be modified at any single lysine with a single Ub molecule (mono-ubiquitination), or at multiple lysines with several independent Ub monomers (multi-mono-ubiquitination), or by chains of lysine-linked Ub moieties (polyubiquitination) (Ikeda and Dikic, 2008; Iwai and Tokunaga, 2009; Kim et al., 2011). The polyUb chains can adopt diverse topologies depending on the Ub linkage type, *i.e.* the specific lysine residue(s) in Ub that participate in the chain formation. For example, structural studies showed that some Ub chains adopt closed or compact conformations where the canonical hydrophobic surface centered at Ub residue Ile44 is partially sequestered in the multi-Ub assembly. The polyUb chains, linked by Lys48, Lys11 and Lys6 result in closed compact structures of Ub assembly with a partially buried hydrophobic surface. In contrast, the Lys63- and linear-linked chains form more extended open conformation with an exposed hydrophobic surface (Dikic et al., 2009; Scott et al., 2015; Weeks et al., 2009). Hence, different Ub chain topologies can provide different binding surfaces and recognition modes, allowing the Ub system to transduce diverse Ub signals that subsequently trigger the varieties of cellular responses.

Ubiquitination has emerged as a key regulatory mechanism for a plethora of cellular processes, including cell cycle progression, transcriptional regulation, apoptosis, signal transduction, protein turnover, endocytosis, and DNA repair (Bergink and Jentsch, 2009; Ciechanover, 1998; Coux, 2002; Goldberg, 2000; Grabbe et al., 2011; Hershko and Ciechanover, 1998; Hirsch et al., 2009; Husnjak and Dikic, 2012; Pickart, 2001; Scott et al., 2015). In general, mono-ubiquitination can lead to nuclear export and functions in DNA repair. Ub chains,

composed of four Ub molecules attached through Lys48, mark the substrate proteins for proteasomal degradation. PolyUb chains that are conjugated through other lysine residues (Lys6, Lys29, and Lys63) are involved in non-proteolytic functions, such as sub-cellular localization, trafficking events, DNA repair, and immune signaling (Ikeda and Dikic, 2008; Iwai and Tokunaga, 2009; Longerich et al., 2014). Mixed or branched polyUb chains, *i.e.* polyUb chain, with different types of lysine residues, were also reported, but their cellular functions are not well characterized (Meyer and Rape, 2014). Finally, polyUb chains that are assembled head-to-tail are known as linear chains or Met1 chains in which Gly76 of the preceding Ub is attached to the first residue (Met1) of distal Ub. This type of Ub chain plays an important role in the activation of NF-κB signaling cascades (Gerlach et al., 2011; Ikeda et al., 2011). So far, eight different homotypic and several heterologous polyUb chains were reported. The known cellular functions linked to the different Ub linkages are depicted in Figure 1.2.

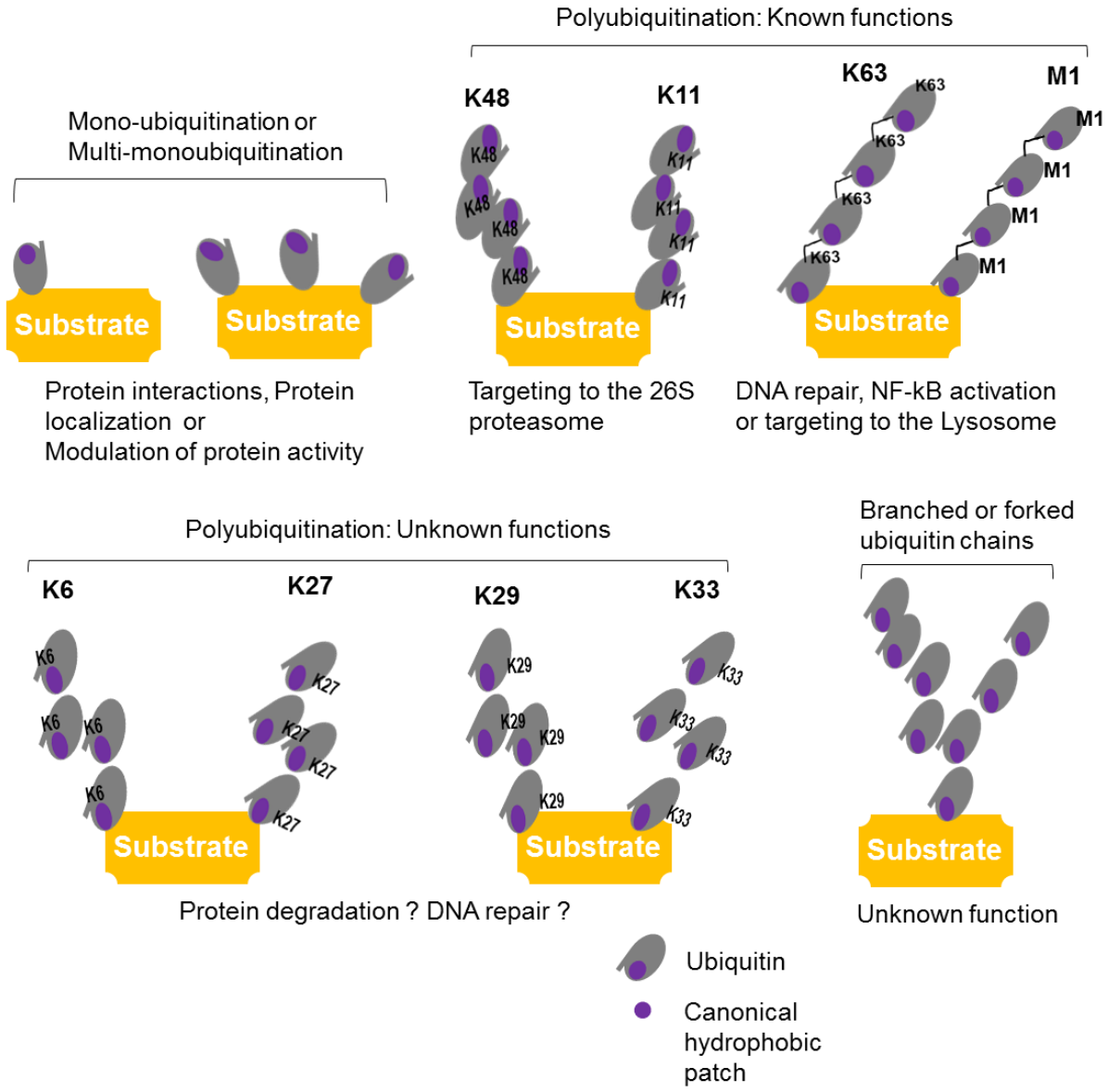

Figure 1.2: A schematic representation of different types of protein ubiquitination. In Ub signaling pathways, proteins are regulated by a single Ub (mono-ubiquitination), multiple single Ub (multi-mono-ubiquitination), or different polyUb chains (polyubiquitination). The substrate could also be modified through linear ubiquitination, Ub attached at N-terminal amino group of distal Ub. To date, eight different types of homotypic and several other heterotypic linkages were reported *in vivo* that regulate various cellular pathways. Figure is adapted and modified from (Ye and Rape, 2009).

1.2 The machinery for Ub signal assembly

The conjugation of Ub to a target protein is a very complex and highly regulated process. Ub conjugation starts from the activation of Ub by the Ub activating enzyme E1. The canonical Ub E1 enzyme possesses an adenylating domain, a Ub fusion domain, and a catalytic cysteine-containing domain. In a typical ubiquitination process, Ub activation requires three steps. Initially, the E1 adenylating domain recognizes the Ub and adenylates its C-terminal glycine residue in the presence of ATP and Mg^{+2}. In the second step, activated Ub encounters a nucleophilic attack by the catalytic cysteine of E1, and forms an E1~Ub thioester. In the third step, E1 interacts with E2, and Ub is then transferred to the catalytic cysteine residue of an E2 conjugating enzyme via a transthiolation reaction. E2s make contact with both E1 and E3s to receive Ub from E1 and to deliver Ub to the E3s. E3 ligases recognize substrates, and mediate substrate Ub conjugation in the final step of ubiquitination.

All E2 conjugating enzymes possess a core catalytic Ub-conjugating (UBC) domain, while some have additional N- or C-terminal extensions that may be required for specific functions. For example, UbE2E1 possesses an approximately 50 residues long N-terminal extension in addition to the core UBC, which is required for substrate recognition and specificity (Sarkari et al., 2013). Similarly, another E2 enzyme, UbE2R1 (Cdc34), possesses a C-terminal extension, which has been shown to be critical in transferring Ub to the substrate in association with its cognate E3 ligase complex (Block et al., 2005). Structural and biochemical studies show that E1 and E3 bind in the same region on E2, implying that E2 could not receive Ub from the next E1~Ub thioester complex before finishing transferring Ub to the E3s. These observations suggest that cycles of sequential Ub receiving and transferring occur through the order of E1, E2, E3, and substrate to ensure polyubiquitination precision and accuracy in the cell. Moreover, E2

plays a critical role in determining the type and outcome of ubiquitination. For example, when pairing with RING E3s, a group of E2s, including UbE2R1, UbE2G1, and Yeast Ubc7 predominantly generate the Lys48-linked chains. In contrast, UbE2N tightly bound with an E2-like subunit (either UbE2V1 or UbE2V2) generates Lys63-linked chains (Stewart et al., 2016) .

The E3 ligases are the key enzymes for the final step of Ub transfer to the substrate. They govern the accuracy and precision of the ubiquitination process. E3s bring and orient E2~Ub conjugates into close proximity with the substrates for proficient Ub conjugation (Bernassola et al., 2008; Pickart and Eddins, 2004; Swatek and Komander, 2016; Varshavsky, 1997). E3s function mostly via two distinct mechanisms: either they form an E3~Ub thioester intermediate before transferring Ub to the substrate, or they directly transfer Ub from the E2~Ub to the substrate. The former is utilized by the HECT-family of E3s and the latter is utilized by the RING-family of E3s. The third group of E3s, termed the U-box E3 ligases, have structures closely resembling the RING domain structures, but lacking the cysteine residues and coordinated zinc ions. Mechanistically, they are also similar to the RING E3s (Bernassola et al., 2008; Metzger et al., 2014; Nagai and Kubori, 2013; Paul and Ghosh, 2014). As HECT-type E3s and RING-type E3s are different in structure and mechanisms of action, these classes of E3 ligases are discussed separately below.

1.3 Ub E3 ligases: The HECT Ub E3 ligases

Members of this family possess a signature HECT (homologous to E6-AP C-terminus) domain at the C-terminus that harbors a conserved catalytic cysteine residue. HECT E3s form an E3~Ub intermediate on the catalytic cysteine prior to transferring Ub to the substrate lysine. HECT E3s require three sequential steps to transfer Ub to the substrate. Initially, the HECT domain binds to the Ub-charged E2, forms an E3~Ub thioester intermediate at the catalytic

cysteine, and transfers Ub from E3~Ub to the substrates. The human genome encodes only 28 HECT-type E3 ligases compared to the large number of RING E3s (approximately 600) that lack the catalytic cysteine (Huibregtse et al., 1995). All HECT E3s share a conserved HECT domain of approximately 350 amino acids at the C-terminus, which was originally identified in the E6-associated protein, E6-AP (Huibregtse et al., 1995). A typical HECT domain consists of two distinct lobes: an N-terminal lobe harboring the E2 binding site, and a C-terminal lobe containing the catalytic cysteine. A flexible peptide hinge connects these two lobes. Structural studies have revealed the crucial role of this peptide hinge in juxtaposing the two lobes in a favorable orientation during Ub transfer. Hence, the catalytic cysteines from both E2 and E3s could come into close proximity to facilitate Ub transfer (Huang et al., 1999; Pandya et al., 2010; Verdecia et al., 2003). The distances between two catalytic cysteines (E2-E3 complex versus Ub-charged E2-E3 complexes) were found to vary significantly. It was measured as approximately 41 Å in the E6-AP-Ubc7H crystal structure (PDB code: 1C4Z) and 16 Å in the case of WWP1 and its cognate E2 (PDB code: 1ND7) (Huang et al., 1999; Verdecia et al., 2003). Interestingly, the distance was found to lessen significantly in the Ub-loaded E2-NEDD4L complex: the distance between the catalytic cysteines of E2 and E3 is only 8 Å (Kamadurai et al., 2009). This suggests that the topologies of HECT domains adopt different conformations depending on whether the HECT domain is free or Ub loaded. The conformational flexibility allows HECT E3s to form polyUb chains with different lengths and types. Further, non-covalent interaction between both lobes of E3 with Ub from the Ub-loaded E2 complex was found to support Ub transfer from E2~Ub to E3. This non-covalent interaction plays a crucial role in elongating the polyUb chains (Ogunjimi et al., 2005; Pandya et al., 2010). A schematic diagram of different HECT domain structures is provided in Figure 1.3.

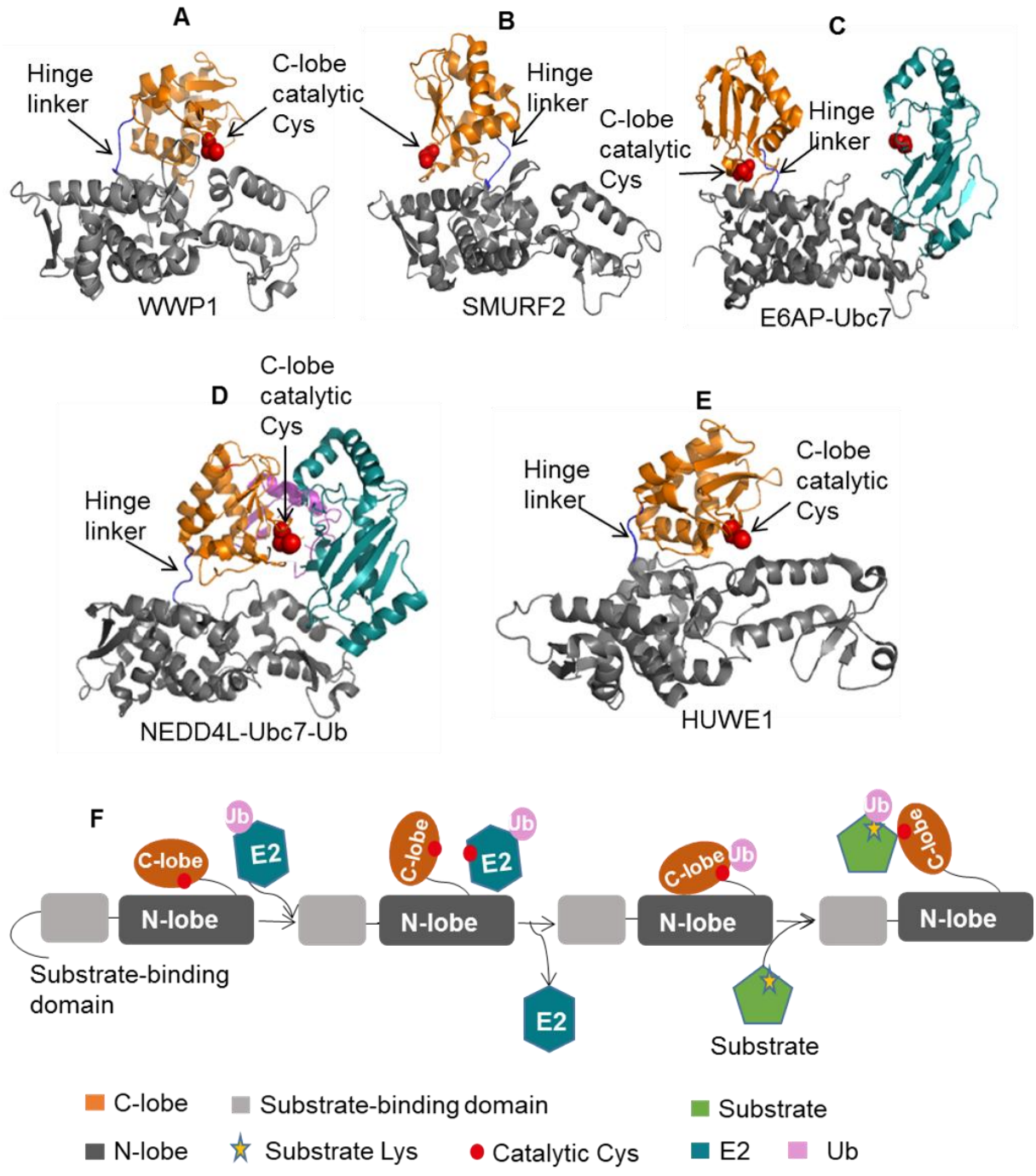

Figure 1.3: Cartoon representation of HECT domain structures. (A) HECT domain of WWP1 (PDB: 1ND7). (B) HECT domain of SMURF2 (PDB: 1ZBD). (C) HECT domain of E6-AP bound with an UbE2L3 (PDB: 1C4Z). (D) HECT domain of NEDD4L bound with an UbE2D2~Ub thioester (PDB: 3JVZ). (E) HECT domain of HUWE1 (PDB: 3H1D), respectively. (F) A schematic diagram of ubiquitination by HECT domain E3 ligases. Figure is adapted and modified from (Buetow and Huang, 2016).

In HECT E3s, while the catalysis is performed by the HECT domain, the substrate recognition is typically done by the N-terminal extensions containing different protein-protein interaction modules. Based on the protein-protein interaction domains, human HECT E3s can be grouped into two large subclasses: (i) NEDD4/NEDD4-like E3s, containing C2, WW, and HECT domain and (ii) "Other" HECT E3s containing neither C2s nor WW domains, referred to as SI(ngle)- HECT E3s (Scheffner and Kumar, 2014; Scheffner and Staub, 2007). A graphic presentation of different HECT E3s is provided in Figure 1.4.

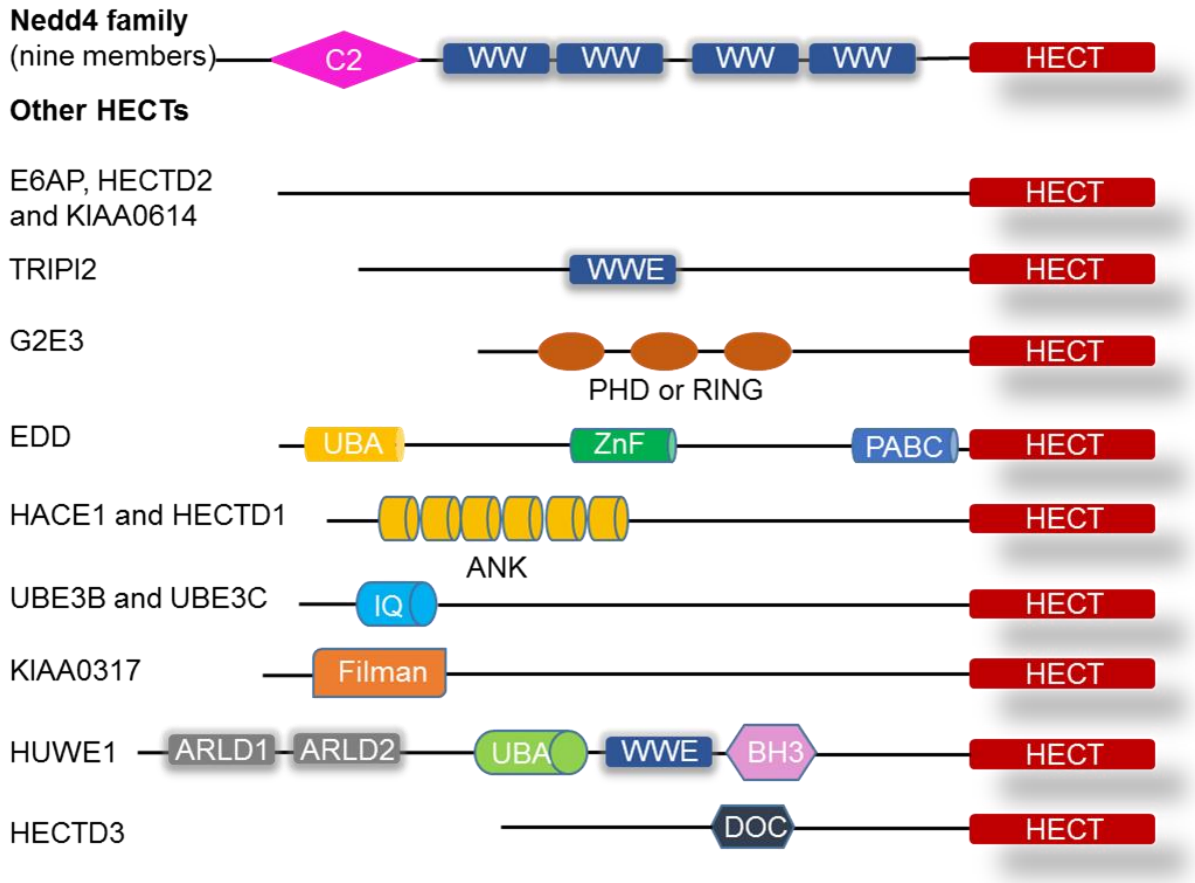

Figure 1.4: The schematic representation of human HECT E3 ligases. HECT E3s are classified in two classes: the NEDD4 family and the Other HECT E3s. The NEDD4 family members contain a unique N-terminal C2 domain, two to four WW domains and a C-terminal HECT domain. Other HECT E3s lack the C2 and WW domains. They possess a variable N-terminal extension with diverse protein-protein interaction modules. All HECT E3 ligases contain signature HECT domains at the C-terminus. Figure is adapted and modified from (Rotin and Kumar, 2009).

NEDD4/NEDD4-like HECT E3s

To date, nine members of this group were reported for the NEDD4/NEDD4-like E3s. Members of this subclass HECT E3 ligases generally consist of an N-terminus protein-kinase C (PKC)-related C2 domain, two to four WW domains (tryptophan-tryptophan), and a C-terminus HECT domain. The sequential C2-WW-HECT is typical for all NEDD4 HECT E3 members. The C2 domain of this HECT E3s is known to bind with Ca^{2+} and phospholipids, and targets E3s to the intracellular membranes. In contrast, the WW domain is known to mediate ligase-substrate association. This domain interacts with a variety of proline-rich motifs and proline-containing phosphoserines. In addition, it also interacts with phosphothreonine motifs (PPXY) of the protein substrate (Bernassola et al., 2008; Dunn et al., 2004; Rotin and Kumar, 2009; Scheffner and Kumar, 2014; Schwarz et al., 1998). Interestingly, several C2 domains of this group are found to regulate self-ubiquitination through intramolecular inter-domain interactions. For example, the C2 domain of SMURF2 prevents auto-ubiquitination through the intramolecular interaction between the C2 domain and the C-lobe of the HECT domain. This interaction sequesters the catalytic cysteine of the C-lobe, blocks Ub-loaded UbcH7 associations, and inhibits Ub thioester formation. This inhibition can be relieved by SMAD7, a TGFβ antagonist, upon TGFβ stimulation. SMAD7 interacts with both the HECT domain and Ub-loaded UbcH7, resulting in conformational changes to the HECT domain, which disrupts the C2-HECT interaction and restores the activity of SMURF2 HECT domain.

Similarly, the WW domain of ITCH, another NEDD4 like HECT E3, was found to interact with the HECT domain, rendering ITCH inactive. This intramolecular inter-domain-mediated inhibition of ITCH can be reversed via stimulus-dependent phosphorylation of the N-terminus residues. These findings are indicative of the importance of the N-terminal C2 and the

WW domain activities in the regulation of HECT E3s functions. These mechanisms ensure the substrate proteins are only targeted for ubiquitination upon activation (Gallagher et al., 2006; Wiesner et al., 2007). The NEDD4/NEDD4-like E3s have been shown to regulate a variety of substrates, including membrane proteins, transmembrane receptors, and intracellular proteins, through mono-and polyubiquitination (Bernassola et al., 2008; Rotin and Kumar, 2009; Scheffner and Kumar, 2014).

Other HECT-type E3 ligases

Members of this class have a conserved C-terminal HECT domain but do not possess defined C2 and WW domains. However, the ligases of this group possess diverse N-terminal extensions, usually containing several protein-protein interaction domains or other functional domains. One example of this class of HECT E3 ligases is E6-AP, and the first reported HECT-type Ub E3 ligase. This protein is about 100 kDa in size and found to interact with an E6 protein of the human papilloma virus (HPV) and tumor suppressor p53. The E6-E6-AP complex binds to p53 and targets p53 for Ub-mediated proteolysis (Huibregtse et al., 1995; Scheffner and Staub, 2007). In this dissertation, we report structural and functional characterization of another member of this family, HUWE1 (HECT, UBA and WWE containing E3 ligase). The general literature review of HUWE1 will be summarized in the second part of the introduction.

1.4 Ub E3 ligases: The RING E3 ligases

The human genome encodes more than 600 E3 Ub ligases (Grabbe et al., 2011), of which the vast majority are the RING domain E3 ligases. Members of this class share a conserved Cys3HisCys4 motif, consisting of about 40 to 60 residues known as the *Really Interesting New Gene* or RING domain. The structure of the RING domain is defined by the consensus sequence Cys-X2-Cys-X(9-39)-Cys-X(1-3)-His-X(2-3)-Cys-X2-Cys-X(448)-Cys-X2-Cys. In the

consensus sequence, Cys and His represent the zinc binding residues, and X is any amino acid. The RING domain forms a cross-brace globular structure coordinating two zinc atoms through the conserved Cys-His residues. This domain is involved in mediating E2-E3 interactions and is responsible for transferring Ub molecules from the E2~Ub thioester to the substrates lysine residue (Deshaies and Joazeiro, 2009; Passmore and Barford, 2004; Pickart, 2001; Pickart and Eddins, 2004). Unlike HECT E3 ligases, RING E3 ligases do not possess intrinsic catalytic activity due to the lack of catalytic cysteine residues. This family of E3s acts as molecular scaffolds that facilitate direct substrate ubiquitination by placing Ub-conjugated E2 into close proximity with the target lysine residues. Moreover, E2-E3 binding induces conformational changes in the E2-E3 complex that facilitate and promote Ub transfer to the substrate. RING E3s also possess other modular domains responsible for substrate recognition and recruitment (Burger and Seth, 2004; Pickart, 2001; Pickart and Eddins, 2004).

Based on the structure and architectural arrangement of RING-type E3s, members of this family can be divided into two classes: **(i) Single subunit RING E3 Ub ligases:** This class of E3s has both the RING domain and substrate binding motifs as part of the same polypeptide, such as MDM2, COP1, and BRCA1/BRAD1 (Metzger et al., 2014). **(ii) Multi-subunit RING E3 Ub ligases:** This class of E3s consists of different protein subunits. The RING domain activities and the substrate binding motifs are provided by distinct subunits within the complex. One such E3 complex is Skip1-Cullin-F-box (SCF) known to ubiquitinate a broad range of substrates involved in cell cycle progression, signal transduction, and transcriptional regulation (Burger and Seth, 2004; Lipkowitz and Weissman, 2011). The SCF complex consists of three core subunits: RING domain protein, CUL1 (scaffold protein), and Skp1 (adapter protein), as well as one variable component, known as an F-box protein. F-box is responsible for substrate

recognition. This ligase complex binds substrates through the interaction of target proteins with the F-box that is recruited to the ligase complex via recognition of F-box motif by Skp1 adaptor protein. Three F-box proteins, SKP2, FBW2, and β-TRCP, have been identified and play important roles in cell cycle control (Bassermann et al., 2014; Berndsen and Wolberger, 2014; Nakayama and Nakayama, 2006).

Member of one unique group of E3 ligases, known as the U-box E3s, are believed to be variants of the RING domain E3 ligases. U-box also mediates ubiquitination by acting as a molecular scaffold. The U-box domains are structurally similar to RING domains but lack Zn^{2+} ions and the chelating Cys and His residues (Lu et al., 2002). A distinct class of E3s, termed RING-in-between-RING (RBR), have recently been reported. These RBR E3s possess a RING1 domain that binds Ub-loaded E2 enzymes and a RING2 domain that harbors a catalytic cysteine similar to HECT-type E3s. Though the Ub transfer mechanisms are not understood well, the RBR E3s are considered to function as RING/HECT hybrids (Dove, 2016).

1.5 Mechanisms of polyUb chain assembly

Although Ub chain topologies are indispensable for the diverse functionalities of the Ub-proteasome system, the mechanistic details of the synthesis of these multifold Ub chain structures are not well understood. For example, how do E3s choose a specific lysine residue on Ub to synthesize a polyUb chain? How are the lengths of the Ub chains determined (Kulathu and Komander, 2012) ? Several models of polyUb chain assembly are discussed below, with the focus primarily on HECT domain E3 ligases.

Structural studies of different di-/tri-Ub chains (Lys63-/Lys48-/Lys11-/Met1-linked linkages) have indicated that lysine selectivity and processivity in chain assembly are two prerequisites in order to assemble a linkage-specific polyUb chain (Castaneda et al., 2013; Cook

et al., 1994; Fushman and Walker, 2010; Komander et al., 2009; Varadan et al., 2004; Varadan et al., 2002). Targeting a single lysine in acceptor Ub (free) would require bringing the donor Ub (E2~Ub or E3~Ub thioesters for RING and HECT E3s, respectively) into close proximity to the acceptor Ub. It also requires correct orientation for Ub catalysis (reviewed in (Kulathu and Komander, 2012). Unlike the RING E3s, whose polyUb chain synthesis is primarily determined by the cognate E2s, HECT E3s directly catalyze the ubiquitination independent of E2s. The structural studies indicate that HECT domains are very flexible and adopt different conformations depending on whether they are bound to Ub (Buetow and Huang, 2016; Scheffner and Kumar, 2014; Scheffner and Staub, 2007). The conformational flexibility allows E3~Ub thioester to accommodate accessible lysine residues on the target, or on a growing polyUb chain. Several models have been proposed for the polyUb chain assembly by HECT E3 ligases, one of which is the "standard sequential-addition model" where single Ub molecules are added sequentially to the distal end of a Ub chain linked to the substrate in a processive reaction. However, this model is unable to account for how long Ub chains are added to the substrate before they dissociate from the E3 (Kim and Huibregtse, 2009; Kulathu and Komander, 2012).

Another possible mechanism is the "Indexation" model. In this model, a polyUb chain is formed on the E3 catalytic cysteine residue by the sequential addition of Ub monomers, and then the polyUb chain is transferred to substrate from the cysteine residue in bulk. This model also fails to address how the E3 ligases determine the chain length and type (Hochstrasser, 2006; Kim and Huibregtse, 2009; Kulathu and Komander, 2012; Verdecia et al., 2003). Furthermore, the fact that a thioester-linked Ub chain on the E3 has not been reported provides additional challenges to prove this model.

A third model of the polyUb chain synthesis is proposed based on the non-covalent Ub-binding capabilities of the E2 and E3s. Several studies have reported that polyUb chains, which are formed by RING-type E3s, are decided mainly by cooperating E2 enzymes. Hence, RING E3-mediated Ub conjugation is E2 dependent. In addition to forming E2~Ub thioester, some E2s were found to bind the donor Ub (the Ub in E2~Ub intermediate) and the acceptor Ub (the Ub at the distal end of a Ub chain) non-covalently in deciding the chain type specificity. These non-covalent bindings were found to orient Ub in such a way that only specific lysine residues would lie in close proximity to the active site cysteines. For example, structural studies of UbE2N-UbE2V2 (Ubc13-MmS2) heterodimer (Ubc13 is an active E2 and has catalytic Cys, MmS2 is an inactive E2 lacking a catalytic Cys) showed that the UbE2V2-Ub-binding places the acceptor Ub in such way that Lys63 is approaching towards the catalytic Cys of UbE2N, hence promoting Lys63-linked chain formation only (Moraes et al., 2001; Wickliffe et al., 2011). In contrast to the RING domain E3 ligases, HECT-types E3s are independent of E2 in determining the outcome of polyubiquitination. Non-covalent interactions of HECT N-and C-lobes with donor Ub are believed to affect linkage specificity and chain length of HECT-mediated polyubiquitination. For example, the co-crystal structure of Yeast HECT domain E3 Rsp5 bound with Ub showed that the N-lobe of Rsp5 makes contact with Ub. This contact orients and tethers the distal end of the growing polyUb chain to facilitate conjugation of the next Ub molecule. These findings suggest that N-lobe-Ub-binding controls the linkage and length of polyUb chain synthesized by Rsp5 (French et al., 2009). Similar non-covalent Ub interactions were also detected for other HECT domain E3s. As in the case of SMURF2, the HECT N-lobe-Ub interaction was shown to be required for SMURF2 auto-and substrate ubiquitination. Accordingly, this HECT N-lobe-Ub-

binding is shown to recruit and orient the mono-ubiquitinated substrate to the HECT domain (Ogunjimi et al., 2005; Ogunjimi et al., 2010).

The current data supports the combined model of the sequential addition and non-covalent Ub-binding for HECT E3s. Remarkably, the co-crystal structure of Rsp5 human orthologue NEDD4 bound with E2~Ub thioester, reveals that the Ub of E2~Ub directly interacts with the C-lobe of NEDD4 HECT domain. This interaction positions the E2 and E3 cysteine into close proximity for the transthiolation reaction and orients the acceptor lysine residue for preferential Lys63-linked chain formation. These results suggest that the C-lobe may be the limiting factor in defining the linkage specificity in HECT-type E3 ligases (Huang et al., 1999; Kamadurai et al., 2009; Kulathu and Komander, 2012; Maspero et al., 2011; Ogunjimi et al., 2010; Pandya et al., 2010; Swatek and Komander, 2016; Verdecia et al., 2003; Wickliffe et al., 2011). Rsp5 preferentially makes Lys63-linked chains both *in vitro* and *in vivo*. To investigate the role of the C-lobe in determining linkage specificity, a domain swapping experiment was performed by exchanging the residues in the C-lobe of Rsp5 with the last 60 residues of E6-AP. E6-AP is known to regulate p53 turnover by catalyzing Lys48-linked p53 ubiquitination. The chimera Rsp5 containing the E6-AP C-lobe is able to synthesize Lys48-specifc chains, suggesting HECT C-lobe-Ub interactions are the key to determining the linkage type, while the N-and C-lobes configuration and distance determine the chain length.

1.6 Ub signal recognition

1.6.1 Ub-interacting domains (UBDs)

Ubiquitination generates diverse Ub signals that can be transduced in the cell and regulate a myriad of cellular processes. The molecular mechanisms that recognize different types of Ub signals are poorly understood. Previous research has shown that differentially

ubiquitinated substrates are recognized by the specialized Ub-binding domains (UBDs) embedded in the multi-domain proteins and Ub signal receptors. UBDs could form transitory, non-covalent interactions with the diverse surfaces of the ubiquitinated proteins and transduce these signals to trigger a specific biological response (Dikic et al., 2009; Scott et al., 2015; Swatek and Komander, 2016).

UBDs are found in more than 200 proteins involved in ubiquitination, deubiquitination, DNA repair, endocytosis, immunity, and autophagy (Grabbe and Dikic, 2009; Grabbe et al., 2011; Husnjak and Dikic, 2012; Ikeda et al., 2010; Kulathu and Komander, 2012; Raasi and Pickart, 2003; Reyes-Turcu et al., 2008; Sokratous et al., 2014; Sprouse et al., 2015; Swatek and Komander, 2016). They function as basic Ub-binding modules, regulating the activity and function of host proteins. The founding member of this family, namely the UBA domain, is a three-helix bundle, characterized as an integral part of the Ub receptor protein S5a/Rpn10, which itself is a subunit of the 26S proteasome. This UBA domain was found to interact selectively with a polyUb chain composed of at least four Ub moieties and to play an important role in recognition of the Ub degradation signal (Deveraux et al., 1994; Scott et al., 2015). Later, similar protein sequences were identified in other proteins involved in proteasomal and lysosomal degradation pathways through bioinformatics. To date, 20 different families of UBDs were reported, involved in a variety of cellular pathways (Dikic et al., 2009; Hochstrasser, 1996; Hofmann and Falquet, 2001; Hurley et al., 2006; Scott et al., 2015). A representative list of UBDs is provided in Table 1.1.

Table 1.1: List of ubiquitin binding domains, structures and their Ub binding modes (Adapted from and modified from (Husnjak and Dikic, 2012)

UBD abbreviations	Structures	Ubiquitin surface	Type of ubiquitin binding	Host proteins
UBA	Three-helix bundle	Ile44	mUb polyUb (>K48) UBl	Cbl-b, hHR23A/B, USP5, HERC2
CUE	Three-helix bundle	Ile44	mUb	Cue2
UIM	Single α-helix, often present as tandem	Ile44	mUb polyUb (K48,K63) UBL	S5a,RAP80, EPS15, ataxin-3
MIU/IUIM	Single α-helix	Ile44	mUb	Rabex-5
DIUM	Single α-helix, binds two Ub monomers	Ile44	mUb	Hrs
VHS	Superhelix of 8 α-helices	Ile44	mUb	STAM, Hrs
GAT	Three-helix bundle	Ile44	mUb	GGA3, TOM1
NZF	Zinc finger, four β-strands	Ile44	mUb polyUb (K63)	TAB2, TAB3
ZnF A20	Zinc finger	Asp58	mUb (Rabex-5) polyUb (K63, A20)	Rabex-5, A20
ZnF UBP	Zinc finger, a globular fold with a deep cleft and pocket to accommodate Ub's tail	Leu8, Ile36	polyUb, unanchored	USP5
UBZ	Zinc finger, ββα-fold	Ile44	mUb	Polymerase η, Polymerase k
UBC	β-sheet	Ile44	mUb	UbcH5C
UEV	Helix-turn-helix, helices separated by an invariant Leu-pro motif	Leu8	mUb	Polymerase ι, Polymerase Rev1
UBM	Helix turn helix, helices separated by an invariant Leu-Pro motif	Leu8	mUb	Polymerase s ι, polymerase Rev1
GLUE	Split-pleckstrin homology domain	Ile44	mUb	EAP45

(Continued)

UBD abbreviations	Structures	Ubiquitin surface	Type of ubiquitin binding	Host proteins
PRU	Pleckstrin homology domain, three loops bind Ub	Ile44	mUb (Ile44) pUb (K48 linker) UBL	Rpn13
Jab1/MPN	Inactive variant of Jab1/MPN domain lacking key residues in the motif	Ile44	mUb	Prp8p
PFU	Four β-strands and two α-helices	Ile44	mUb pUb	Doa1, PLAA
SH3, variant	β-barrel fold, hydrophobic groove binds Ub	Ile44	mUb	S1a1, CIN85, amphiphysin
UBAN	Parallel coiled-coil dimer	Ile44	Met1-di-Ub	NEMO, optineurin, ABIN1-3
WD40 repeat β-propeller	Top surface of WD40 repeat β-propeller	Ile44	mUb	Doa1/UFD3

*mUb denotes mono-ubiquitination
**polyUb denotes polyubiquitination

UBDs are modular structural units which often exist as multiple copies, interacting with Ub non-covalently. Typically, they are 15-60 residues in size and adopt small α-helical structures. Several other structural folds are also reported for UBDs, including zinc fingers (ZnFs), Ub conjugating domains (UBC) and pleckstrin homology folds (PH) (Husnjak and Dikic, 2012). The majority of UBDs bind with Ub through the canonical hydrophobic surface/patch centered in the residue Ile44. The adjacent regions, including hydrophobic residues Leu8 and Val70 in the vicinity of the Ile44 patch, an acidic hydrophilic Asp58 patch, and a hydrophobic Ile36 patch at the C-terminal tail, are also selectively recognized by the UBDs. For example, the Ub-binding motif of Translesion synthesis Y-family polymerases ι and Rev1 interact with the hydrophobic patch centered in Leu8. A20-type ZnF domain (UBD of Rabex-5) of Rabex-5 recognizes the Asp58 centered region, while the ZnF domain of isopeptidase T (IsoT, a deubiquitinase) binds to the Ile36 patch at the C-terminal tail of Ub. These UBDs utilize different Ub-binding modes to facilitate transduction of diverse Ub signals (Dikic et al., 2009; Reyes-Turcu et al., 2006; Reyes-Turcu et al., 2009; Scott et al., 2015). A cartoon representation of different UBDs bound with Ub is provided in Figure 1.5.

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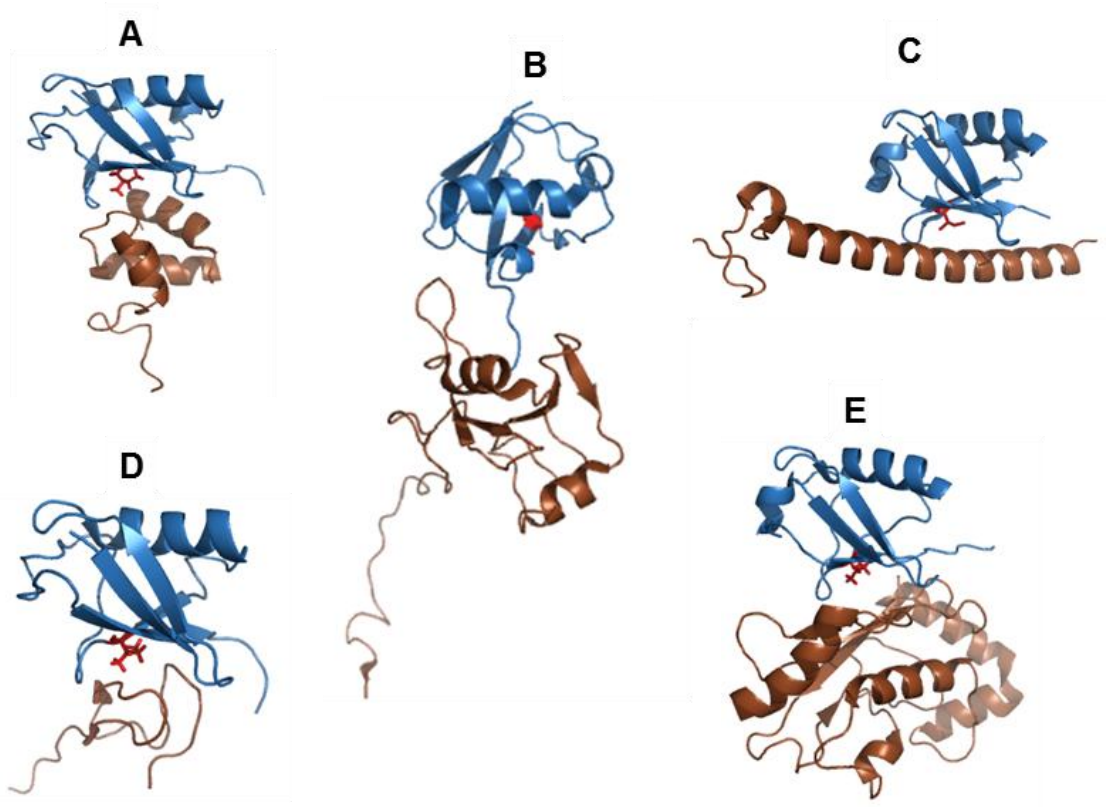

Figure 1.5: Ub is recognized by structurally diverse Ub-binding (UBD) domains. Ribbon representation of Ub-UBD complexes is shown. Ub is shown in blue and UBDs are shown in brown color. (A) Shows the complex structure of UBA domain of ubiquilin1with ubiquitin (PDB: 2JY6). (B) Complex structure of the ZnF domain of the deubiquitinase isopeptidase T with ubiquitin (UBD domain of isopeptidase is known as ZnF domain) (PDB: 2G45). (C) Complex structure of the UBA domain of the Rabex5 with ubiquitin (PDB: 2FIF). (D) Complex structure of UBD domain of A20 protein with ubiquitin (UBD domain of A20 is known as ZnF (PDB: 1Q5W). (E) Complex structure of the UBD domain of E2 Ub-conjugating enzyme with ubiquitin (PDB: 2FUH). This Figure is adapted and modified from (Dikic et al., 2009).

1.6.2 Mechanisms of Ub signal recognition

While a single UBD typically shows weak affinity to Ub, higher affinity Ub-binding could be achieved through different mechanisms, such as oligomerization, multimeric protein-protein interactions and unique Ub-binding modes. Many UIM (Ub interacting motif)-containing endocytic proteins bind mono-Ub very weakly. UBDs of endocytic proteins are referred to as UIM. However, they were found to associate with each other via their UBDs to form oligomers to achieve high affinity and selective recognition of polyUb chains. Furthermore, the tandem or multimeric arrangement of UBDs in Ub receptors also contributed to higher affinity binding. For example, Epsin and Eps15 are signal-transducing adaptor molecules. They possess multiple UIMs and were reported to interact with polyubiquitinated plasma membrane proteins to promote their internalization into clathrin-coated vesicles (Barriere et al., 2006; Chen and De Camilli, 2005; Sugiyama et al., 2005). Finally, some Ub receptors also contain different structural folds of UBDs, which aid in acquiring high-affinity interactions. The hepatocyte growth factor-regulated tyrosine kinase substrate Hrs contains a UIM motif (UBD of Hrs). This UIM folds to a single α helix. The UIM helix was found to bind simultaneously to two Ub molecules via two opposite surfaces. This double sided Ub-binding of UIM was found to be essential in sorting internalized epidermal growth factor receptors by Hrs (Bosanac et al., 2010; Fisher et al., 2003; Hirano et al., 2006; Kang et al., 2003; Lee et al., 2006; Wang et al., 2003).

The preferential recognition of a specific Ub chain by a UBD is another mechanism used by UBDs to transduce diverse Ub signals. For example, UBDs of proteasome shuttle factors were shown to bind Lys48-linked chains more tightly than mono-Ub and Lys63- linked linkages (Varadan et al., 2005). Comparatively, UBDs of DNA repair pathway proteins show preference for the Lys63-linked chains. Several studies report that the order of multimeric UBDs within an

Ub receptor or the linker residues between two UBDs orients them in a specific conformation that favors one type of polyUb chain over the others (Berke et al., 2005; Sims and Cohen, 2009). For example, tandem UIM motifs of RAP80 (UBDs of receptor associated protein 80) preferentially bind with Lys63-linked chains, but not with Lys48-linked chains, to localize the BRCA1/BRAD1 tumor suppression complex to the replication foci during DNA damage response. The underlying key to this selectivity is the linker region between the RAP80 tandem UIM (two UBDs connected by the linker; the UBD is named UIM in RAP80), which place it in a favorable orientation, allowing concurrent interactions with two Lys63-linked Ubs, but not to Lys48-linked Ub moieties (Sims and Cohen, 2009). In contrast, a two-residue linker between two UBDs of Ataxin 3, a deubiquitinase responsible for spinocerebellar ataxia type 3, determines its specificity to the Lys48-linked chain (Berke et al., 2005; Sims and Cohen, 2009). These findings highlight the important roles of multimeric UBDs in recognition of polyUb signals that have distinct consequences in Ub-dependent cellular processes. Structural representations of different UBDs bound with single Ub and di-Ub chains are provided in Figure 1.6.

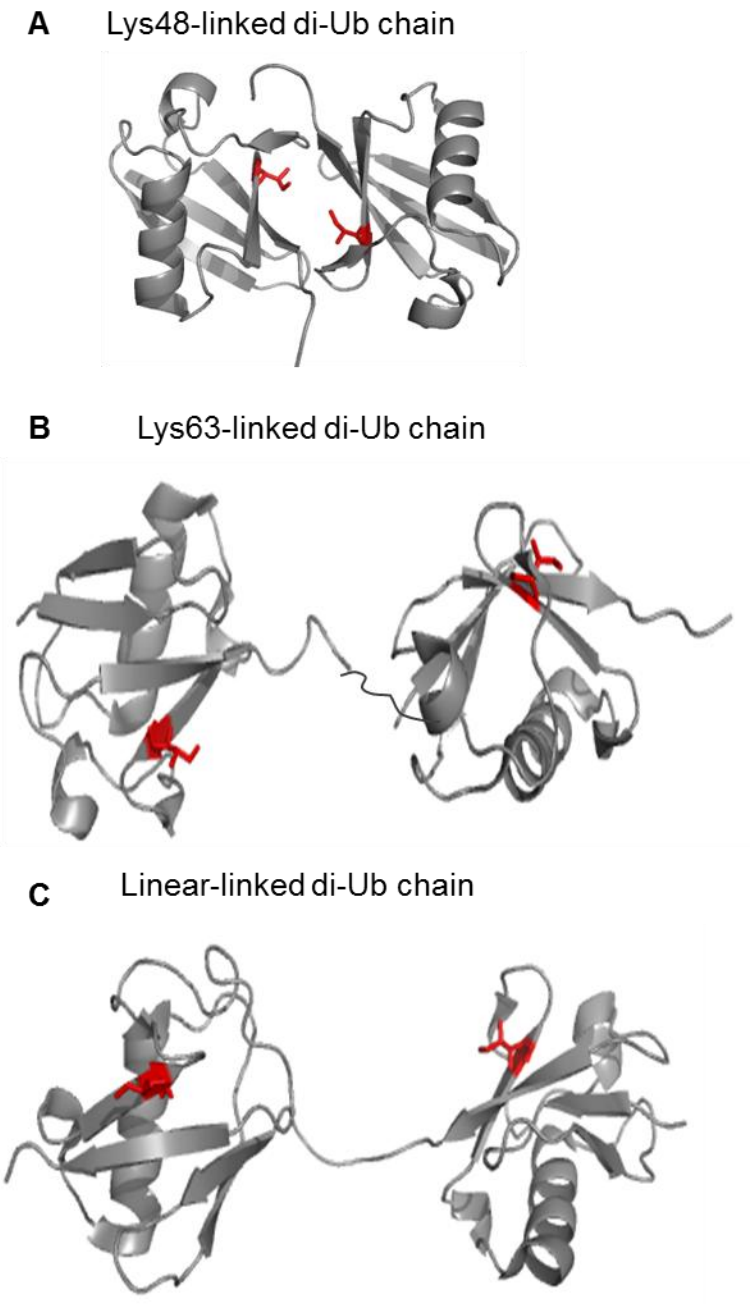

Figure 1.6: Ubiquitin chains adopt different topologies. Di-Ub structure is shown in ribbon representation. Ile44 patches in Ub's are shown as red stick model for all. (A) The closed compact structures of Lys48-linked di-Ub chain (PDB: 1AAR). (B) The open extended structure of the Lys63-linked di-Ub chain (PDB: 2JF5). (C) The extended conformation of linear di-Ub chains (PDB: 2W9N). Figure is generated by RK from the above mentioned PDB files.

1.6.3 Role of UBDs in host protein functions

Ub-binding domains selectively recognize Ub signals (mono- or different polyUb- linked chains) to facilitate Ub mediated cellular events. Thereby UBDs could influence the functions of host proteins in several ways. Some UBDs were found to recognize protein degradation signals consisting of Lys48-linked Ub chains; others provide binding surfaces/docking sites for Ub signals to facilitate non-degradational cellular functions. The important roles of UBDs in regulating host protein functions are well studied in proteasome receptors, endocytic pathways, DNA repair pathways, and NF-κB signaling pathways.

Proteasomal Ub signal recognition by UBDs

Selective proteolysis mediated by the 26S proteasome is signaled by the Lys48-linked polyUb chain. In this process, the ubiquitinated proteins marked for degradation are recognized and delivered to the proteasome by proteasomal shuttle proteins. The key to the specific recognition of the degradation Ub signal is the UBDs present in the proteasome-shuttle proteins. The shuttle proteins, including Yeast Rpn10, RAD23, and Dsk2, contain two unique functional domains: Ub-binding domain (UBD) and Ub-like domain (UBL). UBD is used to recognize ubiquitinated proteins marked for degradation, whereas the UBL domain of shuttle proteins tethers the ubiquitinated protein to the proteasome for proteolysis (Heessen et al., 2005; Husnjak et al., 2008; Kang et al., 2006; Raasi and Pickart, 2003; Wang et al., 2005).

In some instances, UBDs act to regulate the function of UBD-containing proteins through the interaction with mono- or polyUb chains. UBDs can serve as stabilization signals for the host proteins (Clarke et al., 2001; Lambertson et al., 1999; Watkins et al., 1993). It is reported that the UBA domain of RAD23 functions as a *cis-acting* domain, capping the polyUb chain elongation and preventing self-degradation. Similar stabilization effects on host proteins were also

documented for the UBD domains of other proteasome shuttle factors, including Ddi1 and Ede1 proteins, suggesting it may be a general phenomenon for some UBD domain containing-proteasomal proteins (Heessen et al., 2005; Kang et al., 2006; Raasi and Pickart, 2003). The tandem UBD of Yeast transcription factor Met4 also utilizes an analogous mechanism to protect Met4 from proteasomal degradation. UBDs of Met4 were found to inhibit polyubuquitination and degradation of Met4, enabling rapid transcription during nutritional stress (Flick et al., 2004; Flick et al., 2006; Heessen et al., 2005; Tyrrell et al., 2010).

UBDs serve as scaffolds/adaptors for protein-protein interaction

Many UBD containing proteins in the endocytic pathway were shown to form large protein complexes with ubiquitinated proteins and receptors via interacting with UBDs to promote receptor internalization. Multiple binding surfaces and the different structural variations of UBDs expand the Ub signal binding spectrum that facilitates the internalization of receptors into the endosome. For example, two UBDs of Rabex-5, a guanine nucleotide exchange factor of Rab5, interact with two Ub molecules simultaneously to regulate EGFR trafficking. This cooperative action of Rabex-5 UBDs was shown to be essential in regulating Ub-dependent endocytosis of receptor tyrosine kinases (Lee et al., 2006; Penengo et al., 2006).

Several Ub receptors and scaffolding/adaptor proteins were found to form large protein complexes that are involved in cytokine signaling and in activation of the NF-κB signaling pathway. For example, selective recognition of Lys63-linked chain in RIP1 by the TAB domain (UBD of transforming growth factor β-activated kinase 1 (TAK1)) of TAK1 mediates oligomerization and autophosphorylation of TAK1. This event was found to play an important role in the activation of downstream NF-κB signaling upon binding of ligands to the receptors

(Chen and Sun, 2009; Mendoza et al., 2008; Prickett et al., 2008; Sato et al., 2009b; Shinohara et al., 2016).

The gathering of DNA damage repair factors at the stalled replication fork is initiated by different protein-protein interaction modules present in proteins involved in this pathway. UBDs in some of these receptors and proteins were shown to be essential in recruiting the cognate assembly complexes to the DNA damage sites. For example, recognition of Lys63-linked chains on histones H2A/H2AX by the tandem UIM of RAP80 protein (two UBDs of receptor-associated protein 80) is essential in recruiting BRCA1/BRAD1 E3 ligase complex to the foci to start DNA repair. Only then could BRCA1/BRAD1 E3 ligase ubiquitinate chromatin to initiate DNA repair (Al-Hakim et al., 2010; Brzovic et al., 2006; Husnjak and Dikic, 2012; Sims and Cohen, 2009). A recent study further authenticated the crucial role of UBDs in accumulating repair proteins to the damaged chromatin. Tandem UBDs of E3 ligases involved in DNA damage response pathway, RNF168 and RNF169, are indispensable for recruiting proteins to start DNA repair (Doil et al., 2009; Mailand et al., 2007; Panier et al., 2012). Similarly the Ub -binding motifs (UBM) of TLS (Translesion Synthesis polymerases) are essential in binding mono-ubiquitinated proliferating cell nuclear antigen (PCNA), and recruiting the TLS polymerases to the stalled replication foci (Bienko et al., 2005; Bomar et al., 2010; Panier et al., 2012).

1.7 Ub signal termination

Similar to other PTM processes, ubiquitination can be reversed by the family of deubiquitinating enzymes or DUBs. DUBs specifically cleave the amide bond formed between the Ub Gly76 residue and the Ɛ-amine group of substrate lysine residue in an ubiquitinated protein. DUBs function by removal of Ubs to rescue the target protein from degradation, editing the polyUb chains attached to substrate proteins, or processing Ub precursors to maintain the

normal pool of Ub in the cell. Several DUBs are also associated with the 26S proteasome to remove the unanchored polyUb chains (Amerik and Hochstrasser, 2004; Clague et al., 2013; Nijman et al., 2005; Xiao et al., 2016).

Approximately 100 DUBs have been reported in the human proteome, divided into two major sub-classes based on the enzymatic cleavage mechanism: the family of cysteine proteases and the family of zinc-dependent metalloproteases. The cysteine proteases include Ub specific proteases (USPs), Ub C-terminal hydrolases (UCHs), Machado/Joseph protein domain proteases (MJD), and ovarian tumor proteases (OTUs) (Nijman et al., 2005). The cysteine protease family of DUBs harbors a conserved catalytic domain containing a catalytic trio of Cys, His and Asp (or Asn) residues that perform Ub cleavage. Some DUBs also possess N-terminal and C-terminal extensions in addition to the catalytic domain, bearing other functional protein domains to regulate catalytic activity, substrate specificity, and mediate the protein-protein interactions to accomplish a specific function.

DUBs regulate important cellular pathways including cell-cycle progression, chromatin remodeling, DNA repair, signal transduction, and apoptosis. DUBs mediated timely removal of excess Ub signals that control the dynamics of Ub-mediated cellular events. Thereby, DUBs play a crucial role to counterbalance the general E3 ligase activities to maintain cellular homeostasis (Amerik and Hochstrasser, 2004; Clague et al., 2013; Nijman et al., 2005; Xiao et al., 2016).

Part II: The structure and function of the Ub E3 ligase, HUWE1

This dissertation focuses on the characterization of a specific human Ub E3 ligase, HUWE1. A general literature review of the structure and function of HUWE1 is provided in the second part of the introduction.

1.8 HUWE1 Ub E3 ligase

1.8.1 HUWE1 Ub E3 ligase function

HUWE1 is a large multi-domain protein. Encoded by an X-linked gene, it has been implicated in the regulation of multiple cellular processes including apoptosis, DNA repair, and transcription through modulating the function and stability of its substrate proteins. The *HUWE1* gene is located on the short arm of the X-chromosome (Xp11.22) and the protein is conserved from Yeast to humans. Total genetic loss of *HUWE1* in mice is embryonic lethal, underscoring its essential role in embryonic development. A tissue-specific *HUWE1* loss by conditional knockout in mice neuronal and glial cells, or *HUWE1* loss in the mouse cortex, resulted in impaired N-Myc ubiquitination and elevated N-Myc levels, defective programming and differentiation of neural progenitor cells. The *HUWE1* loss was also shown to increase p53 levels in mice brains, which in turn affects cell fate by fine-tuning between cell proliferation and apoptosis (D'Arca et al., 2010; Orivoli et al., 2016; Zhao et al., 2009; Zhao et al., 2008). Further, the *HUWE1* loss in B lymphocyte and pancreatic β cells resulted in abnormal tissue development with elevated p53 levels (Hao et al., 2012; Kon et al., 2012). All these findings suggest that HUWE1 plays an essential role in neurogenesis and tissue homeostasis. In line with the important role of HUWE1 in these physiological processes, *HUWE1* gene duplication and missense mutations in conserved functional domains were discovered in X-linked mental retardation (XLMR), with mild to severe intellectual disabilities. These mutations were found to

co-segregate with XLMR into the next generations within the family (Froyen et al., 2008; Orivoli et al., 2016; Vandewalle et al., 2013). Thus, HUWE1 is an essential protein in neurogenesis and tissue development, and loss or dysregulation of HUWE1 leads to neurological or physiological disorders.

1.8.2 Domain structure of HUWE1

HUWE1 is a large (482 KDa) multiple-domain E3 Ub ligase. It possesses a signature HECT domain at the C-terminus and a large N-terminal extension with several protein-protein interaction modules. The HECT domain was mapped at the end of the C-terminus (residues 4036-4374) that harbors the conserved catalytic cysteine for ubiquitination. Various protein-protein interaction modules were also identified upstream of the HECT domain, believed to confer substrate specificity. These include a UBA domain, a small sequence motif known to be associated with ubiquitination pathways (residues 1316-1355, PDB: 2EAA), a WWE domain predicted to be engaged in protein-protein interactions, and a small BH3 motif known to interact with apoptotic proteins (residues 1976-1990, PDB: 5C6H). Two armadillo repeat-like domains, or ARLD1 (residues 104-374) and ARLD2 (residues 424-815), were also found in the extreme N-terminus through bioinformatics, but not characterized. Recently, a PCNA interacting motif, PIP-box binding sequence (residues 3880-3887), was identified upstream of the HECT domain and found to interact with PCNA (Adhikary et al., 2005; Chen et al., 2005; Choe et al., 2016; Liu et al., 2005; Zhong et al., 2005).

The first crystal structure of the HUWE1 HECT domain (residues 3993-4374, PDB: 3H1D) was reported by Pandya *et al.,* in 2010. Similar to other HECT structures, HUWE1 HECT consists of two lobes, the larger N-lobe that binds Ub-charged E2 and the smaller C-lobe that houses the catalytic cysteine. These two lobes are connected by a flexible linker region

which is known to confer the conformational flexibility during Ub conjugation. A conserved α1 helix within the N-lobe was found to stabilize the structure and restrict self-ubiquitination (Pandya et al., 2010). Recently, another crystal structure of HUWE1 HECT domain has been published containing the same core domain as the previous one, but with an additional 42 residues flanking the HECT domain (residues 3951-4374, PDB: 5LP8, (Sander et al., 2017). In this structure, HUWE1 HECT forms an asymmetric dimer within the crystal through the residues outside of the HECT domain. This dimerization region spans residues 3951-3975, which is found to be crucial to keep the enzyme in the auto-inhibiting state. The C-lobe of HUWE1 is tethered to the dimer interface, such that the hydrophobic residues required to interact with Ub are also buried in the dimer interface. Hence, the HUWE1 dimer hinders the catalytic activity of the HUWE1 HECT domain. An "activation segment" of 50 residues upstream of the dimerization region was also identified (residues 3843-3902), which dissociates the dimer to make the enzyme conformationally active for ubiquitination. Thus, the activation segment serves as a wedge to disrupt the HECT dimer through inter- domain interactions, to activate the HUWE1 HECT domain (Sander et al., 2017). A schematic diagram of HUWE1 functional domains and the identified HUWE1 substrates is presented in Figure 1.7

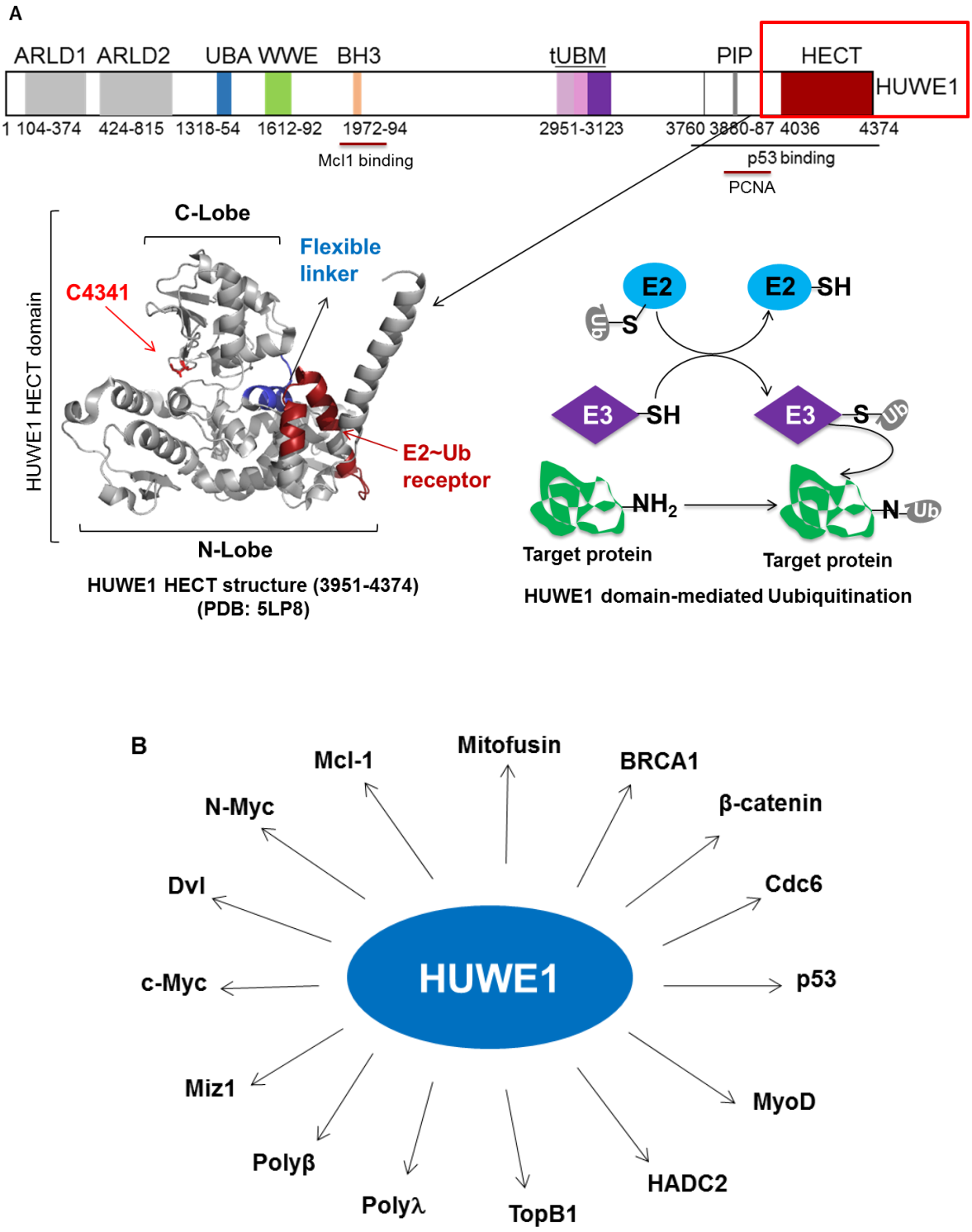

Figure 1.7 legend is provided in next page

Figure 1.7: Human HUWE1 showing functional domains and cellular substrates. (A) (Top) The domain arrangement of HUWE1. The HECT domain is located at the end of the C-terminus (residues 4036-4374) that harbors the conserved catalytic cysteine for ubiquitination. Other identified domains include UBA domain, a small sequence motif associated with ubiquitination pathways (residues 1316-1355), a WWE domain predicted to be engaged in protein-protein interactions and a small BH3 motif reported to interact with apoptotic proteins (residues 1976-1990). Three putative UBM motifs are arranged in tandem depicted as tUBM (residues 2951-3123). Two armadillo repeat-like domain sequences, ARLD1 (residues 104-374), and ARLD2 (residues 424-815) were, also found in extreme N-terminus through bioinformatics but not characterized yet. A PCNA interacting motif, PIP-box binding sequence (residues 3880-3887), is identified upstream of HECT domain. (Down left) Ribbon diagram of HUWE1 HECT domain structure (residues 3951-4374) is generated based on the published PBD file 5LP8 by *Sander et al* (2017) using PyMol. HUWE1 HECT domain is a bilobal structure, consisting of N-lobe and C-lobe, connected by a flexible peptide hinge as indicated. The E2~Ub thioester binding site in N-lobe is indicated in red helices and by a red arrow. The catalytic cysteine, C4341, is shown as red stick and indicated by a red arrow within C-lobe. (Down right) Cartoon representation of HECT domain E3 ligase-mediated ubiquitination processes. (B) Various identified HUWE1 substrates are shown.

1.8.3 HUWE1 regulates multiple cellular proteins

Many HUWE1 substrate proteins have been identified. Interestingly, HUWE1 mediated ubiquitination of tumor suppressors (such as p53, BRCA1), oncoproteins (such as c-Myc, N-Myc, Miz1 and Mcl1) as well as the proteins involved in cell cycle regulation and DNA repair (such as Cdc6, TopBP1, DNA Pols β /λ), regulating diverse cellular processes (Chen et al., 2005; Hall et al., 2007; Hao et al., 2012; Herold et al., 2008; Inoue et al., 2013; Markkanen et al., 2012; Parsons et al., 2009; Wang et al., 2014; Zhong et al., 2005). For example, HUWE1 mediates degradation of p53, a pro-apoptotic protein and Mcl-1, an anti-apoptotic protein (Chen et al., 2005; Zhong et al., 2005), resulting in opposite effects on cell apoptotic regulation, according to specific cellular contexts. Perhaps the more perplexing roles of HUWE1 were reported in the regulation of c-Myc, N-Myc, and Miz1. Adhikary *et al.* (2005) reported that HUWE1 controls the activating and repressing state of c-Myc *in vivo*. Being a transcription factor, c-Myc binds promoters either as a binary activating complex when it partners with MAX, or as a ternary repressing complex that also contains the zinc finger protein Miz1. HUWE1-mediated c-Myc ubiquitination by Lys63-linked chains was found to be essential in recruiting its transcriptional coactivator p300 to promote full activation of c-Myc transcriptional activity. Hence, Lys63-linked polyubiquitination upregulates c-Myc transcriptional activity (Adhikary et al., 2005; Walz et al., 2014). Remarkably, HUWE1 also regulates the stability of c-Myc and N-Myc via Lys48-linked chains in mouse developing brain. HUWE1-mediated Myc protein (c-Myc and N-Myc) ubiquitination and degradation were found to be essential to restrain excess stem cell proliferation and to promote differentiation in neural progenitor cells (D'Arca et al., 2010; Zhao et al., 2009; Zhao et al., 2008). Further, it is known that c-Myc represses transcription when forming a complex with its partner protein Miz1. More recent studies in a Ras-driven skin cancer

model found that HUWE1 regulates the stability of c-Myc and Miz1 through Lys48-linked ubiquitination. This study found that HUWE1 degrades Miz1 more efficiently in c-Myc/Miz1 complex in keratinocytes (Inoue et al., 2013). These findings raise more questions: Does HUWE1 cause c-Myc degradation or activation? How does HUWE1 decide which Ub linkage is needed in the regulation of c-Myc functions? Further structural and functional studies of HUWE1 are warranted to answer these questions (Myant et al., 2017; Peter et al., 2014).

HUWE1 knockout studies confirmed that HUWE1 regulates p53 in a tissue specific manner, which is essential for proper development of these tissues. Conditional knockout of HUWE1 in mouse brain resulted in neonatal lethality and cerebellar abnormalities. Conditional knockout of HUWE1 in B lymphocytes and in pancreatic β cells resulted in elevated p53 levels, and abnormal development of B lymphocytes and pancreas. These findings suggest that HUWE1 indeed regulates its substrates including p53 differentially for tissue homeostasis (D'Arca et al., 2010; Hao et al., 2012; Kon et al., 2012; Zhao et al., 2009; Zhao et al., 2008). Although HUWE1 can modify Myc proteins by two different Ub chains, it regulates p53 only by Lys48- linked polyUb chain to promote its degradation.

Previous research using a HUWE1 knockout mouse model showed that HUWE1 regulates Wnt signaling. The canonical Wnt signaling pathway (Wnt/β-catenin) regulates cell differentiation during embryonic development and preserves cellular homeostasis in many adult tissues. The pathway starts with the secreted Wnt ligands bound to the cell Frizzled (Fz) receptors. Receptors become activated and associated with the coreceptor low-density lipoprotein-related proteins 5 and 6 (LRP5/6). This recruits the cytoplasmic adaptor protein Dishevelled (Dvl) that binds directly to the membrane receptor complex, followed by phosphorylation of LRP 5/6 and multimerization of Dvl. This, in turn, recruits the multimeric

destruction complex (APC/Axin2/ GSK-3β-complex) to the membrane. This recruitment displaces the adenomatous polyposis coli (APC) from the destruction complex that regulates the stability of β-catenin, the key signal transducer of Wnt signaling in the cytoplasm. Activation of Wnt pathway causes β-catenin accumulation in the cytoplasm rendering its translocation into the nucleus. In the nucleus, it binds to the T-cell factor/lymphoid enhancer factor transcription factors (TCF/LEF) to turn on the transcription of a large number of Wnt target genes including *Axin2*, *c-Myc*, *cyclin D1* and *CD44* (Basu et al., 2016; Clevers, 2006; Clevers and Nusse, 2012; de Lau et al., 2007; Gregorieff and Clevers, 2005b; Le Guen et al., 2015; Li et al., 2015). A schematic representation of canonical Wnt/β-catenin signalling pathway is provided in Figure 1.9

HUWE1 was found to regulate Dvl, a cytoplasmic adaptor protein that served as a platform to recruit destruction complex to the receptor complex in the Wnt pathway. HUWE1 was found to ubiquitinate Dvl through Lys63-linked polyUb chains that do not destine Dvl for degradation. The consequence of this ubiquitination inhibits Dvl multimerization, and hampers the Wnt signaling activation. Another study by Dominguez-Brauer *et al.,* using a HUWE1 knockout mouse model showed that HUWE1 regulates Wnt signaling in intestinal tissue development. HUWE1 regulates the proliferation of intestinal stem and progenitor cells through controlling the function of c-Myc. c-Myc is an important Wnt target in intestine as well as the substrate of HUWE1. HUWE1 regulates c-Myc stability directly through ubiquitination and regulates c-Myc expression through modulating the Wnt signaling pathway (Clevers, 2013; Dominguez-Brauer et al., 2016; Myant et al., 2017; Peter et al., 2014).

Recent functional studies of HUWE1 HECT domain mutations in colorectal cancer (CRC) showed that inactivating HUWE1 mutations potentiate tumor initiation through increasing c-Myc and Mcl-1 proteins in an *Apc* heterozygous mouse model. APC is an important

tumor suppressor in destruction complex, which negatively regulates canonical Wnt signaling pathway via destabilization of β-catenin in the cytoplasm. Furthermore, the recent study showed that *HUWE1* loss in intestine resulted in accelerated tumor progression through up-regulation of Wnt signaling pathway in an APC^{min} genetic background (this mouse model contains constitutive *APC* mutation). These studies raise the possibility that HUWE1 may target other proteins in the Wnt signaling pathway. More research is required to explore the mechanisms that connect HUWE1 to the Wnt signaling pathway (Myant et al., 2017; Peter et al., 2014).

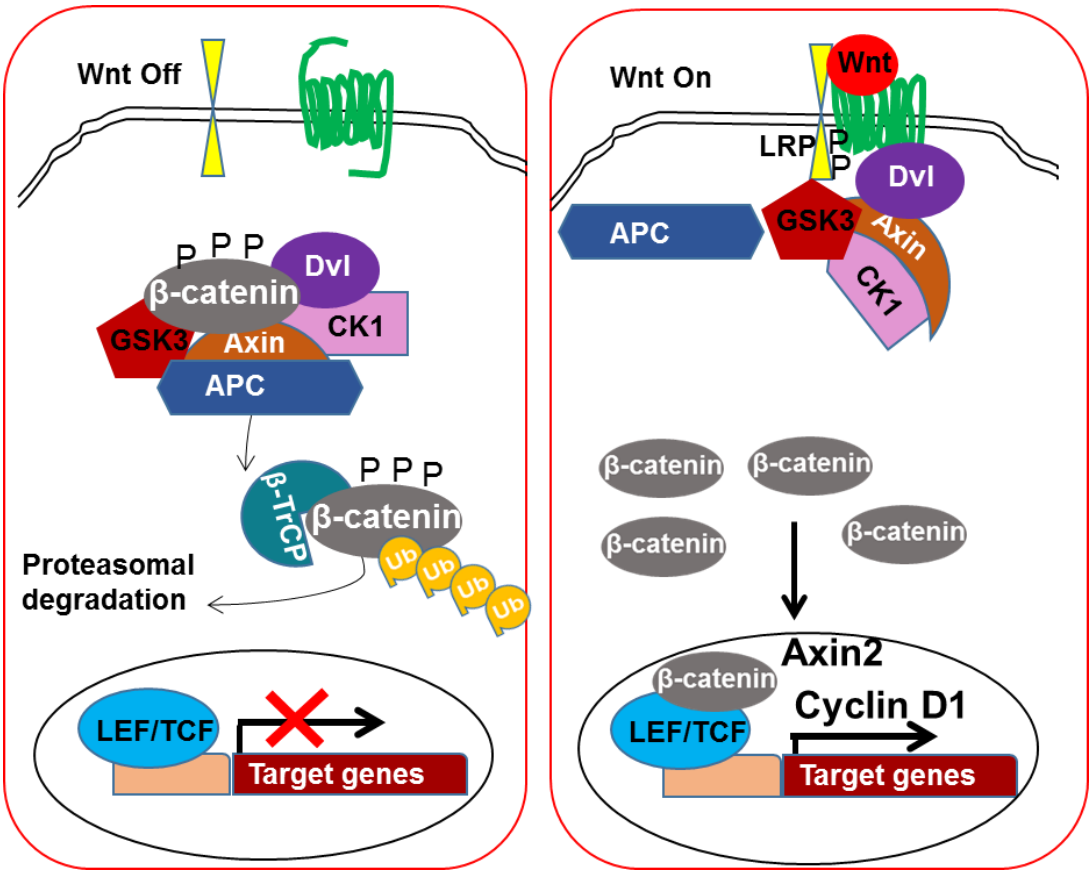

Figure 1.8: A schematic view of the canonical Wnt signaling pathway. In the absence of the Wnt ligand, β-catenin is controlled by the components of the destruction complex and subsequent ubiquitination by β-TrCP in cytoplasm (left panel). β-catenin becomes accumulated in cytoplasm and moves to the nucleus in response to Wnt ligand and regulates transcription of Wnt target genes (eg: *Axin2*, *Cyclin D1*) (right panel). This Figure is adapted and modified from (Yu and Virshup, 2014).

1.9 The proteins that regulate HUWE1 activity

HUWE1 was discovered in an H1299 cell line (a p53 null human non-small cell lung carcinoma cell) as a binding partner of the tumor suppressor ARF (alternative reading frame protein, p14ARF in human and p19ARF in mouse). The same study also showed that HUWE1-mediated p53 ubiquitination was inhibited by ARF. These results imply that ARF acted as an independent regulator of HUWE1 to keep HUWE1 function in check (Chen et al., 2005).

ARF was originally identified as an alternative transcript of the *INK4b/ARF/INK4a* gene locus. This locus also encodes two other INK4 family proteins, p15Ink4b and p16Ink4a, which interact with cell cycle-dependent kinase (CDK) and negatively regulate cell cycle progression (Quelle et al., 1995; Sherr, 2001; Sherr and Weber, 2000). ARF is predominantly a nuclear protein. It can activate and stabilize p53 in the presence of excess oncogenic signals by inhibition of MDM2, the primary p53 E3 ligase. Therefore, ARF modulates p53 activation through two distinct pathways, involving either MDM2 or HUWE1, allowing for precise control of p53 (Chen et al., 2005; Ishibashi et al., 1998). Though the molecular mechanism of the interplay between ARF-MDM2-p53 has been already established, nothing is known about the ARF-HUWE1-p53 axis. Given the fact that p53 is an important tumor suppressor and HUWE1 is overexpressed in many primary tumors containing the *TP53* wild-type gene, it is important to elucidate the mechanistic details about how ARF inhibits HUWE1 activity. A schematic representation of ARF regulated p53 activation pathway is provided in figure 1.9.

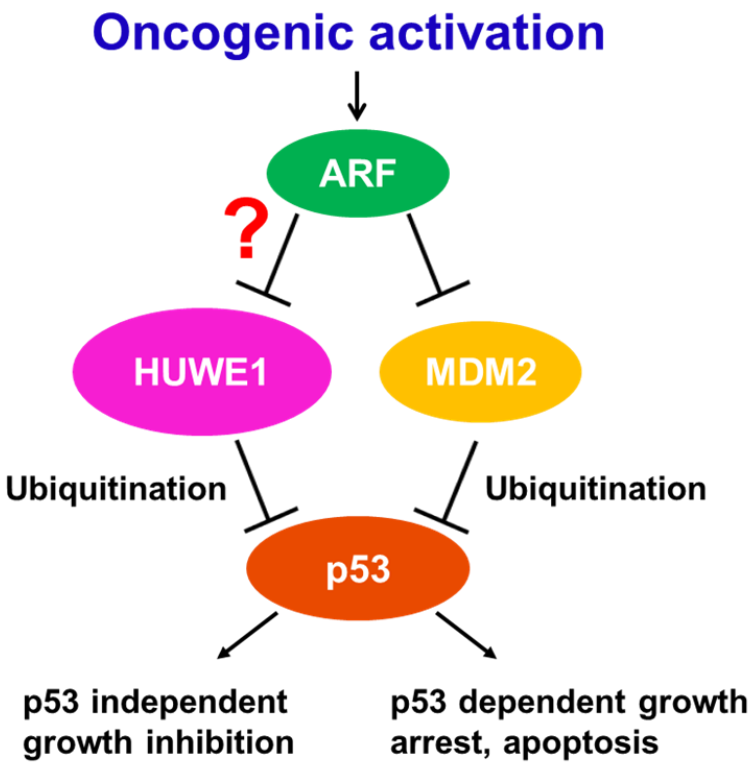

Figure 1.9: A schematic view of the ARF-mediated p53 activation pathway: In response to oncogenic signals, ARF physically binds and sequesters MDM2 (other E3 ligase of p53) to the nucleolus. Thereby p53 become liberated from MDM2 inhibition and is able to execute its normal function. ARF also directly binds and inhibits HUWE1-mediated p53 ubiquitination. However, the molecular mechanism is not known.

HUWE1 E3 ligase activity was also found to be regulated by the Ub -specific proteases or DUBs. Two DUBs,USP4 and USP7S (phosphorylated USP7), were shown to directly bind and deubiquitinate HUWE1. These regulations of HUWE1 were found to stabilize HUWE1 in the cell (Khoronenkova and Dianov, 2013; Zhang et al., 2011). The factors and mechanisms of HUWE1 regulation are still not well understood. Despite its important cellular function, more investigation is needed to understand the regulation of HUWE1 in different cellular processes.

Part III. Rationale and project design

Despite the current knowledge about HUWE1 roles in tumorigenesis and development, molecular mechanisms of its versatile functions are largely lacking. Therefore, our aims and objectives were to investigate the mechanistic details of HUWE1 functions, focusing on three independent goals using multidisciplinary approaches. The specific aims of this project are: (1) Characterization of HUWE1 functional domains; (2) Characterization of HUWE1 regulation by ARF; and (3) Characterization of new HUWE1 cellular substrate proteins.

Project I: Characterization of HUWE1 functional domains

This dissertation focuses on the characterization of the UBD domain of HUWE1. UBD motifs play crucial roles in the interpretation and transmission of the Ub signal by selectively recognizing specific Ub signals (mono- or polyUb chain with specific linkage). Although sequence alignment suggested the existence of three putative UBD (UBM motifs) in HUWE1, there is no information available on the structure and function of these motifs. Moreover, X-linked mental retardation mutation (XLMR), R2981H, was found to be in the UBD domain of HUWE1. No study has been done on how the XLMR mutation, R2981H, affects HUWE1 structure and function. **Therefore, this study is designed to investigate (1) the structure and function of the HUWE1 UBM motifs and their role in HUWE1 function, and (2) the**

molecular basis of R2981H X-linked mental retardation mutation located within HUWE1 UBM.

Project II: Characterization of HUWE1 regulation by ARF

ARF has been shown to inhibit HUWE1-mediated p53 ubiquitination and activates p53 transcription in cancer cells. Several studies also found an inverse correlation between HUWE1 overexpression and p53 levels in many tumors. Recently, development of synthetic peptides targeting and inhibiting protein-protein interactions gained tremendous attention. Several approaches have been used to develop drug-like molecules to activate p53 in the cancer cells (Foster et al., 1999; Kim et al., 1999; Midgley et al., 2000; Rousselle et al., 2000; Selivanova et al., 1999; Wang et al., 2000; Wasylyk et al., 1999). Because ARF inhibits HUWE1-mediated p53 ubiquitination *in vitro* and *in vivo*, ARF-derived peptide may also act to antagonize the E3 ligase activity of HUWE1 and rescue p53 function in the cell. **Therefore, this study aimed to investigate the mechanism of ARF-mediated HUWE1 inhibition and to develop ARF-derived peptides that can serve as an HUWE1 inhibitor to block HUWE1-mediated p53 ubiquitination.**

Project III: Characterization of new HUWE1 cellular substrate proteins

Recent studies in an HUWE1 knockout mouse model showed that HUWE1 regulates Wnt signaling in intestinal tissue development. HUWE1 was found to regulate Dvl, an adaptor protein in Wnt signaling that eventually terminates the Wnt signaling cascade. However, a recent study showed that *HUWE1* loss in intestine upregulates Wnt signaling in an APC mutation genetic background. **As Dvl acts upstream of APC, we hypothesize that HUWE1 may regulate more substrate proteins in the canonical Wnt signaling pathway**. **Therefore, this project is designed to investigate the new HUWE1 substrates in the Wnt signaling pathway.**

In this dissertation, Chapter 2 describes the work related to the identification of three Ub-binding motifs, as well as the structural and functional characterization of these motifs in HUWE1 function (Chapter 2, Project I). Chapter 3 describes the mechanistic investigation of the interaction between HUWE1 and ARF, and the mechanism of ARF-mediated HUWE1 inhibition (Chapter 3, Project II). Chapter 4 describes the identification and characterization of a new HUWE1 substrate, β-catenin, which is the prime signal transducer in the canonical Wnt signaling pathway (Chapter 4, Project III). In Chapter 5, we describe the summary of our work and future directions.

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Chapter 2

Regulation of the Ub E3 ligase HUWE1 by an intramolecular tandem ubiquitin-binding motif

The results presented in chapter 2 were designed by RK and YS. The identification of motifs, all cloning's, biochemical, biophysical and cell biology works were mostly done by RK. Sahar Farhadi (a Masters student in our lab) solved the NMR structure of UBM1. She also worked with RK to analyze the UBM1 structure and data presented in Figures 2.3, 2.4 and 2.5C. All other works were performed by RK.

2.1 Introduction

HUWE1 (HECT, UBA and WWE containing protein 1), also known as HUWE1, ARF-BP1, HectH9 and Lasu1, is a 482 KDa multi-domain E3 ligase comprised of 4374 amino acids (Chen et al., 2005; Zhong et al., 2005). Encoded by an X-linked gene, HUWE1 has been implicated in the regulation of multiple cellular processes including apoptosis, DNA repair, and cell proliferation, through modulating function and stability of its substrate proteins. HUWE1 belongs to the HECT (homologous to E6-AP C-terminus) family E3 Ub (Ub) ligases, containing a catalytic HECT domain at its C-terminus, responsible for ubiquitination of multiple important cellular regulatory proteins such as p53, β-catenin, Mcl-1, KLF4, c-Myc, and histones (Chen et al., 2005; Dominguez-Brauer et al., 2017; Hao et al., 2017; Inoue et al., 2013; Wang et al., 2014; Zhong et al., 2005). Importantly, HUWE1 is capable of catalyzing the formation of different linkage polyUb chains, which has the potential of signaling distinctive biochemical fates for the substrate proteins. For example, HUWE1 preferentially mediates Lys48-linked polyubiquitination of p53, Mcl-1, KLF4, and histones, resulting in proteasomal degradation of the target proteins. HUWE1 is also known to assemble Lys63-linked polyubiquitination on the substrate proteins Dishevelled (Dvl) and c-Myc (de Groot et al., 2014). This mode of ubiquitination is not associated with proteasomal degradation. Ubiquitination of Dvl by HUWE1 leads to the inhibition of multimerization of Dvl and downregulation of the Wnt signaling; while HUWE1 mediated Lys63-linked polyubiquitination of c-Myc, promotes c-Myc transactivation of multiple target genes (Adhikary et al., 2005; Walz et al., 2014). What confers specificity of Ub chain types by HUWE1 is not known. Characterization of the structural determinants and factors that control different modes of HUWE1 ubiquitination is important for delineating the diverse functions of HUWE1 in the cell.

The Ub-binding domains are modular units often found in proteins involved in ubiquination and the deubiquitination processes. Through non-covalent interaction with Ub, the Ub-binding domains act as the readers, and decoders of the protein ubiquitination signals, and play an important role in facilitating Ub mediated cellular events (Hurley et al., 2006; Kirkin and Dikic, 2007). From the protein sequence analysis, we and others found that HUWE1 contains two Ub-binding domains including a Ub associated domain (UBA) in the N-terminal region, and three Ub-binding motifs (UBM) in the C-terminal region upstream of the HECT domain using the bioinformatic approach (Hofmann, 2009). The UBM belongs to a special family of Ub-binding domains found in a group of the translesion polymerases (TLS) including Rev1, polymerase iota (Pol ι) and XPG (Hofmann, 2009). Each Rev1 and Pol ι contains two UBM domains at their C-terminal region, which bind to a Ub canonical hydrophobic surface defined by Ub residues Leu8, Ile44 and Val70 (Bomar et al., 2010; Bomar et al., 2007; Burschowsky et al., 2011). The UBM domains of translesion polymerases Pol ι and Rev1are required for the accumulation of these polymerases at the stalled replication foci, and are important for the functioning of Pol ι and Rev1in translesion synthesis and DNA repair. The UBM domain sequence is highly conserved from yeast to human, but not widespread in proteins (Hofmann, 2009). HUWE1 is the only other protein found to contain the UBM homology domain in Yeast to human proteomes. Despite the lack of polymerase activity, HUWE1 was shown to be part of the DNA repair machinery involving PCNA at the installed replication forks, and plays an essential role in translesion DNA synthesis and repair (Choe et al., 2016).

Germline mutations of HUWE1 have been associated with familial X-linked mental retardation (XLMR) (Friez et al., 2016; Froyen et al., 2008). In a genomic sequencing study of XLMR families, a segregating missense mutation of HUWE1, R2981H, was reported in the

affected males of a severe to profound XLMR family. HUWE1 R2981 is highly conserved from yeast to humans, and locates within the HUWE1 UBM homology domain. As this missense mutation was absent in the control individuals and co-segregated with the respective mental retardation phenotype in the family, it was hypothesized to be deleterious to HUWE1 function and associated with the XLMR phenotype of the affected individuals. Here we report a structural and functional analysis of the HUWE1 UBM domain, and present the molecular basis for the missense mutation R2981H caused impairment to HUWE1 activity and function.

2.2 Experimental procedures

2.2.1 Cloning of HUWE1 constructs used for bacterial and mammalian cell culture studies

The DNA sequences encoding HUWE1: UBM1 $_{2951-3003}$, UBM2 $_{3004-3053}$, UBM3 $_{3054-3123}$, and tUBM $_{2951-3123}$ were amplified using the human HUWE1 cDNA as a template through standard cloning procedure. After digestion, the PCR products were ligated into the bacterial expression pGEX2TK vector (GE Healthcare) between the Nde1 and BamH1restriction sites to produce N-terminal GST-tagged UBM domains. The HUWE1 missense mutation R2981H was prepared using the QuickChange Site-Directed Mutagenesis kit (Stratagene) using UBM1 $_{2951-3003}$ as the template (indicated in Figure 2.2A). The DNA sequences encoding HUWE1 tUBM-CT $_{2951-4374}$, ΔUBM-CT $_{3124-4374}$, HECT $_{3760-4374}$, and tUBM-HECT were cloned into the mammalian pcDNA3.1 vector between the BamH1 and Xba1 restriction sites to create N-terminal FLAG-tagged HUWE1 constructs. Human HUWE1 cDNA was used as a template to produce these constructs. R2981H-CT $_{2951-4374}$ was prepared using the QuickChange Site-Directed Mutagenesis kit (Stratagene) and the tUBM-CT $_{2951-4374}$ construct as a template. The HECT, tUBM-HECT and R2981H-HECT constructs were also made in pET28a (Stratagene) vector between the Nde1 and Xba1 restriction sites to create N-terminal His-tagged HUWE1

constructs for bacterial expressions (indicated in Figure 2.6A). The correct inserts were verified by DNA sequencing.

2.2.2 Expression and protein purification

GST-tagged UBMs were expressed in *E.coli* strain BL21 (DE3; Stratagene) in the LB liquid medium. Proteins were induced using 1 mM IPTG for overnight at 16^0 C. After harvesting, cell pellets were re-suspended in a lysis buffer (20 mM Tris, pH 7.5, 500 mM NaCl, 1 mM benzamidine, 1 mM PMSF, 0.1% Triton and 10% glycerol). Cells were lysed via sonicating at 40% amplitude, 0.5 seconds on, 2 seconds off for 4 min in ice. Clear supernatants were obtained by centrifugation at 17,000 rpm at 4^0 C. Clear supernatants were incubated with freshly prepared glutathione agarose beads (GE Healthcare) for 1 hr at 4^0 C in a rocker. After binding, beads were washed 5 times with washing buffer (lysis buffer without Triton) and eluted using reduced glutathione (50 mM Tris, pH 7.5, 500 mM NaCl, 30mM GSH, 1 mM benzamidine, 1 mM PMSF, and 10% glycerol). For His-tagged proteins, constructs were expressed in *E.coli* strain BL21 (DE3) in LB liquid medium and induced the same as described earlier. Cell pellets were lysed in the same lysis buffer supplemented with 10 mM imidazole. Clear supernatants were obtained by centrifugation and incubated with fresh Ni^{2+}-NTA beads for 1 hr at 4^0 C in a rocker. Protein-bound beads were washed 5 times with washing buffer containing 15-20 mM imidazole. Proteins were eluted with elution buffer containing 500 mM imidazole.

For the $^{1}H^{15}N$ and $^{15}N^{13}C$-labelled UBM proteins, and $^{1}H^{15}N$-labelled Ub, which were used in NMR mapping and structural analysis, *E.coli* strain BL21 bearing the corresponding constructs were grown in M9 minimal medium, includingNa_2HPO_4 (42 mM), NaCl (8.6 mM), KH_2PO_4 (22 mM), $CaCl_2$ (100 µM), $ZnSO_4$, (50 µM), $MgSO_4$ (2 mM), and 1X

thiaminsupplemented with $^{15}NH_4Cl$ (0.8 g 1^{-1}) for ^{15}N labelling or $^{15}NH_4Cl$ (0.8 g 1^{-1}) and ^{13}C glucose (2 g 1^{-1}) for ^{15}N-^{13}C double labelling, respectively. Protein induction and purification were done as described earlier. GST-UBMs were dialyzed overnight in thrombin cutting buffer, and GST tags were cleaved off from the UBMs, using thrombin before NMR analysis (10 units/mg of protein for overnight in 4^0 C).

2.2.3 *In vitro* binding assays

For GST pull-down assays, purified GST-UBMs and His-Ub proteins were mixed (4:1) in the dialysis tube to a volume of 500 µl with a binding buffer and dialyzed in the binding buffer (20 mM Tris pH 7.5, 100 mM NaCl, 1 mM DTT and 5% glycerol) for 2 hrs at 4^0 C. Freshly prepared glutathione agarose was added to the dialysis tube and dialyzed for one more hour. Protein-bound beads were washed 5 times with GST wash buffer as described earlier, and the interaction complexes were eluted with reduced glutathione (GSH). UBM-Ub interaction complexes were resolved in SDS-PAGE and subjected to immunoblotting using GST (Cat # 71097-6, Novagen) and His (Cat # 34530, Qiagen) antibodies, respectively. GST alone was used as a control.

2.2.4 Fluorescence Polarization assays (FP)

GST-UBMs and GST alone were purified as described above, followed by gel filtration (size-exclusion chromatography by a Hi-Load 26/60 Superdex-75 column, GE Healthcare) in 20 mM Tris pH 7.5 and 150 mM NaCl at 4^0 C. N-terminally labelled FITC-Ub was obtained from Boston Biochem. Assay buffer (20 mM Tris pH 7.5, 100 mM NaCl, 1 mM DTT and 5% glycerol) was dispensed in a 12 well microtiter plate (1-11 wells). Most concentrated GST-UBMs were placed in well 12. A two-fold serial dilution of GST-UBM was made via transferring the equal volume of protein from well 12 to 11, 11 to 10 and so on until well 2. As a

result, well 2 had the most diluted proteins, and well 12 had the most concentrated ones. Freshly made 40 nM FITC-Ub in the assay buffer was dispensed in every well. Plates were centrifuged at 1000g at 4^0 for 5 min in at intermittent intervals in order to mix everything properly. 10 µl of the reaction mixture was added in a black 384 well FP plate in duplicates (cat # PCR-384-BK, AXYGEN®). Well 1 and 2 had the assay buffer and served as control. Polarized emitted FITC signals were recorded at 480 and 520 nm respectively, using a Synergy H4 Hybrid Reader (BioTeK®). The polarized data were plotted and one-site specific binding were analyzed using GraphPad Prism version 5.0. The binding constants/dissociation constants (K_d) were determined using non-linear regression analysis. Experiments were repeated three times for each UBM and Ub chains. Average K_d values and standard deviation were calculated from three independent experiments.

2.2.5 Chemical shift perturbation experiments

All NMR experiments were performed at 25^0 C using a Bruker AVANCE 700 MHz Nuclear Magnetic Resonance (NMR) Spectrometer equipped with a cryo- probe™. NMR protein samples were prepared in PBS buffer (pH 6.5) supplemented with 10% v/v D_2O. Chemical shift mapping of UBM was performed by observing the ^1H-^{15}N HSQC spectra of the ^{15}N-labeled UBMs (200 µl of 0.2 mM UBMs in a 3 mm NMR tube). Then ^1H-^{15}N HSQC spectra were recorded again by adding the unlabeled Ub until no changes was observed in chemical shifts. Reciprocally, ^1H-^{15}N HSQC spectra were acquired using ^{15}N-labelled Ub and unlabeled UBMs. Spectra were processed using NMRPipe. Backbone resonances of reference Ub (Vajpai et al., 2013) and UBM1 from the current study were assigned to identify the interacting residues on Ub and UBM1, respectively. The values shown in Supplementary Figure 2.S1 were calculated using the equation $\Delta_{comp} = [\Delta\delta^2_{HN} + (\Delta\delta_N/5)^2]^{1/2}$.

2.2.6 NMR Spectroscopy and data analysis for UBM1 structure

For the UBM1 structure, the assignments of ^{1}H, ^{15}N and ^{13}C resonances were obtained by an ABACUS approach using the following experiments: backbone directed resonances were assigned by standard 3D triple-resonance experiments HNCO, CC(CO)NH-TOCSY, HC(CO)NH-TOCSY, (H)CCH-TOCSY and H(C)CH-TOCSY. NOE peaks were picked using manual procedures. The restraints for backbone φ and ψ torsion angles were derived from chemical shifts of backbone atoms using TALOS. Automatic NOE assignment and structure calculations were performed using CYANA (version 2.1) through its standard protocol. The final 20 lowest-energy structures were refined within the CNS package by performing a short constrained molecular dynamics simulation in explicit solvent. Resulting structures were analysed using MOLMOL, PROCHECK-NMR, and PSVS validation software. Figures were prepared using MOLMOL and Pymol (DeLano Scientific LLC) (Grishaev et al., 2005; Guntert et al., 1997; Koradi et al., 1996; Laskowski et al., 1996)

2.2.7 Circular Dichroism (CD) Spectroscopy

GST tags were cut off from GST-UBM1 WT and R2981H UBM1 mutant proteins using thrombin as described earlier after purification. 10 µM proteins were placed in a rectangular cell with a 0.1 cm path length. Far-UV CD spectra were collected with a Jasco J-810 instrument. Spectra were obtained at a scan rate of 50 nm/min and a 1.0 nm bandwidth from the 190-250 nm regions. The baseline was corrected using the sample buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 1 mM DTT and 5% glycerol).

2.2.8 *In vivo* HUWE1 expressions and turnover assays

HEK293T cells (ATCC) were cultured in DMEM H21 (Invitrogen) supplemented with 10% FBS, and penicillin/streptomycin. Nearly confluent cells were co-transfected using vectors encoding FLAG-tagged HUWE1 and HA-Ub proteins through a polyJet transfection reagent (SignaGen® laboratories) according to the manufacturer's instructions. Cells were harvested at 48 hrs and lysed in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl and 1X protease inhibitor (Roche)). Cell lysates were sonicated for 1-3 seconds (at 10% amplitudes, 0.5 second on and 2 seconds off) and clear lysates were prepared via centrifugation at 13,000 rpm for 15 min at 4^0 C. Immunoblotting was performed for total cell lysate (TCL), and HUWE1 proteins were probed with FLAG antibody (Cat # F3165, Sigma). For turnover assays, HEK293T cells were grown as explained above and transfected with vectors encoding FLAG-tagged HUWE1. Cells were treated with 10 µg cycloheximide (CHX) (10mg/ml) for the indicated times. Cell lysates were prepared as explained above and immunoblotting was performed for TCL using the FLAG antibody (Cat # F3165, Sigma.). The protein stability was determined by the percent of HUWE1 remaining at indicated time points compared to the initial time point.

2.2.9 *In vivo* ubiquitination assays

HEK293T cells were grown as mentioned earlier and co-transfected using pcDNA3.1 vectors encoding FLAG-tagged HUWE1 and HA-Ub proteins. Cells were treated with 20 µM MG132 for 4 hrs before harvesting. Cell lysates were prepared using lysis buffer supplemented with 20 mM N-Ethylmaleimide (NEM, Cat # E3876, Sigma) and a 1X protease inhibitor, (cat # 05056489001, Roche). HUWE1 proteins were immunoprecipitated from TCL using FLAG antibody (Cat # A190-102A, Bethyl), then subjected to immunoblotting with FLAG (Cat # F3165, Sigma) and HA (Cat # H3663, Sigma) antibodies obtained from Sigma. To investigate

the effect of HUWE1 on p53, endogenous p53 proteins were also immunoprecipitated using p53 antibody (Cat # sc-99, Santa Cruz) from the same TCL, subjected to immunoblotting with HA (Cat # H3663, Sigma) and p53 antibodies (Cat # DO-1/sc-126, Santa Cruz). GAPDH (Cat # 47724, santa cruz) and Vinculin (Cat # 4650, cell signaling) antibodies were used to show the abundance of proteins.

2.2.10 *In vitro* ubiquitination assays

The *in vitro* ubiquitination assays were performed in a 20 µl reaction volume in the assay buffer using purified recombinant proteins (50 mM Tris pH 7.6, 5 mM $MgCl_2$, 2 mM ATP, 2 mM DTT and 1X protease inhibitor cocktail (Roche)). The reaction mixtures were made using 10 µM of E1, E2, E3 (HUWE1 proteins as E3 ligases) and Ub for auto-ubiquitination unless otherwise specified. For p53 ubiquitination, 10 µM of p53 was used as a substrate in the aforementioned reaction mixtures. After incubation at 30^0 C for 90 mins, the reactions were stopped with SDS-PAGE sample buffer and resolved in 7.5% SDS-PAGE gels. Ubiquitinated HUWE1 and p53 proteins were visualized and evaluated by immunoblotting using His, Ub, Fk2 and p53 antibodies respectively [His (Cat # 34530, Qiagen), Ub (Cat # P4G7 MMS-258R, Covance), Fk2 (Cat # 04-263, sigma) and p53 (Cat # DO-1/sc-126, Santa Cruz)].

2.3 Results

2.3.1 Human HUWE1 possesses three unique Ub-binding motifs (UBM)

The sequence alignment of human HUWE1 with other UBM containing proteins, namely polymerase ι and Rev1, reveals HUWE1 has three putative UBM appearing in tandem order located upstream of the HECT domain (Figure 2.1 (A & B)) (ref). We named the three UBMs as UBM1 (residues 2951-3003), UBM2 (residues 3004- 3052), and UBM3 (residues 3053-3123) respectively, and the entire region as tandem UBM (tUBM, residues 2951-3123). Importantly,

the residues essential for structural integrity, and function of the UBM domains of polymerase ι and Rev1, were also conserved in three HUWE1 UBMs (Figure 2.1B). The missense HUWE1 mutation, R2981H, reported in the inherited X-linked mental retardation patients, was located within the UBM1 domain of HUWE1 (Figure 2.1(A & B) (Froyen et al., 2008). This Arg residue (R2981 in human HUWE1) was found to be conserved in the UBM1 domains of the HUWE1 orthologs implying its structural or functional importance.

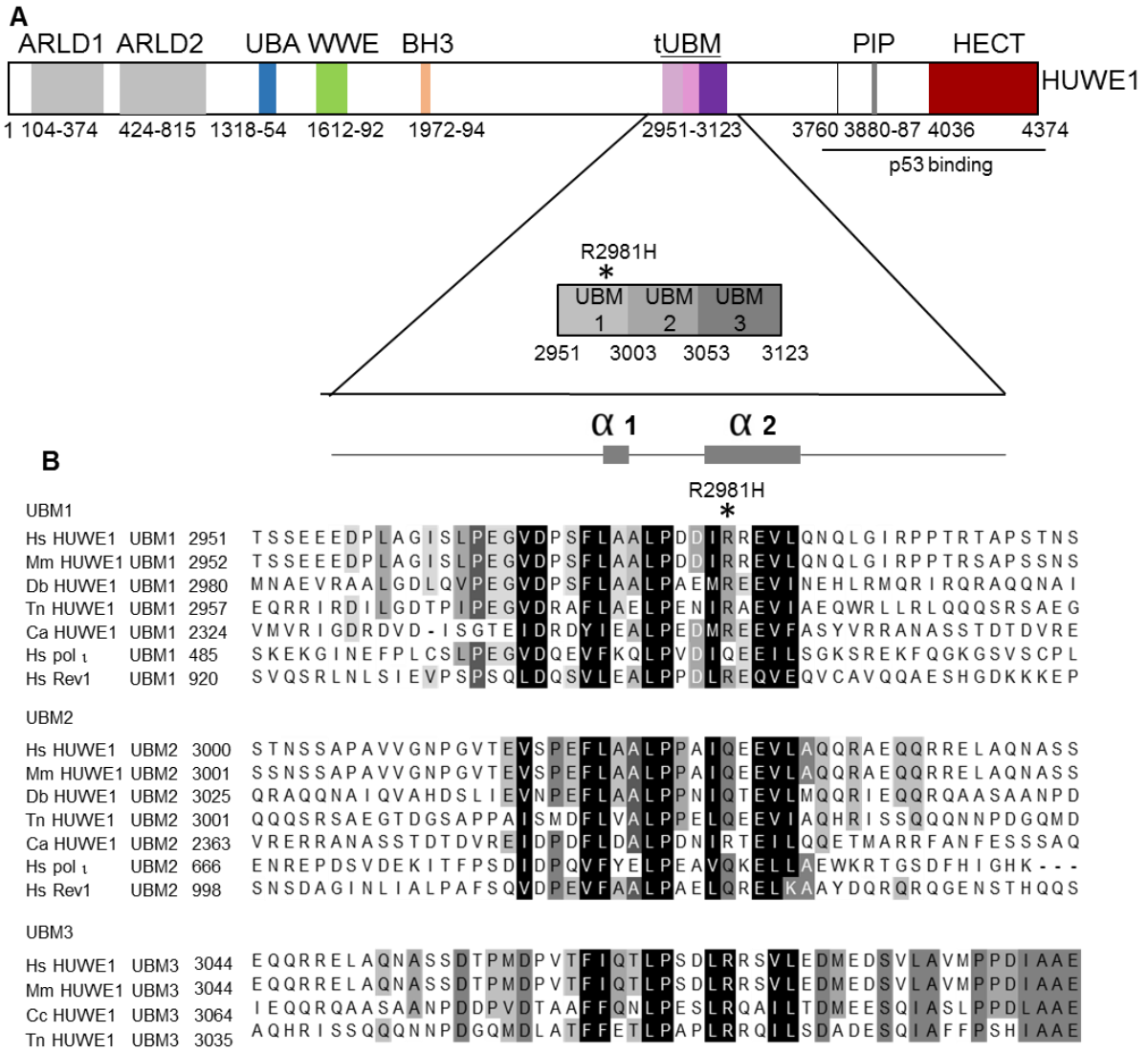


Figure 2.1: A schematic representation of HUWE1 domain structure and sequence alignment of HUWE1 UBM residues. (A) HUWE1 domains. ARLD: Armadillo-repeat-like domain 1 & 2, UBA: Ub-associated domain, WWE: protein-protein interaction domain, BH3: Bcl2-homology 3 domain, tUBM: tandem UBM domain, PIP: PIP-box domain, HECT: homologous to the E6-EP carboxyl terminus. (**B) Amino acid sequence alignment of HUWE1 UBMs.** The identical and conserved residues are highlighted in black and gray, respectively. The reported X-linked mental retardation mutation, R2981H, of human HUWE1 is indicated as an asterisk. Hs HUWE1: *Homo sapiens* HUWE1 (Ac: NP_113584.3), Mm HUWE1: *Mus musculus* HUWE1 (Ac: NP_067498.4), Db HUWE1: *Drosophila busckii* HUWE1 (Ac: XP_017850182.1), Tn HUWE1: *Trichinella nativa* HUWE1 (Gb: KRZ60489.1), Ca HUWE1: *Candida albicans* HUWE1 (Gb: KHC82168.1), Hs pol ι *Homo sapiens* polymerase ι (gene ID: 11201), Hs Rev1: *Homo sapiens* Rev1 (gene ID: 51455).

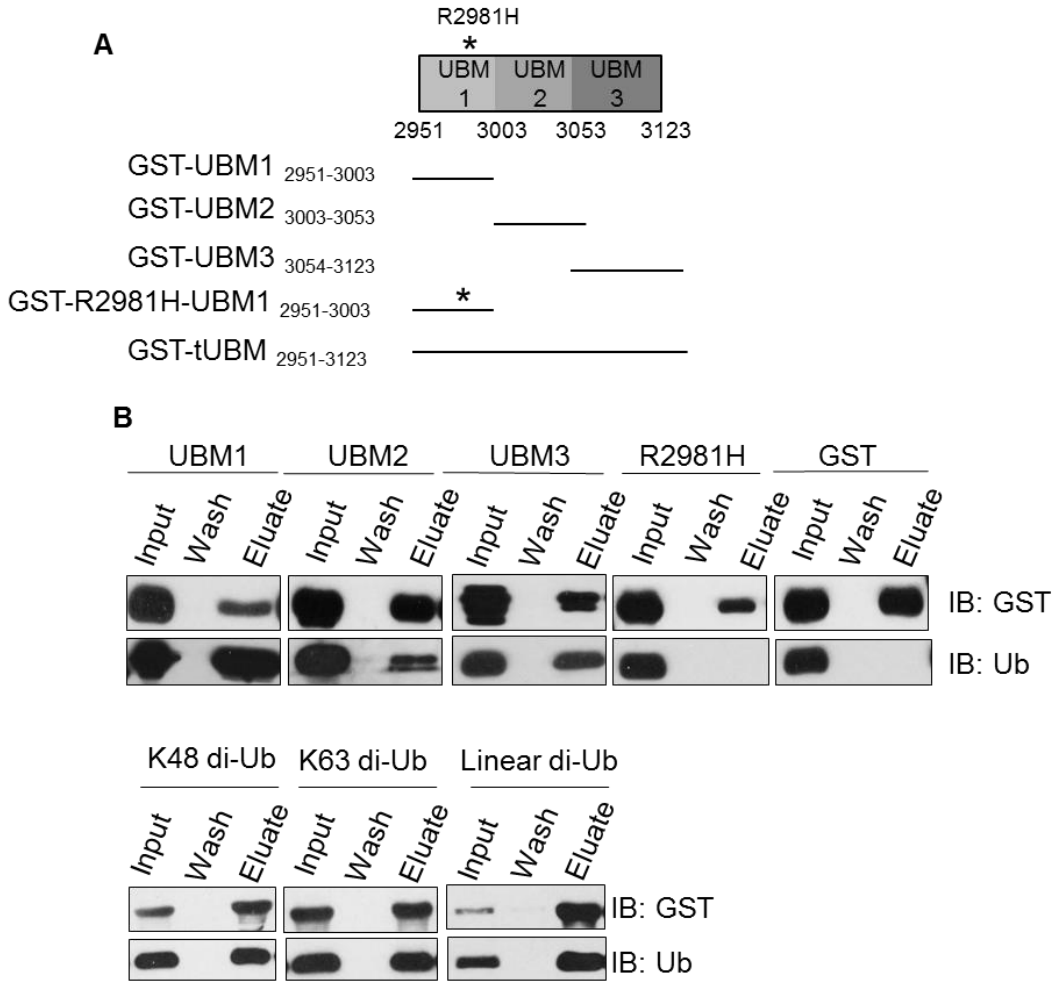
The binary interaction between three putative HUWE1 UBMs and Ub was examined using the GST pull-down assay. We expressed three HUWE1 UBMs as GST-fusion proteins and immobilized them on glutathione-Sepharose beads. The immobilized GST or UBM GST-fusion proteins were used to pull down Ub. Ub was found to co-elute with three UBM GST-fusion proteins but not GST alone, indicating direct physical interactions between HUWE1 UBMs and Ub (Figure 2.2B, upper panel). As the mutation R2981H is located in HUWE1 UBM1, we expressed the mutant UBM1 R2981H as a GST-fusion protein and examined whether this mutation affected UBM1 binding with Ub. As shown in Figure 2.2B (upper panel), UBM1 R2981H GST-fusion protein could not pull down Ub, indicating that the mutation R2981H impaired the interaction between UBM1 and Ub.

Next, fluorescence polarization experiments were performed to assess the binding affinity of UBMs with the N-terminally labeled FITC-Ub. UBM1 showed the strongest binding to Ub among all three UBMs of HUWE1 with a Kd_{UBM1} value of 55 μM, while UBM2 and UBM3 demonstrated weaker interactions (Kd_{UBM2} 164 μM and Kd_{UBM3} 305 μM). The tandem UBM bound to Ub with an affinity (Kd_{tUBM} 84 μM) similar to what was observed for the interaction between a single copy of UBM and Ub (Figure 2.2C), suggesting that tandem UBM repeats could not increase binding affinity when interacting with mono-Ub. This result is consistent with the previously published data on the RAP80 protein that demonstrates tUIM binds to single Ub with an affinity similar to single UIM (Kim et al., 2007; Sims and Cohen, 2009; Sobhian et al., 2007). We also tested the ability of the mutant UBM1 R2981H binding to FITC-Ub using fluorescence polarization, but could not detect any interaction. This is consistent with what we observed for UBM1 R2981H using the GST-pull down assay, further demonstrating that the mutant is defective in Ub binding and supporting the essential role of R2981 in structural and

functional integrity of UBM1. Taken together, HUWE1 contains three *bona fide* UBM motifs and each UBM motif is capable of interacting with Ub individually.

2.3.2 The tandem UBM motif of HUWE1 interacts with Ub chains

Emerging evidence suggests that the tandem arrangement of multiple copies of Ub-binding domain confers binding affinity for polyUb chain over mono-Ub, and show preferences to a specific polyUb chain linkage (Dikic et al., 2009; Scott et al., 2015; Weeks et al., 2009). As HUWE1 has three UBM motifs arranged in tandem (tUBM), we examined (1) whether tandem UBM interacts with polyUb chains and (2) whether it shows any linkage preferences. The GST pull-down assay was employed to study the interactions between the tandem UBM GST-fusion protein and Lys48-, Lys63-linked, or linear di-Ub chains. Tandem UBM was found to interact with all three specific linkage di-Ub (Figure 2.2B, lower panel). To further investigate the interaction between tandem UBM and different length or linkage Ub chains, the *Kd* values of tandem UBM interacted with fluorescein labeled di-Ub or tri-Ub were measured using the fluorescence polarization method. Tandem UBM preferentially interacted with tri-Ub, followed by di-Ub. The binding affinity of tandem UBM for di-and tri-Ub linked chains were more than 4-fold stronger than the affinity for mono-Ub regardless of linkage type (Figure 2.2D). Moreover, although tandem UBM interacted with all three tested linkage Ub chains, it bound more tightly to the linear (*Kd* 6 μM for di- and tri-Ub) and Lys63-linked (*Kd* 11 μM for di- and 8 μM for tri-Ub) Ub chains than the Lys48-linked chains (*Kd* 19 μM for di- and 10 μM for tri-Ub). These findings support the synergistic and cooperative binding of the three UBM motifs of HUWE1 when interacting with Ub chains, as found in UBDs of RAP80 and Yeast transcription factor Met4 (Flick et al., 2004; Flick et al., 2006; Kim et al., 2007; Sims and Cohen, 2009; Sobhian et al., 2007; Tyrrell et al., 2010).



C

UBM versus mono-Ub

UBMs	Mono-Ub*
UBM1	55 ± 6.0
UBM2	164 ± 5.0
UBM3	305 ± 8.4
tUBM	84 ± 4.0
R2981H	nd**
GST	nd**

D

tUBM versus Ub Chains

Ub chains	di-Ub*	tri-Ub*
Linear	6 ± 0.8	5.9 ± 0.2
K48	19 ± 3.0	10 ± 1.0
K63	11 ± 2.0	7.7 ± 1.0

* Results are presented as K_d (μM) values in the format of mean ± SD (n = 3).

** denotes binding not detected.

Figure 2.2 legend is provided in next page

Figure 2.2: HUWE1 UBMs interact with Ub. (A) GST-UBM fusion proteins used in *in vitro* binding assays. (B) GST pull-down assays. Equal amounts of purified GST-UBMs and mono-Ub or di-Ub proteins were incubated (I). The interaction complexes (E) were resolved by SDS-PAGE and subjected to immunoblotting using antibodies as indicated. GST alone served as control. I, W, E denote for samples from input, wash and eluate, respectively. Upper panel shows single UBM binding with mono-Ub and lower panel shows tandem UBM binding with di-Ub-linked Ub chains. (C) Fluorescence polarization assays. 40 nM FITC-Ub were incubated with increasing concentrations of UBMs. The fluorescence polarization values were detected using a Synergy plate reader. The binding constants (*Kd*) were determined by nonlinear curve fittings using Prism 5 GraphPad. (D) Fluorescence polarization assays to detect binding affinities between tandem UBM, and di-and tri-Ub-linked Ub chains. Assays were performed as described in C. The results are the mean ± SD (n=3). nd* denotes for interaction not detected.

2.3.3 NMR Solution Structure of HUWE1 UBM1

To investigate the structural basis of HUWE1 UBM-Ub interaction, we solved the solution structure of the UBM1 motif (Figure 2.3 (A & B)). The structure of UBM2 and UBM3 were not pursued due to their poor solution behavior (These two proteins did not give well dispersed ^{1}H-^{15}N HSQC spectra). The structure of UBM1 (residue 2951-3003) is well defined from the residue 2956 to 2993 with overall RMSD value of 0.8 Å and 1.2 Å for the backbone and heavy atoms, respectively in 20 structure ensemble. The statistics of the 20 structure ensemble are given in Table 2.1. The UBM1 structure consists of two helices, joined by the signature turn composed of highly conserved Leu-Pro (Leu2976-Pro2977) motif. The N-terminal helix includes the residues from Pro2970 to Ala2975, while the C-terminal helix spans from Asp2978 to Gln2988. The residues Pro2958 to Ala2960 at the N-terminus folded into a 3_{10} helix. The N-terminal helix residues Phe2972, Leu2973, Ala2974 and Ala2975 and the C-terminal helix residues Ile2980, Val2984 and Leu2985 form the central hydrophobic core. The residues Pro2958, Ile2959 and Ala2960 from the 3_{10} helix and the residues Leu2989 and Ile2991 from the C-terminal loop pack against the central core, providing additional structural support to the domain (Figure 2.3C). The topology of HUWE1 UBM1 is similar to the UBM motifs of polymerase ι (Figure 2.3 (D). Aligning HUWE1 UBM1 with human polymerase ι UBM1 (2KHU) and UBM2 (2KHW) gives RMSD values of 2.7 and 2.9Å for 32 backbone Cα atoms, despite lacking of sequence similarities between HUWE1 and polymerase ι UBMs.

To understand the molecular basis of the UBM1 and Ub interaction, we performed ^{1}H-^{15}N HSQC NMR-based binding experiments. We monitored the NMR resonance changes of the ^{15}N-labeled Ub after addition of unlabeled HUWE1 UBM1. Using the published assignments and structures of Ub (Huang et al., 2014), we were able to map the interaction surface of Ub

based on the perturbed Ub residues upon binding to HUWE1 UBM1. The most perturbed Ub residues cluster in the regions on one side of the β-sheet: Lys6-Thr9, Arg42-Glu49, and Val70-Leu73. Interestingly, Ub Lys6, Lys11, and Lys48 backbone amides, showed strong perturbation upon binding UBM1, indicating these Lys residues are affected when Ub interacts with UBM1. However, little perturbation was observed for the other four Ub Lys residues Lys27, Lys29, Lys33, and Lys63, suggesting that these lysine residues reside away from the interaction surface (Figure 2.4 (A, B, C) and 2.S1).

Next, we mapped the HUWE1 UBM1 interaction surface upon binding to Ub using the same method. The perturbed region of the ^{15}N-labeled HUWE1 UBM1 after binding to Ub includes: Val2968-Leu2973 from the N-terminal helix, Ile2980-Gln2988 from the C-terminal helix and the Leu-Pro linker between the two helices (Figure 2.4 (A, B, C) and 2.S1). The interaction mode of HUWE1 UBM1 and Ub is similar to what has been reported for the interaction of polymerase ι UBMs and Ub (Burschowsky et al., 2011). We used the complex structure of human polymerase ι UBM1-Ub (PDB: 2MBB) and UBM2-Ub (PDB: 2KHW) to build the model of HUWE1 UBM1-Ub interaction. As shown in Figure 2.4D, Ub Leu8 residue forms hydrophobic contact with the UBM1 N-terminal helix residues Val2968 and Phe2972. Ub Arg42 and Ile44 residues interact with UBM1 residues Glu2983 and Leu2976 respectively. Ub Val70 makes contact with UBM1 Val2984 and Gln2986 from the C-terminal helix. Although Ub Lys residues Lys6, Lys11, and Lys48, do not directly participate in binding UBM1, they are located contiguous to the UBM1 binding surface. This explains the resonances shift of these residues when Ub interacts with HUWE1 UBM1. To compare the Ub binding modes of the three HUWE1 UBM motifs, we further mapped the chemical shift perturbation of Ub upon binding to HUWE1 UBM2 and UBM3. As shown in Figure 2.S1 (Supplementary), the same set of Ub

residues interact with UBM2 and UBM3 as in the interaction between Ub and UBM1 (Figure 2.4A). Collectively, these results suggested that the three HUWE1 UBM motifs bind Ub in a similar mode, with the interaction surface involving the central hydrophobic core of UBMs and the canonical hydrophobic patch of Ub.

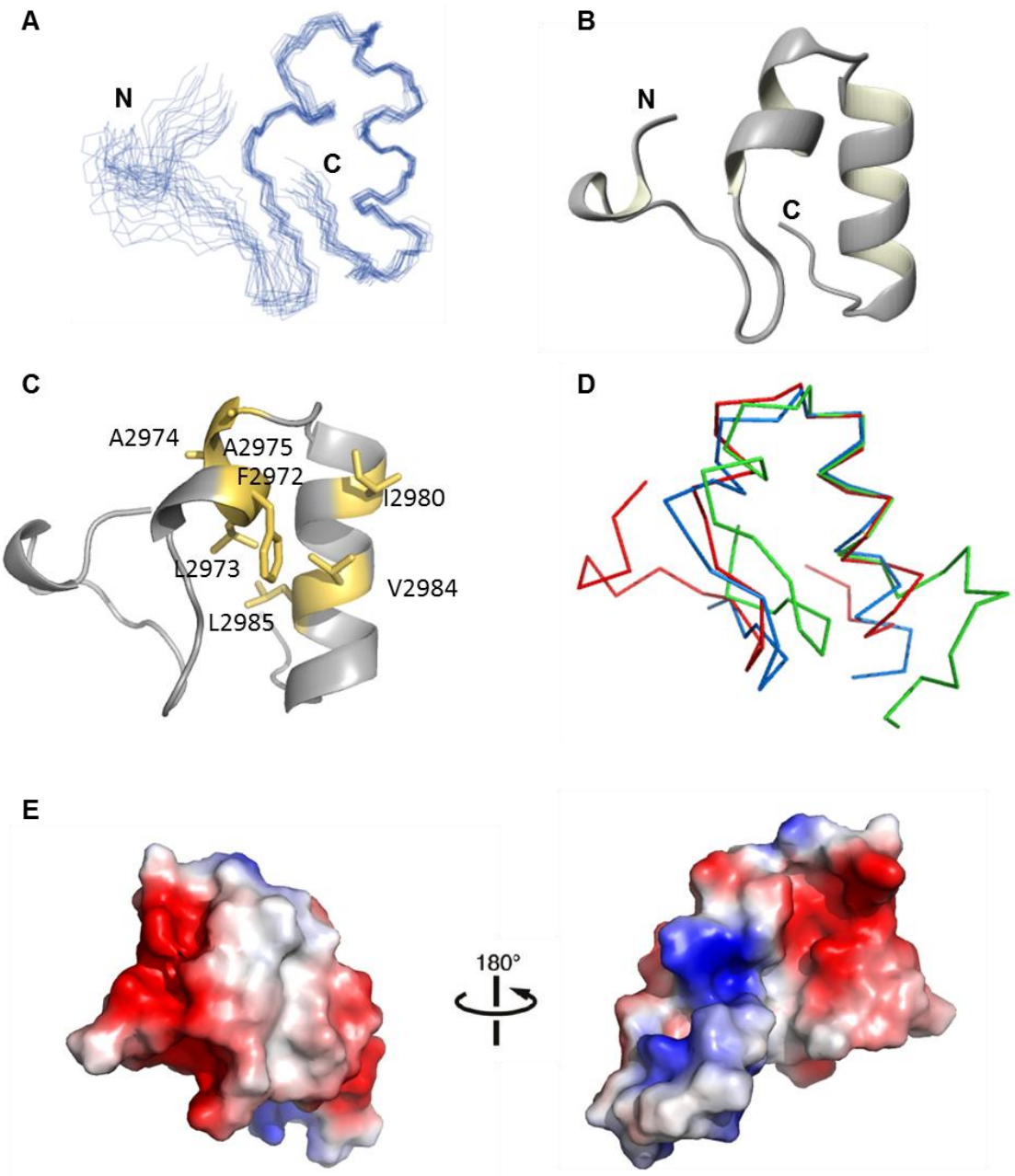


Figure 2.3: Solution structure of human HUWE1 UBM1 domain. (A) The backbone traces of the 20 lowest energy NMR structures of UBM1 (residues 2956-2993 are shown). (B) The cartoon diagram of UBM1. (C) The core hydrophobic residues of UBM1, the core hydrophobic residues are shown as yellow sticks. (D) The ribbon diagram of superposition of the human HUWE1 UBM1 (PDB: 2MUL) (red), human polymerase ι UBM1 (PDB: 2MBB), and UBM2 (PDB: 2KHW) (green and blue respectively). (E) Electrostatic surface representation of human HUWE1 UBM1.The structural diagrams are prepared using Molmol and Pymol, respectively.

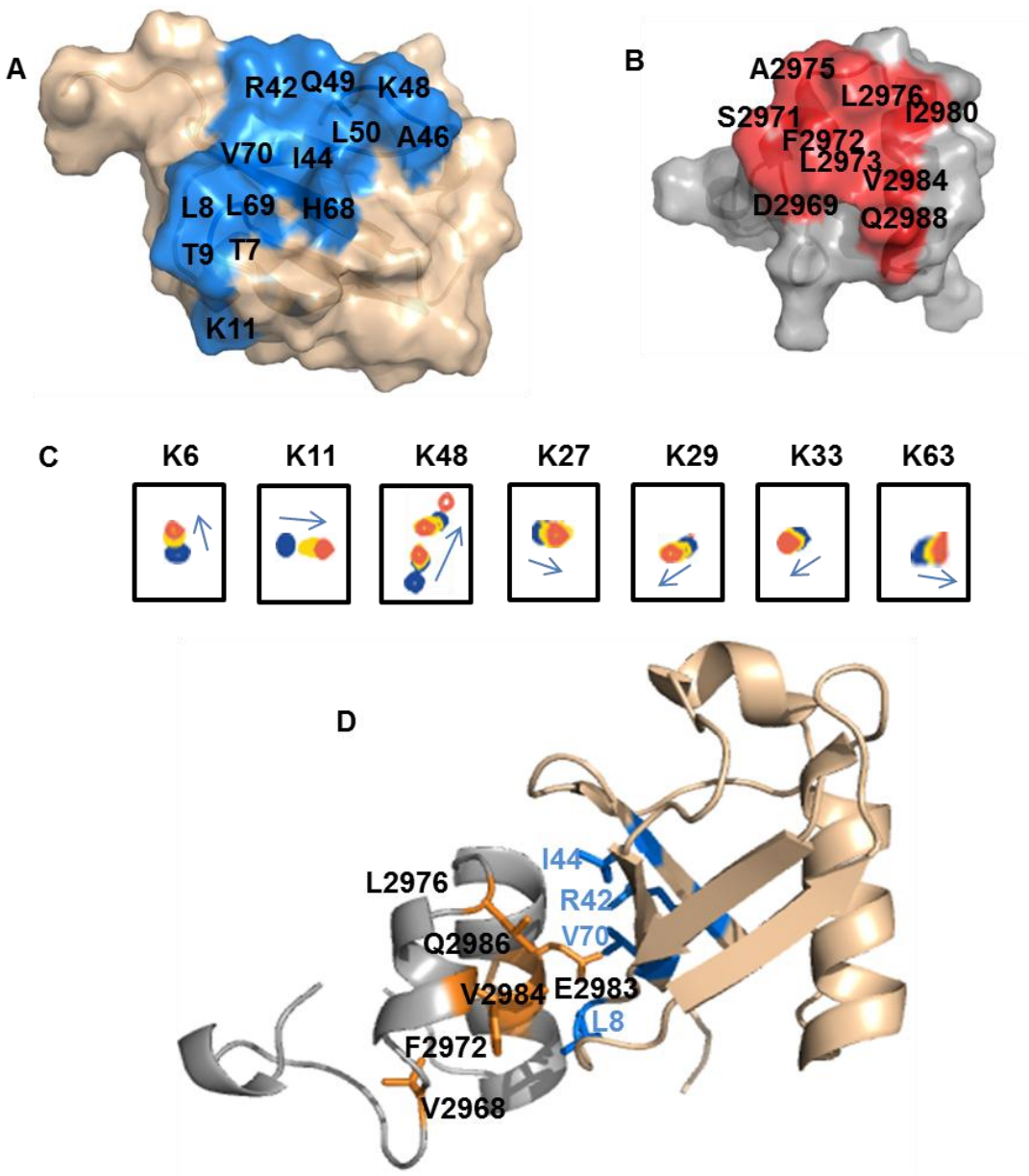


Figure 2.4: Interacting interface of Ub and UBM1 domain of HUWE1. (A) Surface representation of the Ub structure with HUWE1 UBM1 binding residues indicated in cyan. **(**B) Surface representation of HUWE1 UBM1 with Ub-binding residues indicated in red. The interactions are determined by NMR chemical shift perturbation assays. (C) Perturbations of seven lysine residues in Ub upon binding with HUWE1 UBM1 analyzed from NMR chemical shift perturbation assays. Cyan: 0 mM UBM1, yellow: 0.5 mM UBM1 and red: 1 mM UBM1. The cyan arrows indicate the directions of the chemical shift changes upon binding to UBM1. (D) The cartoon model of HUWE1 UBM1 bound with Ub, based on the complex structure of Ub-UBM1 from human pol ι (PDB: 2MBB). The residues involved in interaction between Ub (Leu8, Ile44, Arg42 & Val70; cyan) and HUWE1 UBM1 (L2976, Q2986, V2984, E2983 & V2968; brown) are shown as stick models. The diagram is generated using Pymol.

Table 2.1: Structural Statistics for HUWE1 UBM1(20 structures)

NOE distance restraints	658
Intra-residue	184
Sequential (|i-j| =1)	243
Medium-range (|i-j| ≤4)	187
i, i+2	73
i, i+3	76
i, i+4	38
Long-range (|i-j| ≥ 5)	44
Hydrogen bonds	22
Dihedral angle constraints	72
Ramachandran plot	
Favoured regions	98.8%
Allowed regions	1.2%
Deviations from ideal geometry	
Bonds	0.020 Å
Angles	1.2°
RMSD (2956-2993)	
Backbone	0.8 Å
Heavy Atoms	1.2 Å

2.3.4 R2981H mutation within UBM1 of HUWE1 is a structural mutation

We have shown that the HUWE1 UBM1 R2981H mutant was defective in binding Ub. To understand the structural basis of functional impairment in this mutation, we use the far-UV circular dichroism (CD) to compare global folding of the wild-type UBM1 and R2981H mutant. The CD spectrum of the wild-type UBM1 gives two depressions, at ~208 and ~220 nm, suggesting the helical nature of the protein. R2981H showed remarkable changes in the CD profile, lacking the two depressions found in the wild-type spectrum, indicating the mutant protein is partially unfolded (Figure 2.5A). The 2D NMR ^{1}H-^{15}N HSQC spectrum of UBM1 R2981H further supports a significant structural change of this mutant protein, implying an essential role of R2981 in the structure of UBM1 (Figure 2.5B). Analyzing the solution structure of UBM1, we found that the amide side chains of Arg2981 formed H-bond with the side chain of the residue Asp2978 (OD2) at the N-cap position of the C-terminal helix. There are more NOEs observed between side chain of Leu2973 from the N-terminal helix and the side chain of Arg2981, suggesting the residue Arg2981 is important for formation of the C-terminal helix and maintaining the overall structure stability of the motif (Figure 2.5 (C & D).

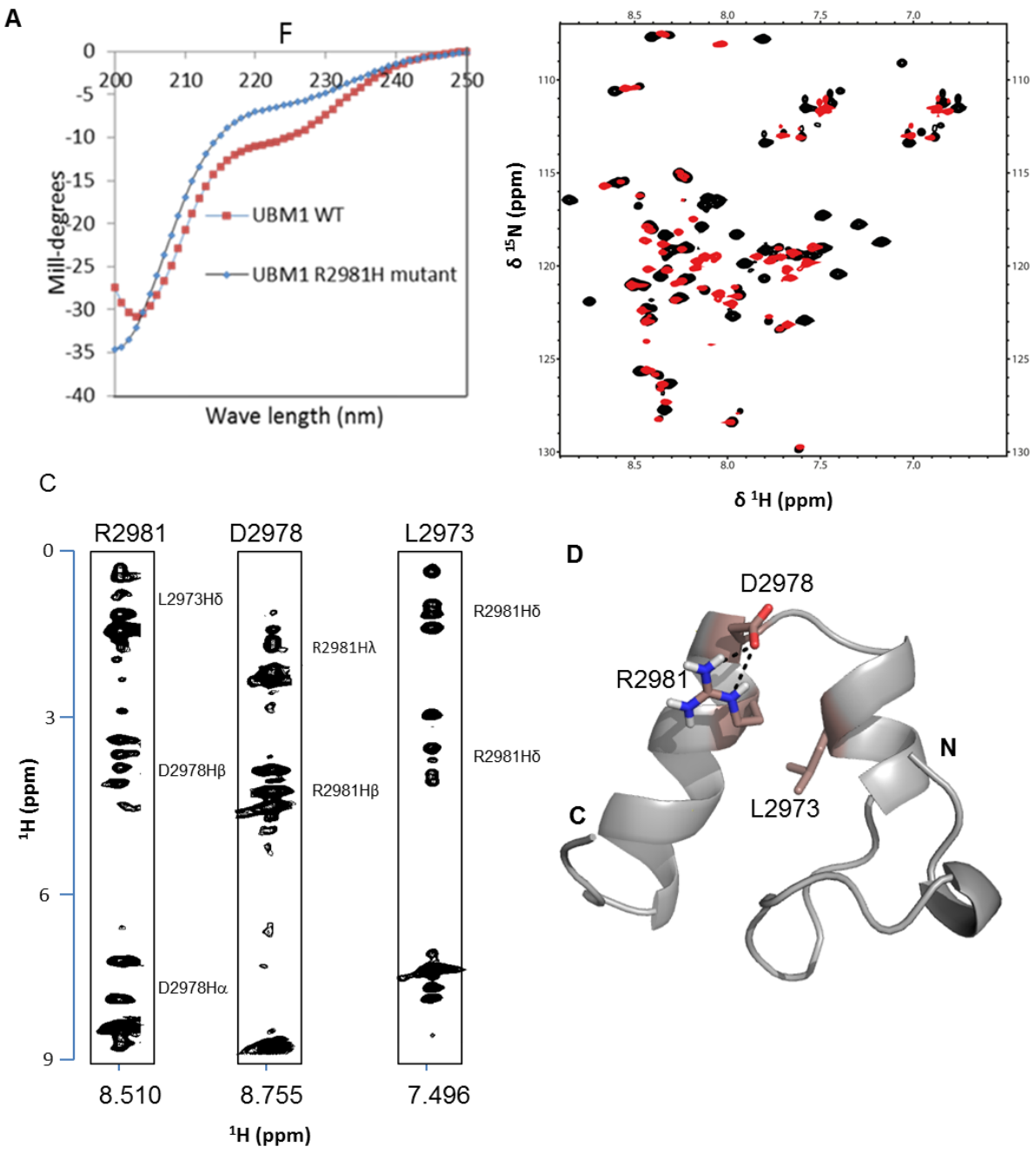


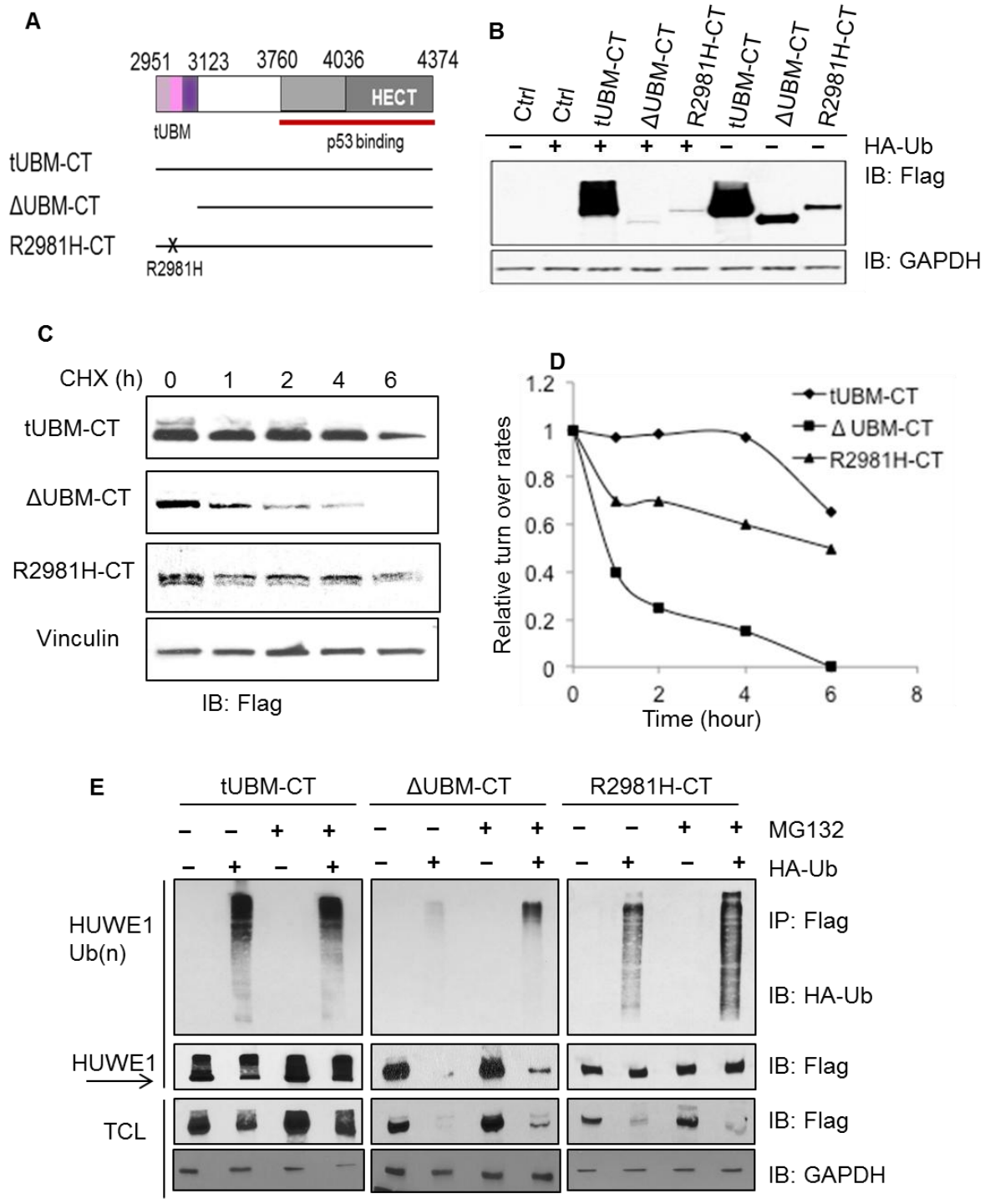
Figure 2.5: R2981H mutation in human HUWE1 UBM1 domain (2 MUL) is a structural mutant. (A) CD spectra of UBM1 WT (red square) and the R2981H mutant (blue square). (B) Overlay of ^{1}H-^{15}N HSQC spectra showing chemical shift resonances of backbone amide groups of UBM1 WT (black) and R2981H mutant (red). **(**C) The strips constructed from ^{1}H-^{1}H NOESY spectrum showing intermolecular NOEs between R2981, D2978 and L2973. (D) The cartoon representation of HUWE1 UBM1 structure showing H-bond between residues R2981 and D2978. The diagram is prepared using Sparky and Pymol.

2.3.5 Tandem UBM (tUBM) enhances HUWE1-mediated polyubiquitination

To address the functional role of HUWE1 tandem UBM in a cellular context**,** we overexpressed FLAG-tagged HUWE1 C-terminus containing wild type tandem UBM (tUBM-CT), the tandem UBM deletion mutant (ΔUBM-CT) or the R2981H mutant (R2981H-CT) in HEK293T cells (schematics of HUWE1 proteins provided in Figure 2.6A). The three constructs showed very different protein expression profiles: tUBM-CT was expressed as intense smeared bands, indicating the protein was highly modified in the cell, whereas ΔUBM-CT and R2981H-CT appeared as an unmodified single band. Co-expression of Ub in HEK293T cells destabilized ΔUBM-CT and R2981H-CT, but not tUBM-CT, suggesting that the tUBM motif protects HUWE1 from degradation (Figure 2.6B). To further examine whether tUBM affects protein stability, a cycloheximide chase experiment was carried out with ectopically expressed tUBM-CT, ΔUBM-CT and R2981H-CT. tUBM-CT was the most stable protein until 6 hours, while tUBM deletion or R2981H mutation resulted almost 60% and 40%, respectively decrease of ΔUBM-CT and R2981H-CT proteins (Figure 2.6 (C & D)). As proteins marked by Lys48-linked polyUb chains are recognized and degraded by 26S-proteasome, we asked whether tUBM protected the protein from 26S-proteasome mediated degradation. 293T cells overexpressing tUBM-CT, ΔUBM-CT and R2981H-CT were treated with MG132, a specific inhibitor of 26S-proteasome. MG132 stabilized polyubiquitinated ΔUBM-CT and R2981H-CT, but had little effect on polyubiquitinated tUBM-CT. This result suggests that intact tUBM motif may protect polyubiquitinated proteins from 26S-proteasome-mediated degradation (Figure 2.6E).

2.3.6 Tandem UBM favors Lys63-linked Ub chain formation

To examine whether tandem UBM plays a role in the linkage specificity of HUWE1-mediated polyubiquitination, we performed *in vivo* ubiquitination assays using wild-type and linkage specific Ub mutants Lys63O and Lys48O, both of which contain only one lysine residue at the indicated position, with the other six lysine residues mutated to alanine. HEK293T cells were co-transfected with FLAG-tagged tUBM-CT, or ΔUBM-CT and with HA-tagged WT, or mutant Ubs (Lys48O and Lys63O). Ubiquitinated tUBM-CT or ΔUBM-CT was detected by immunoprecipitation with a FLAG antibody and immunoblotted for HA-Ub. Consistent with the previous data, more polyubiquitined products were observed with tUBM-CT compared to ΔUBM-CT. Moreover, tUBM-CT was more efficient in producing Lys63-linked than Lys48-linked Ub chain, while no linkage difference was observed in ΔUBM-CT mediated polyubiquitination (Figure 2.6F). These findings suggesting that tUBM could affect tUBM containing E3 ligase mediated ubiquitination and linkage selectivity.



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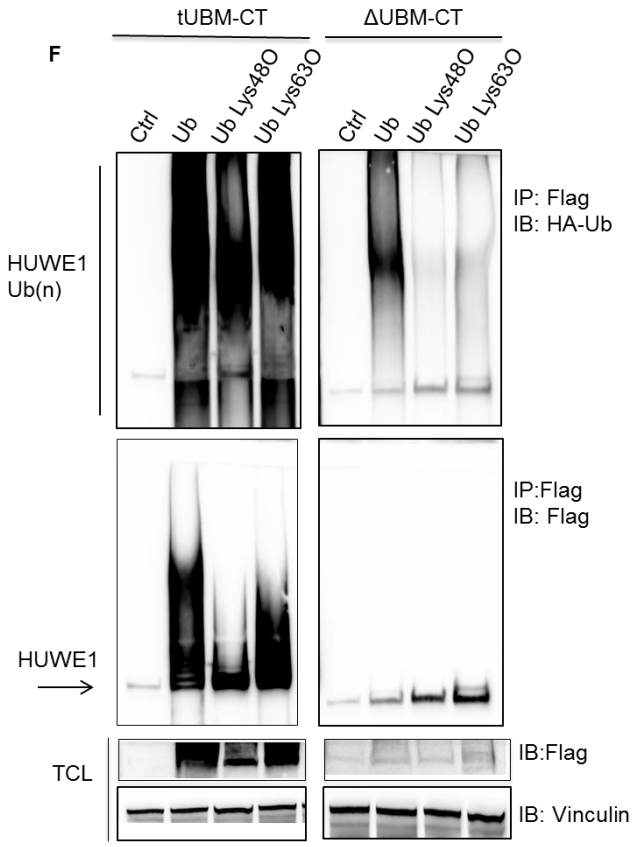
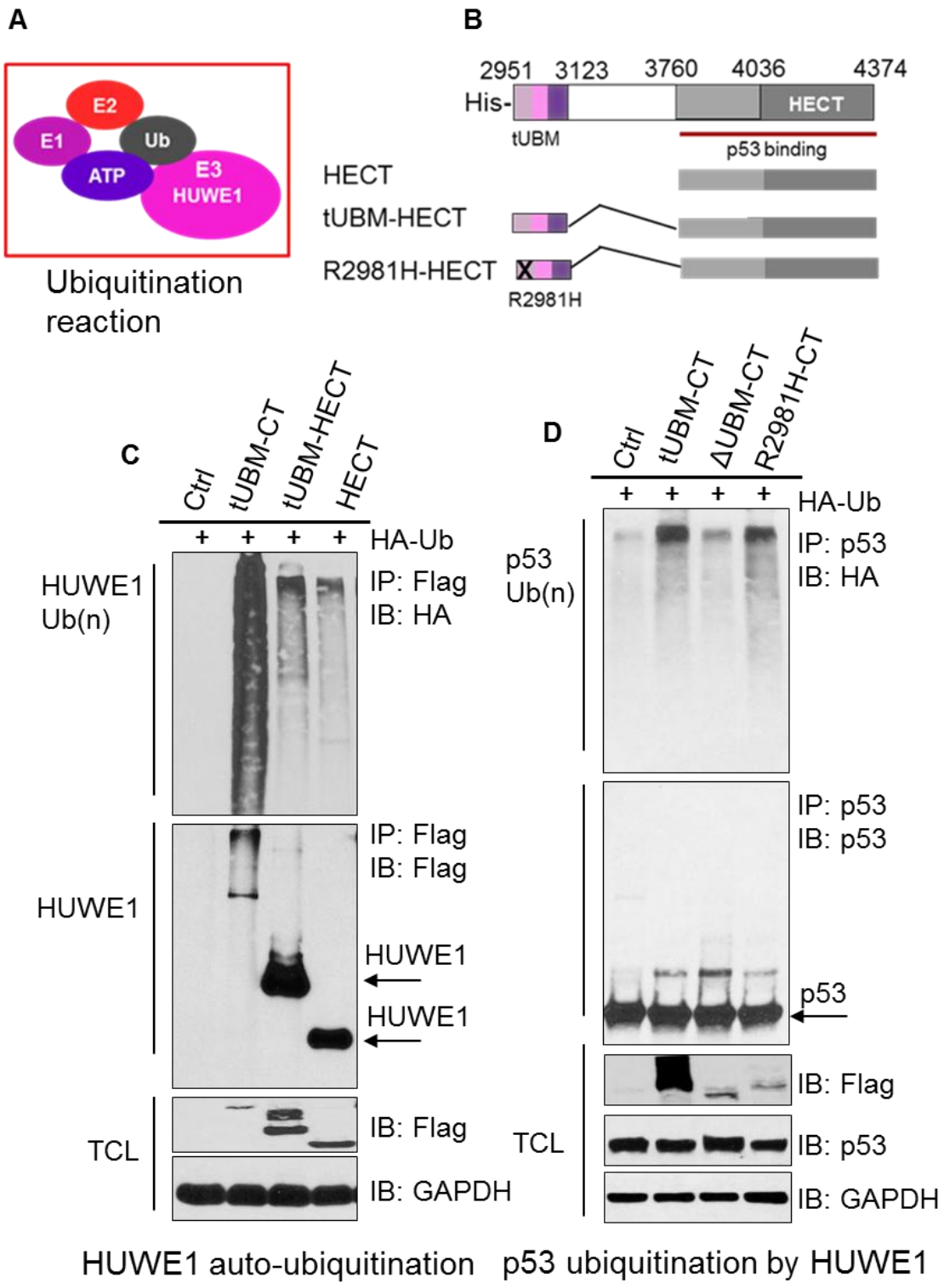


Figure 2.6: Tandem UBM enhances HUWE1-mediated polyubiquitination. (A) Schematics of HUWE1 proteins used in *vivo* ubiquitination assays. (B) Immunoblots show HUWE1 proteins are modified in cell when overexpressed with tandem UBM motif. Total cell lysates from HEK293T cells with overexpressed FLAG-tagged HUWE1 proteins and HA-Ub were subjected to immunoblotting using FLAG antibody. Empty vector served as a control (Ctrl). (C) Immunoblots show the cycloheximide chase assay of HUWE1 proteins**.** HEK293T cells were transfected with plasmids expressing FLAG-tagged HUWE1 and treated with cycloheximide (CHX) for the indicated times. Total cell lysates was immunoblotted using the indicated antibodies. Vinculin was used as the loading control. (D) Relative turnover rates of ectopically expressed HUWE1 proteins. Densitometric quantification of HUWE1 proteins were performed using Image J from the experiment C and normalized with time zero (t = 0) as 1. (E) *In vivo* auto-ubiquitination of HUWE1 proteins. HEK293T cells were co-transfected with plasmids expressing FLAG-tagged tUBM-CT, ΔUBM-CT, or with R2981H-CT mutant and HA-Ub. Cells were treated with 25 μM MG132 before harvesting. Lysates were immunoprecipitated and immunoblotted with FLAG and HA antibodies to detect ubiquitinated HUWE1. (F) *In vivo* auto-ubiquitination of HUWE1 with WT or mutant Ub. HEK293T cells were co-transfected with plasmids expressing FLAG-tagged tUBM-CT, ΔUBM-CT, and with WT HA-Ub, or the mutants Lys48 only (Lys48O) or Lys63 only (Lys63O). After treatment with MG132, cell lysates were immunoprecipitated and immunoblotted with the indicated antibodies to identify the ubiquitinated HUWE1. Empty vector was used as a control (Ctrl).

2.3.7 Tandem UBM increases the E3 ligase activity of HUWE1 HECT domain

As tandem UBM increases HUWE1-mediated polyubiquitination in cells, we asked whether tUBM acts as a *cis*-element to enhance the E3 ligase activity of HUWE1. To investigate this, we generated a fusion construct, tUBM-HECT, placing the tUBM motif directly in front of the catalytic HECT domain (3760-4374), which is sufficient for HUWE1 auto- and p53 ubiquitination. We compared the E3 ligase activity of tUBM-HECT with HECT domain through co-expression of these constructs with HA-Ub in 293T cells. Notably, a cellular ubiquitination assay showed that tUBM-HECT is more active in forming polyubiquitination than HECT domain alone, but the amount of polyUb products were less than that of tUBM-CT (Figure 2.7C). This result indicates that tUBM enhances polyubiquitination by HUWE1 HECT domain. To test whether tUBM could enhance HUWE1 HECT domain-mediated substrate ubiquitination, a cellular p53 ubiquitination assay was carried out to compare the amount of p53 ubiquitinated by tUBM-CT, the mutant ΔUBM-CT and R2981H-CT in 293T cells. tUBM-CT was the most active form in HUWE1 mediated p53 polyubiquitination, while ΔUBM-CT and R2981H-CT were less efficient in mediating p53 polyubiquitination (Figure 2.7D). To further validate the effect of tUBM on HUWE1 E3 ligase activity, an *in vitro* ubiquitination assay was performed using recombinant HECT domain, tUBM-HECT or R2981H-HECT mutant as E3 ligases. Addition of the recombinant activating enzyme E1, conjugating enzyme E2, His_6-tagged-Ub and ATP in the reaction promoted E3-specific auto-ubiquitination and allowed us to evaluate the E3 ligase activity of three HUWE1 HECT domain recombinant proteins. Consistent with the cellular effect of tUBM, tUBM-HECT was more active in mediating polyubiquitination, while R2981H-HECT showed similar activity as the HECT domain alone (Figure 2.7E). Though our structural data clearly showed that R2981H mutant (R2981H-CT) disrupt Ub-binding property and the

overall structural stability of UBM1, we observed very little effect of this mutant in *vivo* HUWE1 ubiquitination assays (Figure 2.6E). This HUWE1 protein possesses the intact UBM2 and UBM3 motifs along with the single amino acid substitution (R2981H). As these two motifs are fully still functional, it is possible that these two motifs participating in ubiquitination. Next, we examined p53 ubiquitination using recombinant HECT domain, tUBM-HECT or R2981H-HECT mutant as E3 ligases and p53 as substrate. Similar to the auto-ubiquitination results, tUBM-HECT was more active in mediating p53 ubiquitination compared to HECT domain alone or R2981H-HECT (Figure 2.7F). Taken together tUBM enhances HUWE1 E3 ligase activity and promotes HUWE1-mediated polyubiquitination.



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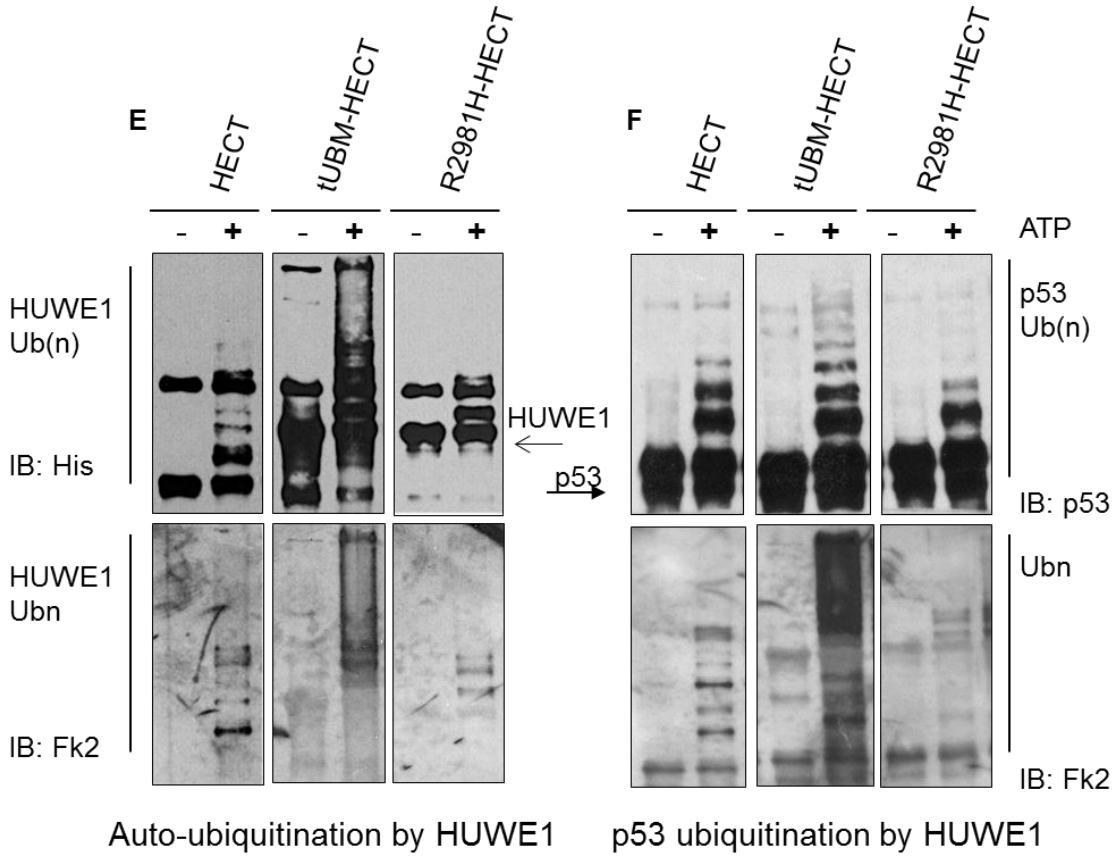

Figure 2.7: Tandem UBM enhances HUWE1 HECT domain-mediated polyubiquitination. (A) A schematic of ubiquitination reaction components. (B) Schematic representation of HUWE1 proteins. (C) *In vivo* auto-ubiquitination of HUWE1. HEK293T cells were co-transfected with plasmids expressing FLAG-tagged HECT domain, tUBM-HECT, or the mutant R2981H-HECT, and with HA-tagged WT Ub. Auto-ubiquitinated HUWE1 was immunoprecipitated with FLAG antibody and immunoblotted as indicated. Empty vector served as control (Ctrl). (D) *In vivo* p53-ubiquitination mediated by HUWE1. The assay was performed by overexpressing the FLAG-tagged-HUWE1 proteins in HEK293T cells. HEK293T cells were co-transfected with plasmids encoding tUBM-CT, ΔUBM-CT, or R2981H-CT mutant, and HA-Ub (WT). The ubiquitinated p53 was immunoprecipitated and immunoblotted from TCL using p53 and HA antibodies respectively. Vector alone served as control (Ctrl). (E) *In vitro* auto-ubiquitination of HUWE1 using purified recombinant proteins of HUWE1 (HECT, tUBM-HECT or the mutant R2981H-HECT). The ubiquitination reaction contains E1, E2, Ub, ATP, and HUWE1 proteins as E3 ligases. Ubiquitinated HUWE1s were detected by immunoblotting using His and fk2 antibodies. (F) *In vitro* p53 ubiquitination by HUWE1 proteins as E3 ligases and p53 as substrate. The p53 ubiquitination reaction contains all the proteins as explained in Figure 2.7C and purified recombinant p53 as substrate. The p53 ubiquitination was detected via immunoblotting using p53 and fk2 antibodies, respectively.

2.4 Discussion

Ubiquitin (Ub) signals are recognized and transduced by the proteins containing specialized Ub-binding domains in the cell. The uniqueness and complexity of Ub recognition by these specialized Ub-binding domains play an important role in Ub signaling and its downstream biological functions. In this study, we report a tandem Ub-binding motif identified in human E3 ligase HUWE1, which enhances HUWE1-mediated polyubiquitination through interacting with both mono- and polyUb chains. We also provide structural evidence that a HUWE1 mutation R2981H, identified in inherited mental retardation patients, impaired the function of HUWE1 through destabilizing the structure of Ub-binding motif.

The HUWE1 tandem Ub-binding motif is located the upstream of HECT domain. It contains three independently folded Ub-binding motifs. Despite of limited sequence similarity, the solution structure of the first UBM of HUWE1 resembles polymerase ι UBMs and adopts a helix-turn-helix topology. Similar to murine polymerase ι UBMs, HUWE1 UBM1 interacts with Ub canonical hydrophobic surface delineated by the residues Leu8, Ile44 and Val70. This mode of Ub interactions appears to be uniform among all three HUWE1 UBMs, as the NMR HSQC titration experiments showed that the same Ub surface was involved in all three UBM interactions (Figure 2.S1). Importantly, this mode of interaction between UBMs and the Ub provides a mechanism for Ub linkage preference of HUWE1 tandem UBM. HUWE1 tandem UBM prefers to bind linear or Lys63-linked Ub chains as these Ub chains adopt more extended open conformation with fully exposed hydrophobic surface. In contrast, Lys48-linked chains possess more closed or compact conformations with the canonical hydrophobic surface partially sequestered at inter-domain interface of the two conjugated Ub molecules (Figure 1 & 2 (Ryabov and Fushman, 2006). In order to interact with UBMs, Lys48-linked Ub chain must open to

expose the hydrophobic UBM-binding surface by repositioning of Ub. This leads to a less efficient interaction of Lys48-linked Ub chain with UBM. Indeed, our biochemical data showed that tandem UBM preferred linear or Lys63-linked over Lys48-linked di- and tri-Ub chain (Figure 2.2D).

The linkage preference of HUWE1 tandem UBM in binding Ub also affected HUWE1 mediated polyubiquitination. HUWE1 HECT domain containing upstream tandem UBM is more efficient in generating Lys63-linked Ub chains. Tandem UBM deletion or mutation resulted in less-stable HUWE1 proteins, confirming the importance of tandem UBM as a crucial determinant of HUWE1 activity and stability. HUWE1 is known to catalyze substrate specific Lys63- and Lys48-linked ubiquitination. HUWE1 mediated Lys63 polyubiquitination is essential for transcriptional activation of cMyc as wells as the negative regulation of the WNT signaling by Dvl. HUWE1 mediated Lys48-linked ubiquitination of p53, Cdc6 and KLF4 marks substrate protein for proteasomal degradation. While it is not clear how HUWE1 differentiates two types of ubiquitination, the role of HUWE1 tandem UBM in different HUWE1 mediated substrate ubiquitination warrants further investigation.

A protein or protein domain must fold appropriately to achieve proper conformation and function following synthesis. Our structural and biophysical data established that R2981H mutation that lies within UBM1 motif is a structural mutant. It impairs the structural fold by destabilizing C-terminal helix within the structure. This notion is supported by the CD and NMR ^{1}H-^{15}N HSQC which showed significant structural changes of R2981H mutant protein in secondary and tertiary levels. Consequently, R2981H disrupted the binding and affinity of UBM1 to Ub *in vitro* biochemical and biophysical assays (Figure 2.5). These findings suggest

the importance of structural stability of individual Ub-binding motif in Ub-dependent cellular processes.

Overall, our structural and biochemical studies of HUWE1 tandem UBM presents a regulatory function of tandem UBM on HUWE1 E3 ligase activity, the mode and specificity of HUWE1 mediated polyubiquitination. This can be achieved through several mechanisms. First, tandem UBM appears as the *cis*-element upstream of the catalytic HECT, which could serve as a Ub hub that recruits free Ub to enrich effective Ub concentration in the catalytic center of HUWE1. Second, tandem UBM could bind the nascent growing Ub chains and prevent it from being degraded by 26S-proteasome or Ub specific proteases. Lastly, tandem UBM interacts with the Ile44-centered hydrophobic surface of Ub, which could result in discrepancies in access of different lysine residues of acceptor Ub. The interaction of tandem UBM and acceptor Ub will make Lys63 more accessible than Lys48 for conjugation, which will prime the substrate for Lys63-specific ubiquitination (Figure 2.8). In conclusion, our study presents structural and functional characterization of a new tandem UBM, which modulates HUWE1 E3 ligase activity and is critical for the overall function of HUWE1.

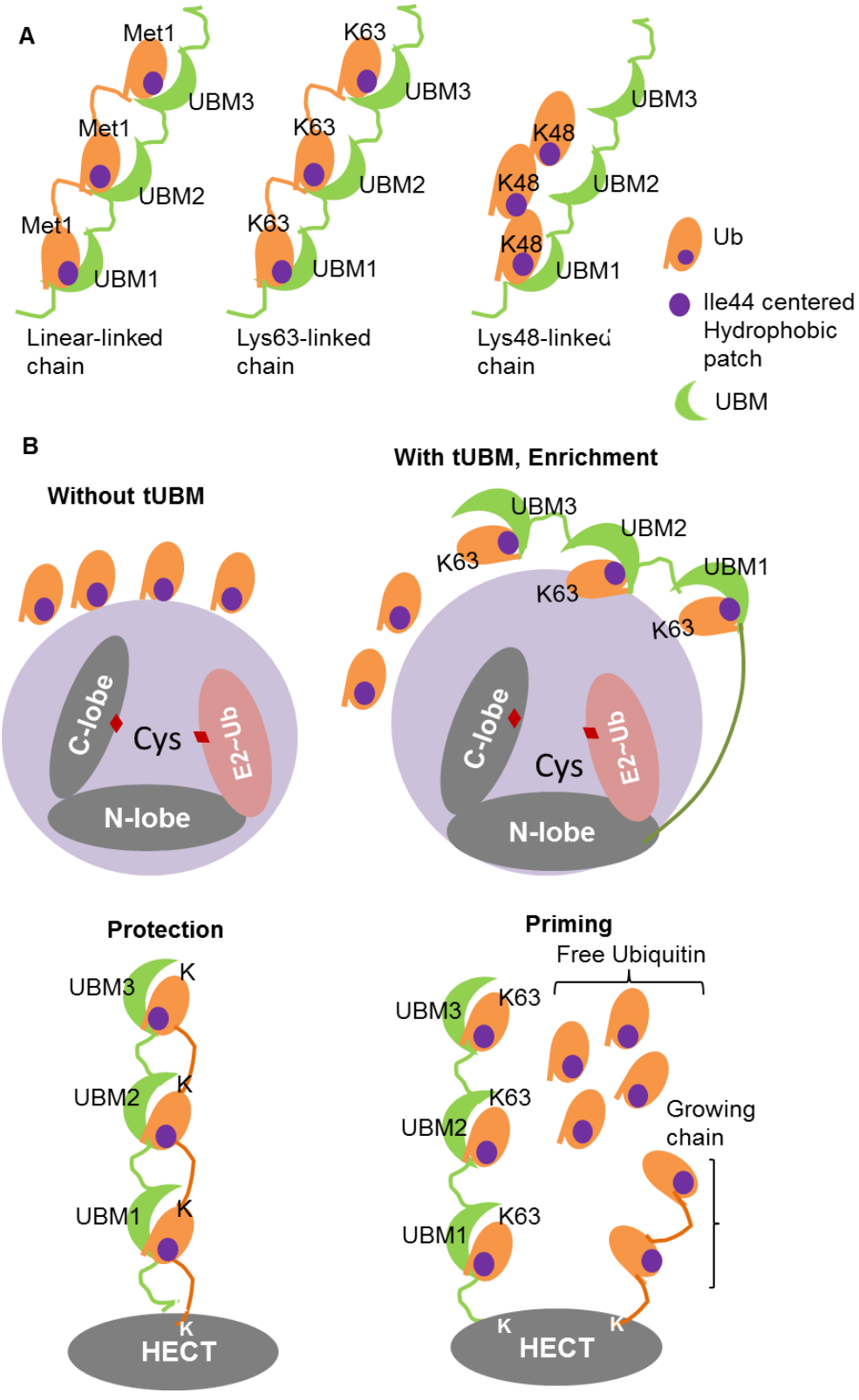

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Figure 2.8: Schematic models for tandem UBM interactions with Ub chains and the role of tandem UBM in HUWE1-mediated ubiquitination. (A) Schematic representations of HUWE1 tandem UBM interactions with different Ub-linked Ub chains. (B) Schematic models describing the roles of tandem UBM in HUWE1-mediated polyubiquitination. Tandem UBM plays a regulatory role in modulating HUWE1 E3 ligase activity, the mode and specificity of HUWE1-mediated polyubiquitination. The results suggest that tandem UBM appears as the *cis*-element upstream of the catalytic HECT, which could serve as a Ub hub that recruits free Ub to enrich effective Ub concentration in the catalytic center of HUWE1. The catalytic centre is indicated as purple circles in upper panel in Figure B. Tandem UBM also could bind the nascent growing Ub chains and prevent it from being degraded by 26S-proteasome. Tandem UBM interacts with the Ile44-centered hydrophobic surface of Ub, which could result in discrepancies in access of different lysine residues of acceptor Ub.

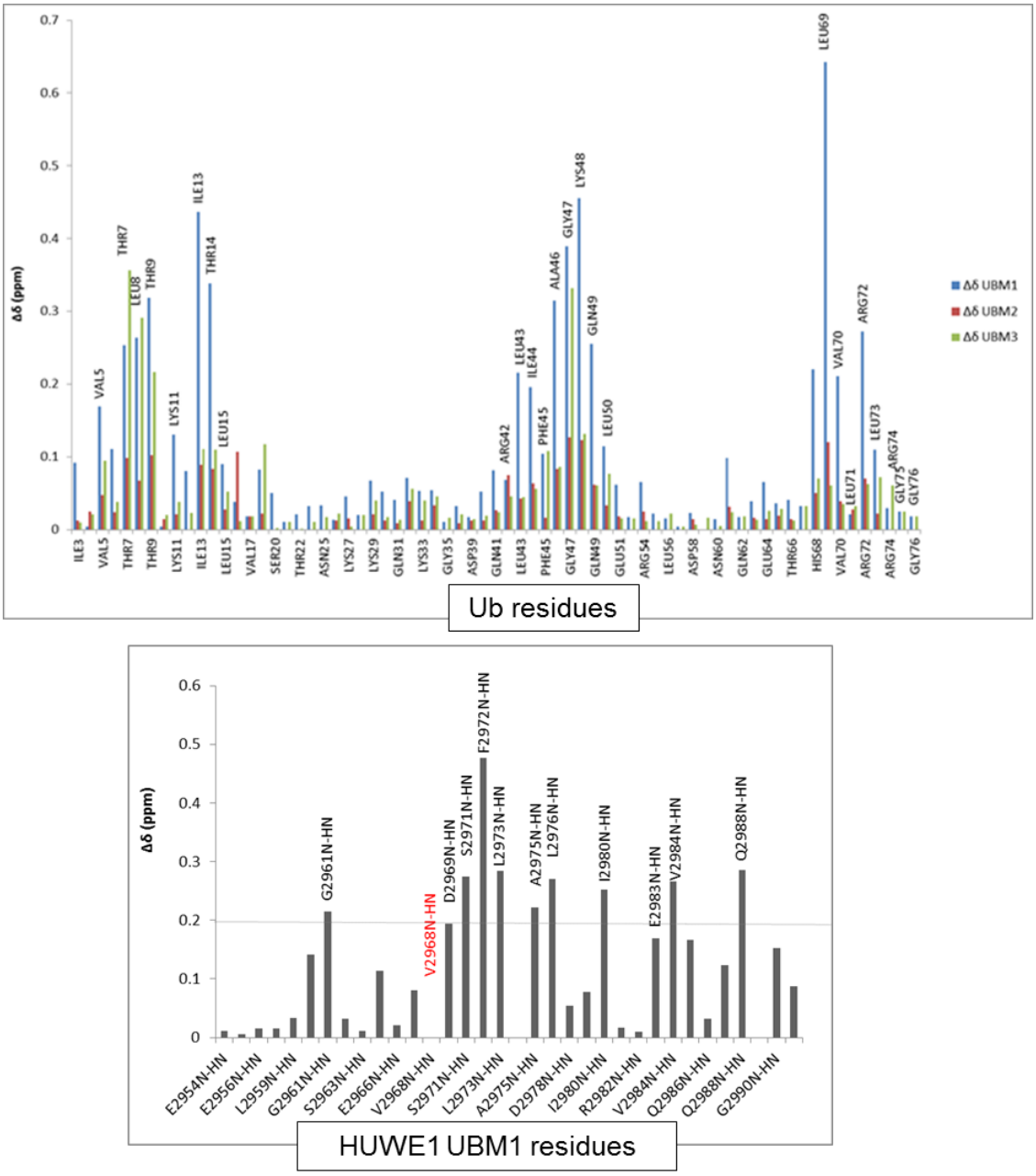

Figure 2.S1: Mapping Ub-UBM binding interface using 2D HSQC NMR. Upper panel: Composite chemical shift changes (Δδcomp) versus residue number of Ub upon binding with UBM1, UBM2 and UBM3. Blue, red and green denote for chemical shift for UBM1, UBM2 and UBM3 respectively. Spectra were processed using NMRPipe software. The backbone resonances of reference Ub and UBM1 (lower panel) from the current study were assigned to identify the interacting residues on Ub and UBM1, respectively. The values shown were calculated using the equation $\Delta_{comp} = [\Delta\delta^2_{HN} + (\Delta\delta_N/5)^2]^{1/2}$. Lower panel: 2D ^{1}H-^{15}N HSQC spectra were acquired for ^{1}H-^{15}N UBM1 and unlabeled Ub as described above.

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Chapter 3

Molecular basis of human ARF-mediated inhibition of ubiquitin E3 ligase HUWE1

The results presented in chapter 3 were designed by RK and YS. Except for the Mass Spectrometry data presented in Figure 3.5A, all other experiments were done by RK. The MS data was performed by SZ, a PhD student from Dr Wilson lab.

3.1 Introduction

Alternate Reading Frame protein (p14ARF in human and p19ARF in mice) was originally identified as an alternative transcript of the *INK4b/ARF/INK4a* gene locus. This locus also encodes two other INK4 family proteins, $p15^{Ink4b}$ and $p16^{Ink4a}$, which interact with cyclin dependent kinase (CDK) to negatively regulate the cell cycle progression. ARF shares the coding sequence of exons 2 and 3 with *INK4a*, but expresses from a separate promoter with a distinct first exon (exon 1ß) upstream of the exon1α of *INK4a*. Therefore the ARF protein sequence is completely unrelated to that of $p15^{Ink4b}$ and $p16^{Ink4a}$ (Carrasco-Garcia et al., 2017; Chen et al., 2006; Matheu et al., 2008; Quelle et al., 1995; Sherr, 2001; Sherr and Weber, 2000). A schematic diagram of the human *ARF-INK4a* locus is provided in Figure 3.1A. ARF is predominantly a nucleolar protein. It can activate and stabilize p53 by inhibiting the ubiquitin ligase activity of MDM2, and sequestering MDM2 into the nucleolus in response to oncogenic stimuli. Thus, p53 becomes liberated and executes its tumor suppressive functions. ARF was shown to interact with another p53 E3 ligase HUWE1 (also known as ARF-BP1, Mule, HECTH9), by directly binding and repressing HUWE1 mediated p53 ubiquitination. Therefore, ARF modulates p53 activation through two distinct pathways, involving either MDM2 or HUWE1, allowing for precise control of p53 (Chen et al., 2005; Zhang et al., 1998).

The exon 1ß encodes for a conserved N-terminal region (residues, ARF_{1-64}) which is essential for MDM2 and HUWE1 binding, and the induction of p53-dependent cell-cycle arrest and apoptosis (Weber et al., 1999). ARF_{1-64} was also shown to be essential to interact with other ARF targets such as E2F1, c-Myc, FoxM1b and E1A onco-proteins, thereby suppressing their transcriptional activity and cell proliferation (Datta et al., 2004; Eymin et al., 2001; Gusarova et al., 2007; Iaquinta et al., 2005; Kalinichenko et al., 2004; Martelli et al., 2001; Shen et al., 2011;

Weber et al., 1999). Previous research suggests that ARF utilizes different sequence motifs to target and regulate its interacting proteins. While ARF residues 1-25 (ARF_{1-25}) were shown be essential in binding MDM2, residues 26-37 (ARF_{26-37}) were indispensable for nucleolar localization of MDM2 and p53 activation. Notably, extreme N-terminal residues 1-14 (ARF_{1-14}) were also shown to be the key site for binding and targeting c-Myc protein into the nucleolus (Datta et al., 2004; Weber et al., 1999). Further, a forkhead box family Transcription factor FoxM1b was found to bind ARF between residues 26-44. These residues (ARF_{26-44}) were found to be sufficient to bind and inhibit FoxM1b-mediated transcriptional activities. Additionally, a membrane transducing synthetic ARF peptide based on this ARF_{26-44} sequence inhibited both FoxM1b driven transcriptional activity and anchorage-independent cancer cell growth (Gusarova et al., 2007; Kalinichenko et al., 2004). These findings support the role of ARF in tumor suppression by targeting multiple cellular regulators.

HUWE1 is a large, multi-domain HECT E3 ubiquitin ligase that was initially identified in ARF-containing protein complexes from p53 null H1299 cells (Chen et al., 2005). Further investigation revealed that it is a *bona fide* ubiquitin (Ub) E3 ligase for p53, which promotes p53 degradation through proteasome (Chen et al., 2005). The relationship between p53 and HUWE1 is further validated by the genetic studies. Conditional HUWE1 knockout in B lymphocytes was found to cause p53 accumulation, impaired B lymphocyte development, and homeostasis which was rescued via p53 knockout. Similarly, partial loss of HUWE1 in pancreatic β cells resulted in p53 elevation and β cell losses, with severe diabetic phenotypes that were also recovered by concomitant knocking out of p53 (Hao et al., 2012; Kon et al., 2012). These findings suggest that HUWE1 is directly controlling p53 to fine-tune tissue homeostasis in physiological conditions. Significantly ARF_{1-64} was shown to inhibit HUWE1-mediated p53 ubiquitination, which

stabilize and activate p53 function in an MDM2-independent manner (Chen et al., 2006). Together these suggest that HUWE1 is the major E3 ubiquitin ligase of p53 in human cells and is also a key target for ARF-mediated tumor suppression.

However, the mechanistic details of ARF mediated inhibition of HUWE1 activity have not been characterized previously. Therefore, we employed biochemical and biophysical approaches to map and identify minimal sequences of human ARF that directly bind to HUWE1. Here, we report an ARF peptide which inhibits HUWE1 activity, elevates p53 transcript levels, and suppresses cancer cell growth harboring the wild-type p53.

3.2 Experimental procedures

3.2.1 Cloning and protein purification

Expression vectors encoding His-tagged (pET15b) and FLAG-tagged HUWE1 HECT domains (pcDNA3.1) (HUWE1 $_{3760-4374}$ and HUWE1 $_{3951-4374}$) were generated according to the standard cloning method as explained in chapter 2. Expression vectors encoding GST-ARF (pGEX2TK) and YFP-ARF fusion peptides (pcDNA3.1) were generated using human full-length ARF as template. Recombinant proteins used in *in vitro* binding and ubiquitination assays were purified using standard chromatography methods as explained in chapter 2.

3.2.2 *In vitro* binding assays

For GST pull-down assays, equal amounts (500 µg) of purified GST-ARFs and His-HUWE1 proteins were mixed in a dialysis tube in 500 µl volume in a binding buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 1 mM DTT and 5% glycerol) and dialyzed for 2 hrs at 4^0 C. Freshly prepared glutathione agarose was added to the dialysis tube and dialyzed for one more hour. Protein-bound beads were washed 5 times with GST wash buffer and interaction complexes were eluted with reduced GSH. ARF-HUWE1 interaction complexes were resolved

using 7.5% SDS-PAGE and probed using GST (Cat # 71097-6, Novagen) and His (Cat # 34530, Qiagen) antibodies, respectively. GST protein was used as a control.

3.2.3 Peptide arrays

Two peptide SPOT arrays (8mer and 14mer long) corresponding to human ARF protein sequence were synthesized on cellulose membrane by Fmoc chemistry through a Multipep synthesizer as previously reported (Sarkari et al, 2010). After blocking with 5% milk in PBS-T (phosphate-buffered saline with Tween-20), the membrane was incubated with 1 µM His-tagged HUWE1 $_{3760\text{-}4374}$ protein. Following washes in PBS-T, the interaction was detected using His antibody via western blotting technique. 14mer Histidine peptide was used as a control.

3.2.4 Fluorescence Polarization Assays (FP)

HUWE1 protein was purified using standard chromatography method, followed by gel filtration (size-exclusion chromatography by a Hi-Load 26/60 Superdex-75 column, GE Healthcare) in 20 mM Tris pH 7.5, 150 mM NaCl and 2 mM DTT at 4^0 C. HUWE1 proteins were titrated (0 to 1.3 mM) with 40 nM N-terminally labelled FITC-ARF peptides (obtained from GenScript®) in a micro-titre FP plate as described in chapter 2 (cat # PCR-384-BK, AXYGEN®). Polarized emitted FITC signals were obtained at 480 and 520 nm, respectively, using a Synergy H4 Hybrid Plate Reader (BioTeK®). The polarized data were plotted and analyzed using GraphPad Prism version 5.0 and binding constants (*Kd* values) were determined using non-linear curve fitting and based on one site-specific binding using the model: $Y = Bmax*X/(K_d + X)$. Experiments were repeated and three independent experiments were used to calculate the average K_d values and standard deviation (SD).

3.2.5 *In vitro* ubiquitination assays

The *in vitro* ubiquitination assays were performed in a 20 µl reaction volume in the assay buffer using purified recombinant proteins (assay buffer: 50 mM Tris pH 7.6, 5 mM $MgCl_2$, 2 mM ATP, 2 mM DTT and 1X protease inhibitor cocktail (Roche)). 10 µM of HUWE1 were incubated with increasing amounts of FITC-ARF peptides for 10 min in ice in assay buffer. The HUWE1-ARF complex was added to the reaction mixtures containing 10 µM of E1, E2 and Ub for auto-ubiquitination, unless otherwise specified. For p53 ubiquitination, 10 µM of p53 were used as a substrate in the aforementioned reaction mixture. After incubation at 30^0 C for 90 min, the reactions were stopped with SDS-PAGE sample buffer and resolved in 7.5% SDS-PAGE gels. Ubiquitinated HUWE1 and p53 proteins were visualized and evaluated by immunoblotting and probed with Ub (cat # P4G7 MMS-258R, Covance) and p53 (Cat # DO-1/sc-126, Santa cruz) antibodies. The effects of ARF peptides in HUWE1 activity were determined by considering the HUWE1 protein as 1 in '0" ARF *i.e* this lane does not have ARF.

3.2.6 HDX exchange of HUWE1 $_{3951-4374}$-ARF $_{45-52}$ peptide complex

100 µM of HUWE1 HECT domain (HUWE1 $_{3951-4374}$) was mixed with D_2O (1:3) in the rapid mixing module to achieve flow rate 1µl/min for protein and 3µl/min for D_2O. Similarly, 100 µM of HUWE1 HECT domain-ARF $_{45-52}$ peptide complex (1:5) was labeled with D_2O as explained above. Following D_2O labeling, the reaction was quenched with acetic acid and digested with trypsin. The digested samples were electro sprayed at 3.5 kilovolts in the positive ion mode with curtain gas of 30psi and declustering potential of 60 volts. The m/z range of 400-1500 was acquired for all conditions. The deuterium uptake percentage by HUWE1 $_{3951-4374}$ peptides were analysed using custom built FORTRAN software as explained in *Shaolong et al* (2015) (Zhu et al., 2015). A difference plot of deuterium uptake by HUWE1 $_{3951-4374}$ upon

binding with ARF $_{45-52}$ peptide was generated and modeled in HUWE1 HECT domain structure from PDB file (5LP8). Deuterium uptake by apo HUWE1 HECT domain is served as control.

3.2.7 *In vivo* ARF peptides and HUWE1 expressions and ubiquitination assays

HCT116 and U2OS cells were grown in McCoy supplemented with 10% FBS and penicillin/streptomycin. Nearly confluent cells were co-transfected with vectors encoding FLAG-tagged HUWE1HECT domain (residues between 3951-4374) and YFP-ARF peptides through polyJet transfection reagent (SignaGen® laboratories) according to the manufacturer's instructions. Cells were harvested at 48 hrs and lysed in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl and 1X protease inhibitor (cat # 05056489001, Roche)). Immunoblotting was performed for total cell lysate (TCL). YFP-ARF peptides, Fagged-HUWE1, endogenous p53 and p21proteins were probed with YFP (Cat # G163, abm), FLAG, (Cat # F3165, Sigma) p53 (Cat # DO-1/sc-126, santa cruz), and p21 (Cat# sc-397, santa cruz) antibodies. For ubiquitination assays, HCT116 cells were grown as explained above and co-transfected using vectors encoding FLAG-tagged HUWE1, YFP-ARF, and HA-Ub proteins. Cells were treated with 20 μM MG132 for 4 hrs before harvesting. Cell lysates were prepared using lysis buffer supplemented with 20 mM N-Ethylmaleimide (NEM, cat # E3876-5G, Sigma) and a 1X protease inhibitor (Roche). HUWE1 proteins were immunoprecipitated with FLAG antibody from TCL, and then subjected to immunoblotting and probed with FLAG, YFP and HA antibodies (Cat # H3663). Endogenous p53 proteins were also immunoprecipitated with p53 (Cat # sc-99, santa cruz) antibody from the same TCL, subjected to immunoblotting, and probed with HA (Cat # H3663, Sigma) and p53 antibodies (Cat # DO-1/sc-126, santa cruz).

3.2.8 Colony formation assay

Cancer cell lines (HCT116 and U2OS) stably expressing YFP-ARF peptide (8mer ARF $_{45-52}$) were grown in McCoy, supplemented with 400 µg/mL of G418, until single colonies were formed. Single colonies were pooled together and grown for three more generations. 6000 to 8000 cells were plated in a 6-well plate in triplicates. The growth of the survival colonies were chased for 10-14 days in growth medium supplemented with G418. At the end, colonies were washed with 1X PBS and stained with methylene blue dye. Empty vector (YFP only) served as a control. Cells were counted using Image J software. The image was converted to a binary black and white image at 8-bit and only black colonies were counted.

3.3 Results

3.3.1 ARF N-terminal domain contains two binding sites for HUWE1 interaction

Previous research showed that HUWE1 HECT domain (residues 3760-4374, HUWE1$_{3760-4374}$) is sufficient to promote HUWE1 auto-ubiquitination and p53 ubiquitination by HUWE1 (Chen et al., 2005). This HECT domain was also found to interact directly with the N-terminus of p14ARF (human ARF, residues ARF $_{1-64}$). To identify the minimal region of ARF required for HUWE1 interaction, we produced three GST-ARF fusion proteins, spanning different lengths of the ARF N-terminal domain (as indicated in Figure 3.1C). We examined their interactions with His-tagged HUWE1 $_{3760-4374}$ through GST pull-down assay. HUWE1 $_{3760-4374}$ was found to co-migrate with all three GST-ARF fusion proteins but not GST alone, indicating that there are two HUWE1 binding sites within the N-terminal domain of ARF (Figure 3.1D).

To further map the ARF sequences required for the interaction, a peptide spot array was developed with synthetic overlapping 14mer peptides spanning the entire human ARF protein sequence on a cellulose membrane (Nady et al., 2008). This array was used to examine the

interaction with His-tagged $HUWE1_{3760-4374.}$ Immunoblotting was used to detect the His-tagged $HUWE1_{3760-4374}$ bound to the peptides on the membrane. The peptides from two regions within the N-terminal domain of ARF were found to interact with HUWE1. The first region was between ARF residues 17 to 30, and the second one spanned from ARF residues 45 to 58 (Figure 3.1E). We repeated the experiments with an array of overlapping 8mer ARF peptides and found that the same regions interact with HUWE1 (Figures 3.1F).

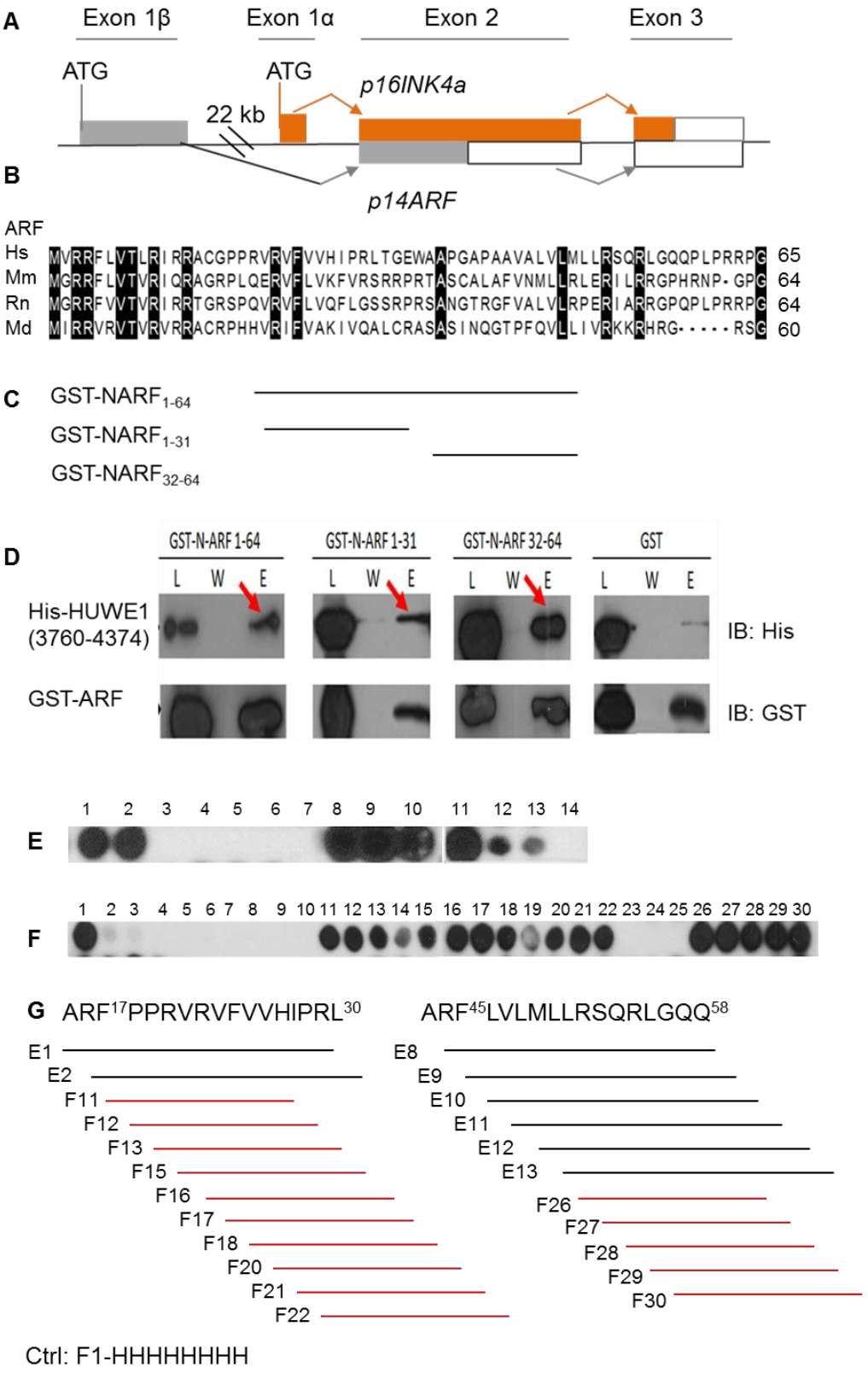

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Figure 3.1: ARF possesses two HUWE1 interaction sites: (A) A schematic diagram of the *ARF-INK4a gene* locus. (B) Sequence alignment of N-terminus of ARF proteins from different species. Hs: *Homo sapiens*, gene ID: 1029, Ms: *Mus musculus*, gene ID: 12578, Rn: *Rattus norvegicus*, gene ID: 25263, Md: *Monodelplus domestica*, gene ID: 554242. (C) His-tagged HUWE1 and GST-tagged ARF fusion proteins used in *in vitro* binding assays. (D) GST pull-down assay to examine the interaction between ARF and HUWE1. Equal amounts of purified GST-ARFs and His-tagged HUWE1 proteins were incubated (L). The interaction complexes (E) were resolved by SDS-PAGE and subjected to immunoblotting using antibodies as indicated. GST alone served as a control. L, W, E denote samples from input (incubation), wash and eluate, respectively. GST alone served as control. Red arrows indicate Elution samples. (E) Mapping of the interaction between HUWE1 and ARF. A peptide SPOT array with overlapping 14mer peptides spanning the human ARF protein sequence was incubated with His-tagged HUWE1. Interaction was detected through immunoblotting using His antibody. (F) A peptide SPOT array with overlapping 8mer ARF peptides for interaction of ARF peptides and HUWE1. The experiment was done as explained for E. The positive control (Ctrl) is poly Histidine peptide located at F1 in blot F. (G) A sliding window shows the positive interaction for indicated spots and corresponding ARF sequences. Black lines indicate 14mer and red lines indicate 8mer ARF peptides.

3.3.2 ARF peptides show micro-molar affinity to HUWE1

We synthesized three N-terminally, FITC-labelled ARF peptides (ARF $_{17-30}$, ARF $_{45-58}$ and ARF $_{45-52}$) based on the spot array screening results, and measured the binding affinity with HUWE1 $_{3760-4374}$ and HUWE1 $_{3951-4374}$ proteins using fluorescence polarization assays. As ARF peptides showed comparatively stronger affinity to HUWE1 $_{3951-4374,}$ data for HUWE1 $_{3760-4374}$ is not shown. All three peptides showed binding to HUWE1 $_{3951-4374}$, while ARF $_{45-52,}$ a 8mer peptide showed strongest affinity among three peptides with a dissociation constant (K_d) value of 6.5 µM. In comparison, ARF $_{17-30}$ and ARF $_{45-58}$ bind HUWE1 $_{3951-4374}$ with weaker affinities, as reflected by their K_d values of 22 µM and 18 µM, respectively (Figure 3.2A and 3.2B). Altogether, synthetic ARF peptides are capable of binding HUWE1 and have a strong affinity to HUWE1. These results show that the ARF N-terminal domain contains two binding sites for HUWE1, and each can interact with HUWE1 independently.

A

ARF	Peptide sequences	K_d (μM) ±SD
ARF_{17-30}	Fitc-PPRVRVFVVHIPRL-ac	22.5±2
ARF_{45-58}	Fitc-LVLMLLRSQRLGQQ-ac	18±1.2
ARF_{45-52}	Fitc-LVLMLLRS-ac	6.5±1.5

B

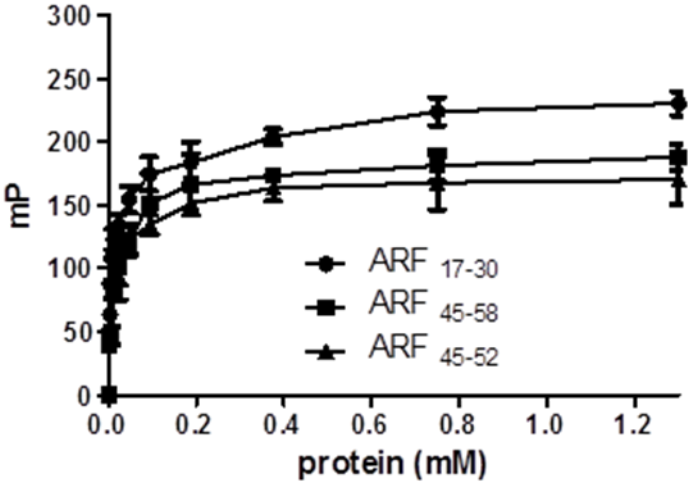

Figure 3.2: Fluorescence polarization assays: (A) Binding affinities of synthetic FITC-labelled ARF peptides to HUWE1 determined through fluorescent polarization assays. FITC-ARF peptides were incubated with increasing concentrations of HUWE1 HECT proteins. The fluorescence polarization values were detected using a Synergy plate reader. The binding constants (*Kd*) were determined by nonlinear curve fittings using Prism 5 GraphPad. The values were normalized with blank. The results are the mean ± SD (n=3). (B) A representative binding curve of ARF peptides and HUWE1 is shown.

3.3.3 Mapping regions of HUWE1 required for p53 ubiquitination

To optimize the minimal region of HUWE1 required for p53 binding and ubiquitination, we carried out an *in vitro* p53 ubiquitination assay using different lengths of HUWE1 recombinant proteins. As shown in Figure 3.3, HUWE1 $_{3760\text{-}4374}$, HUWE1 $_{3902\text{-}4374}$ and HUWE1 $_{3951\text{-}4374}$ were sufficient to ubiquitinate p53, while the HUWE1 $_{3760\text{-}4000}$ protein, lacking HECT domain (residues 4000-4374), was unable to ubiquitinate p53 (Figure 3.3). This result suggests that p53 regulating HUWE1 residues lie within the HUWE1 $_{3760\text{-}4374}$ protein and the minimal required region is 3951-4374. As ARF peptides showed strongest affinity to HUWE1 $_{3951\text{-}4374}$ and that it is also capable of ubiquitinating p53, we used this HUWE1 protein in the rest of the assays.

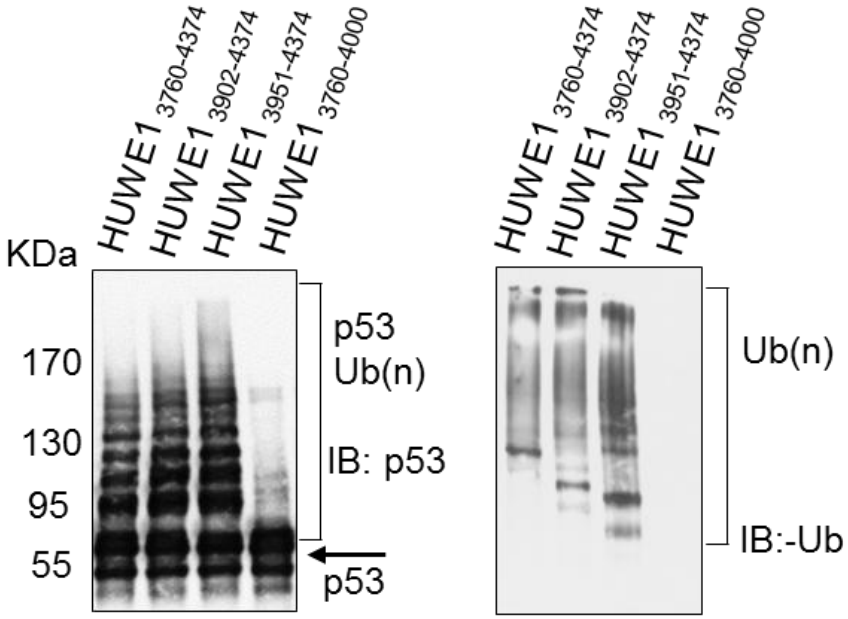

Figure 3.3: Mapping regions of HUWE1 required for p53 ubiquitination. Reaction mixture contains 10 µM of E1, E2, ubiquitin, HUWE1 HECT domain proteins as E3 ligases, and p53 as substrate. After adding ATP, p53 ubiquitination reactions were performed in 30^0 C for 90 min and resolved in SDS-PAGE. p53 ubiquitination was detected via immunoblotting using the indicated antibodies.

3.3.4 Synthetic ARF peptides inhibit HUWE1 activity and p53 ubiquitination

As ARF was shown to inhibit HUWE1-mediated auto-and p53 ubiquitination, we next examined whether the identified ARF peptides hamper HUWE1 ubiquitination. Three synthetic ARF peptides were examined for their inhibitory effect on HUWE1 E3 ligase activity using HUWE1 auto-ubiquitination assay. Results showed that all three peptides inhibited HUWE1 $_{3951-4374}$ activity in a dose-dependent manner (Figure 3.4A). ARF $_{17-30}$ shows half inhibition at 50 µM, whereas ARF $_{45-52}$ shows inhibition at 20 µM. Three peptides completely block HUWE1 ubiquitination at 100 µM. These results suggest that synthetic human ARF peptides were able to inhibit HUWE1 E3 ligase activity directly. Since the 8mer ARF $_{45-52}$ shows stronger inhibition, next we examined whether it also could impair p53 ubiquitination by HUWE1. For this, different doses of ARF $_{45-52}$ peptides were pre-incubated with HUWE1 $_{3951-4374}$ and added to the p53 ubiquitination reaction mixtures. Ubiquitinated p53s were resolved and probed through immunoblotting with p53 and Ub antibodies, respectively. As shown in Figure 3.4B, ARF $_{45-52}$ peptide inhibited HUWE1-mediated p53 ubiquitination in a dose dependent manner. This result is consistent with the inhibitory effect of the peptide shown in the HUWE1 auto-ubiquitination assay (Figure 3.4A). Together, synthetic 8mer ARF $_{45-52}$ peptide is proficient to inhibit HIUWE1 mediated auto-and p53 ubiquitination by HUWE1. We also examined the effect of the peptide on the ubiquitination activity of another HECT domain recombinant protein (HUWE1 $_{3760-4374}$) and found that this peptide demonstrated similar inhibitory effects and attenuated HUWE1-mediated p53 ubiquitination (data not shown).

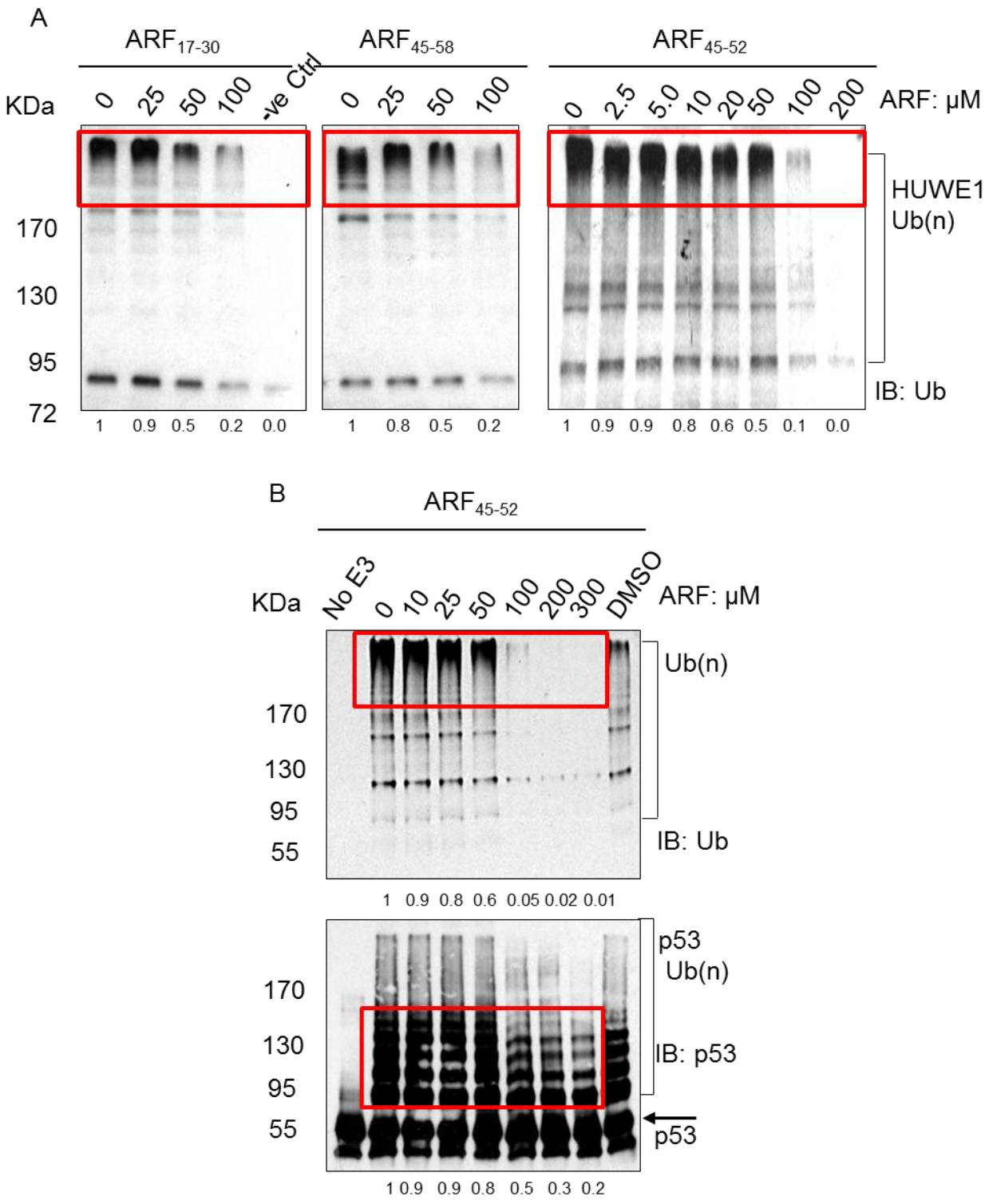

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Figure 3.4: Human ARF peptides inhibit HUWE1 activity and p53 ubiquitination by HUWE1 in a dose-dependent manner: (A) ARF peptides inhibit HUWE1 $_{3951-4374}$ auto-ubiquitination. A serial dilution of ARF peptides were incubated with 10 µM of HUWE1. The HUWE1-ARF peptide complexes were added to the reaction mixtures containing 0.25 µM E1, 10 µM E2, 30 µM Ub and 2mM ATP. The HUWE1 ubiquitination was detected through immunoblotting using indicated antibodies. (B) A serial dilution of 8mer ARF peptide was incubated with 10 µM of HUWE1 $_{3951-4374}$. The HUWE1-ARF peptide complexes were added to the reaction mixtures containing E1, E2, Ub, ATP and 10 µM p53 as substrate. The p53 ubiquitination was detected via immunoblotting using p53 and Ub antibodies as indicated. The polyubiquitination of HUWE1 $_{3951-4374}$ (Figure A) and p53 (Figure B) were quantified using ImageJ. The quantified areas were indicated in red boxes. The relative quantity of ubiquitination in the presence of ARF peptide was indicated below the blots in comparison of ubiquitination in the absence of ARF peptide in "0" lanes in every blot.

3.3.5 ARF $_{45\text{-}52}$ directly binds to the α1 helix of N-lobe of HUWE1 HECT domain

Amide hydrogen exchange with deuterium is a powerful technique in investigating a protein's dynamics and interaction in solution. Hydrogen to deuterium (H/D) exchange relies on the solvent accessibility/hydrogen bonding of the amide hydrogen. Consequently, amide hydrogens located on the surface or involved in weak interactions are exchanged rapidly into deuterium. However, those involved in strong interactions or buried in the interior, exchange more slowly. Therefore the functional state of a protein could be extracted by measuring the HDX exchange rates of backbone amides of a protein. To investigate the mechanisms of ARF $_{45\text{-}52}$ effects on HUWE1 activity, we collected hydrogen to deuterium exchange (HDX) data of HUWE1 $_{3951\text{-}4374}$ protein upon incubation with ARF $_{45\text{-}52}$ via Mass Spectrometry (MS). The HDX data showed that one particular region of HUWE1 HECT domain protected HUWE1 from exchanging with deuterium upon interaction with ARF $_{45\text{-}52}$ peptide. The HUWE1 residues are located between 3984 - 4002 and 4007- 4017 in HUWE1 $_{3951\text{-}4374}$ protein respectively, and three peptides were found to be protected from changing with deuterium (Figure 3.5A, residues are indicated as red). These residues are highly conserved and essential to form a α1 helix of N-lobe within HUWE1 HECT domain structure. The N-lobe is the also the acceptor site for Ub-charged E2 during ubiquitination. Therefore, if ARF $_{45\text{-}52}$ peptide pre-occupied the site in N-lobe including the α1 helix, Ub-charged E2 would not be able to contact the HUWE1 HECT domain, which is required for transthiolation reaction between Ub-loaded E2 and E3 ligase (indicated in red in Figure 3.5B). Most importantly, the hinge peptide linker that connects two lobes of HECT domains was also prevented HDX exchange upon binding to ARF$_{45\text{-}52}$ peptide (indicated in red in Figure 2.5B). All HECT domain structures including HUWE1 were reported to be conformationally flexible due to this peptide linker. This linker confers the rotation of C-lobe

(contain catalytic cysteine) towards the N-lobe which houses the Ub-loaded E2 during transthiolation; and closes the distances between the catalytic cysteines of E2 and E3, which is required for polyUb chain synthesis. Therefore, our data suggest that due to ARF $_{45-52}$ binding, HUWE1 HECT domain lost conformational flexibility and restricted Ub-loaded E2 to make contact with HUWE1 N-lobe. Consequently HUWE1-mediated auto-and p53 ubiquitination will be hampered. Together, consistent with what has been observed in *in vitro* assays, HDX data showed that ARF $_{45-52}$ peptide causes allosteric hindrance to keep the HUWE1 in an inactive state.

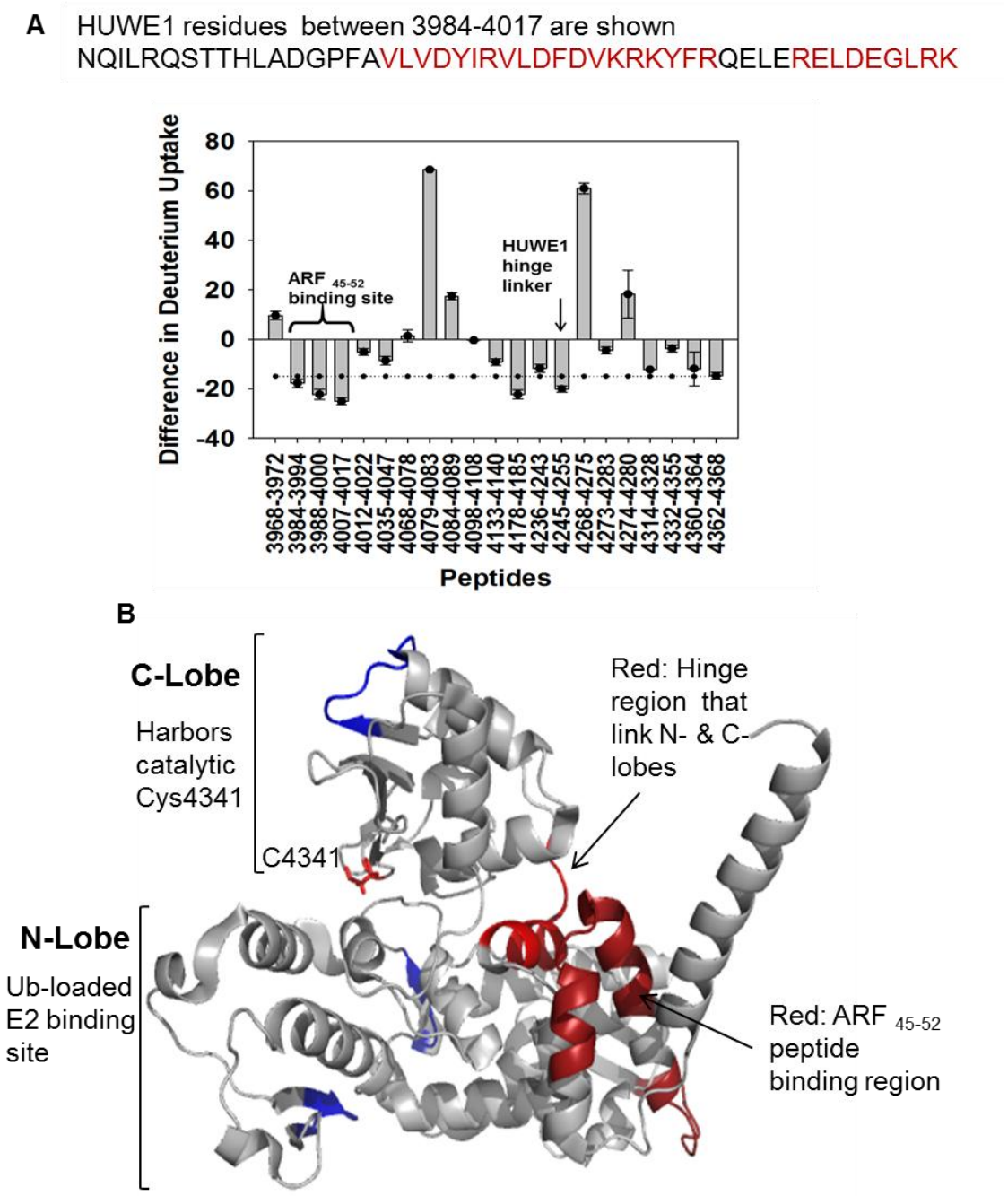
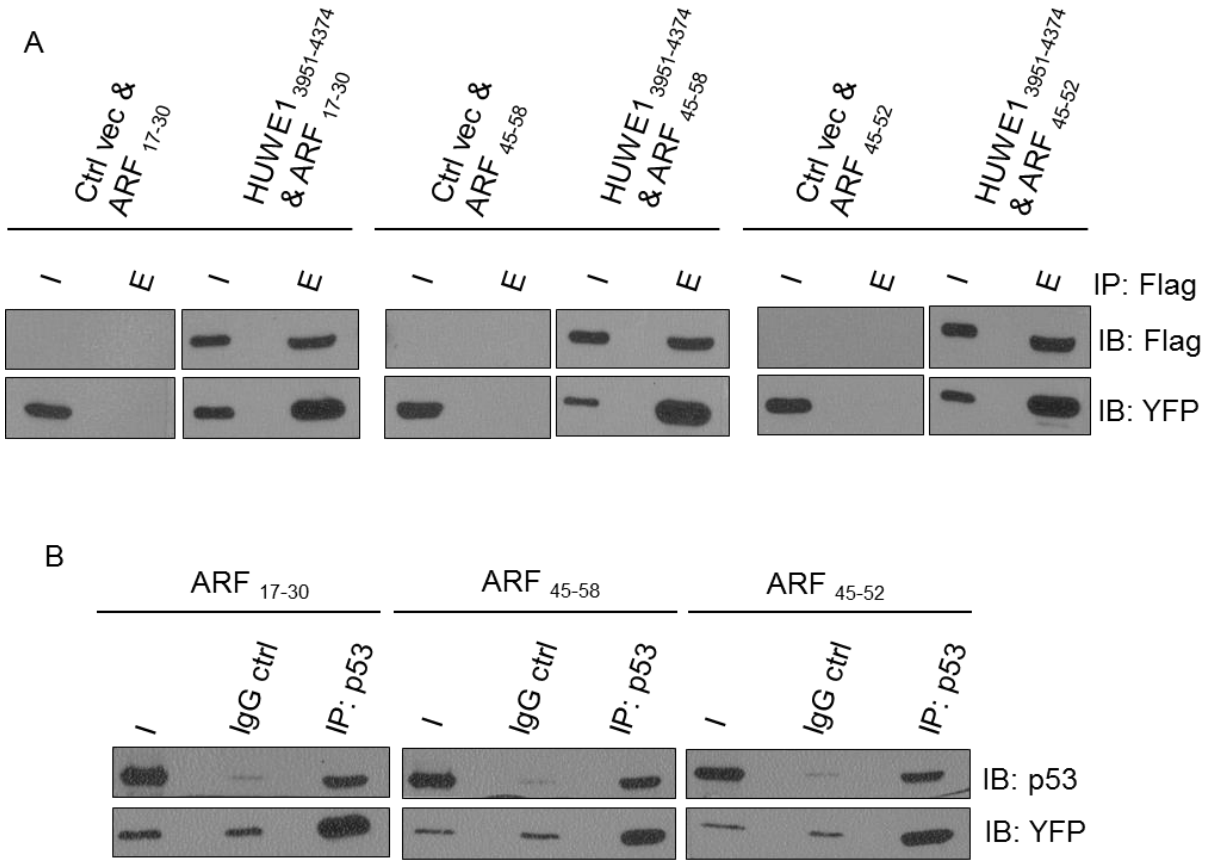


Figure 3.5: HDX MS mapping of the interaction between ARF peptide and HUWE1. (A) A difference plot of deuterium uptake by HUWE1 upon binding with ARF peptide. The ARF binding HUWE1 regions (HUWE1 residues) with decreased deuterium (values below zero) are indicated in red. (B) The cartoon representation of HUWE1 HECH domain structure showing difference in deuterium uptake upon binding with ARF peptide. Red colour denotes the α1 helix and other helix in the N-lobe, and also the hinge linker of HUWE1 HECT domain, other affected area are also indicated as blue. Catalytic Cys4341 is indicated as red stick. Structure is modeled using Pymol based on PDB: 5LP8 domain structure of HUWE1.

3.3.6 ARF peptides bind physically with HUWE1 $_{3951-4374}$ protein *in vivo* to inhibit HUWE1-mediated p53 ubiquitination

Next we investigate whether ARF $_{45-52}$ peptide could physically interact with HUWE1 *in vivo*. Total cell lysates (TCL) from HCT116 cells (co-transfected by expression vectors encoding FLAG-HUWE1 $_{3951-4374}$ and three YFP-ARF peptides) were immunoprecipitated by FLAG and immunoblotted with FLAG and YFP antibodies. Empty vector (expressing YFP only) served as a control in each experiment. The results show that all three ARF peptides were co-eluted with HUWE1 HECT domain probed by FLAG and YFP antibodies, indicating that all three peptides are capable of binding to HUWE1 *in vivo* (Figure 3.6A). To investigate whether the ARF peptides interact with p53, endogenous p53 was immunoprecipitated using p53 antibody from the same lysate and probed with p53 and YFP antibodies. The results showed that ARF peptides were also co-eluted with p53, suggesting that ARF peptides interact with p53 *in vivo* (Figure 3.6B). The next thing to examine is whether these peptides could block HUWE1 E3 ligase activity and inhibit p53 ubiquitination by HUWE1.HCT116 cells were co-transfected by vectors expressing FLAG-HUWE1 $_{3951-4374}$, HA-Ub and YFP-ARF peptides. The TCL were subjected to immunoprecipitation by p53 antibody and immunoblotted by p53 and HA antibodies respectively. The results showed that all ARF peptides (YFP-ARF $_{45-52,}$ YFP-ARF $_{17-30}$ and YFP-ARF $_{45-58}$) inhibited HUWE-mediated p53 ubiquitination (Figure 3.6C). However, ARF $_{45-58}$ and ARF $_{45-52}$ peptides abolished p53 ubiquitination by HUWE1 completely. ARF $_{17-30}$ also inhibited p53 ubiquitination, but at a lesser extent compared to the other two peptides. All three peptides also elevated p53 protein levels (Figure 3.6C). Vector encoding YFP only served as a control. These results are consistent with the *in vitro* inhibition results that also showed ARF $_{17-30}$ to be weaker compared to the other two peptides (Figure 3.4A). These results confirmed that ARF $_{45-58}$

and ARF $_{45\text{-}52}$ have the potential to diminish HUWE1 E3 ligase activity and prevent p53 ubiquitination by HUWE1 *in vivo*. We repeated the experiments and found the same negative effect of ARF peptides inhibiting p53 ubiquitination by HUWE1.



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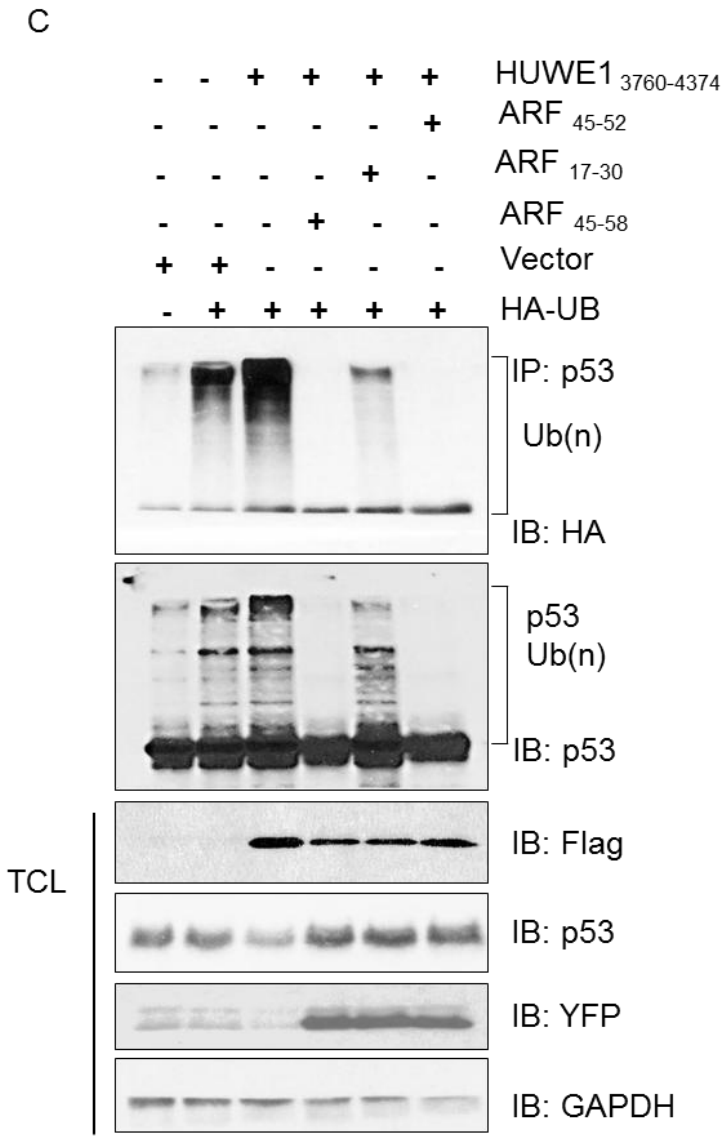

Figure 3.6: ARF peptides interact with HUWE1 $_{3951-4374}$ and p53 proteins *in vivo* and inhibit HUWE1-mediated p53 ubiquitination. (A) Immunoblots show ARF peptides interaction with HUWE1 HECT domain *in vivo*. TCL were immunoprecipitated from HCT116 cells (co-transfected with vectors encoding FLAG-HUWE1 $_{3951-4374}$ and YFP-ARF peptides) using FLAG antibody and immunoblotted with FLAG and YFP antibodies as indicated. Empty vector served as a control (Ctrl). (B) Immunoblots show ARF peptides interaction with p53 *in vivo*. Endogenous p53 from the experiment (A) was immunoprecipitated using p53 antibody and immunoblotted with p53 and YFP antibodies. IgG served as a control. (C) Immunoblots show ARF peptides inhibit HUWE1-mediated p53 ubiquitination and increase p53 protein levels *in vivo*. Endogenous p53 was immunoprecipitated using p53 antibody from TCL, prepared from HCT116 cells (co-transfected with plasmids expressing FLAG-HUWE1 $_{3951-4374,}$ HA-Ub, and YFP-ARF peptides), and immunoblotted with p53 and HA antibodies as indicated. TCL from this experiment were also immunoblotted using indicated antibodies.

3.3.7 ARF $_{45-52}$ peptide inhibits cancer cell growth in colony formation assays

p53 is an important tumor suppressor. Our previous results showed that ARF $_{45-52}$ peptide elevated p53 and its transcriptional target p21. It also abolished p53 ubiquitination by HUWE1 $_{39521-4374}$ in our previous experiments. Therefore, we investigated whether this peptide could inhibit cancer cell growth. The growth of two ectopically expressed stable cell lines (transfected with ARF $_{45-52}$ encoding or empty vector) (HCT116 p53 WT and HCT116 p53 null cells) was chased for 10-14 days in growth medium supplemented with G418. The results showed that more than 70% of the colonies were diminished in case of HCT116 p53 WT, compared to the empty vector. However, ARF $_{45-52}$ has no effect on growth of HCT116 p53 null cells (Figure 3.7). These results suggest that ARF $_{45-52}$ is proficient to suppress growth of HCT116 cells which retain wild-type p53. It also inhibited cancer cell growth in ARF $_{45-52}$ overexpressing stable U2OS cells (Figure 3.7S) indicating that ARF $_{45-52}$ exerts its growth suppressing role in a p53-dependent manner.

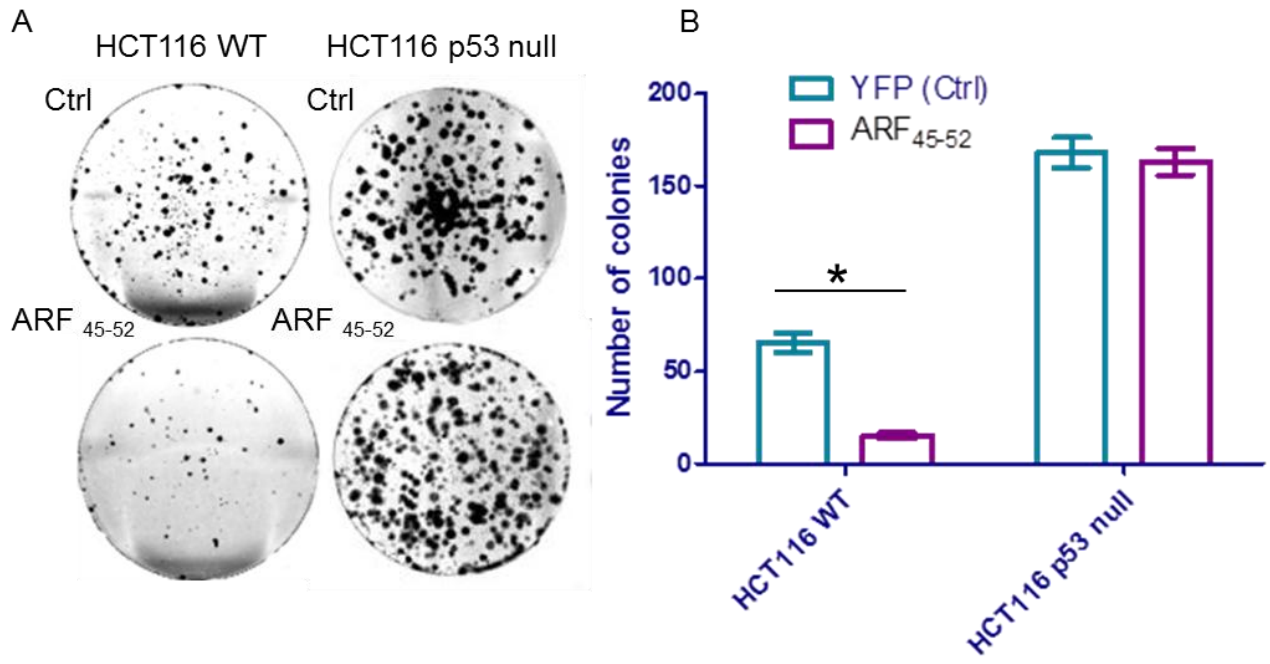

Figure 3.7: ARF $_{45\text{-}52}$ peptide inhibits cancer cell growth in colony formation assay: (A) The colony formation assay showing cell growth inhibition of cancer cells. HCT116 cells were transfected with pcDNA3.1 encoding YFP-ARF $_{45\text{-}52}$ peptide or with empty vector (YFP, Ctrl) and selected with 400µg/ml G418. 6000 cells/well were seeded as triplicate and cell growths were chased for two weeks. Plates were stained with crystal violet solution containing methanol and were scanned. (B) The number of colonies of HCT116 cells , WT or p53 null cells , expressing YFP or ARF peptide from experiment A. Results are the mean ± SD (n = 3). ∗P<0.05.

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3.4 Discussion

The use of short peptides or peptidomimetic inhibitors has emerged as a key method in perturbing the protein-protein interaction in cell signaling or inhibiting the enzymatic activity in the development of drug-like molecules. Here we describe the identification of two ARF sequences that are responsible for the interaction with the HUWE1 HECT domain and inhibition of its E3 ligase activity. Importantly, the synthetic peptides derived from these two regions of ARF inhibited HUWE1 activity in the ubiquitination assays. These peptides also upregulated p53 levels and its transcriptional activity in cancer cell when overexpressed as YFP-fusion peptides. Furthermore, a 8mer ARF peptide, ARF $_{45-52}$ inhibited cancer cell growth in HCT116 cells, which is dependent on the wild type p53.

The HUWE1 protein bears additional residues upstream of the HECT domain where some of its important substrates including p53 were found to bind (Chen et al., 2005; Hall et al., 2007; Noy et al., 2012; Wang et al., 2014). *In vitro* p53 ubiquitination assays from current study, by different HUWE1 proteins as E3 ligases, also suggest that p53 regulating HUWE1 residues located within HUWE1 residues lie between 3760-4374 (HUWE1 $_{3760-4374}$), and the minimal required region is between residues 3951-4374 (HUWE1 $_{3951-4374}$)(refer to Figure 3.3). The biochemical mapping of the interaction between HUWE1 and ARF indicate that there are two functional regions within the N-terminus of human ARF $_{1-64}$ protein interacting with HUWE1: one spanning residues ARF $_{1-31}$ and the other spanning residues ARF $_{32-64}$. The first region (ARF $_{1-31}$) overlapped with the MDM2 and c-Myc binding region, previously mapped as ARF $_{1-14}$ (Datta et al., 2004; Lohrum et al., 2000; Weber et al., 1999). This extreme N-terminus was found to be essential in binding MDM2. However ARF residues 26-37 (ARF $_{26-37}$) were found to be essential in targeting MDM2 to the nucleolus and in activating the p53 function. Using a peptide

array, we identified the first ARF site that interacts with HUWE1 and is between ARF residues 17-30. We also found the second HUWE1 interacting site in the peptide array, which resides between residues 45-58 in ARF. The synthetic peptides based on these two regions, ARF $_{17-30}$ and ARF $_{45-58,}$ showed micro-molar affinities to HUWE1 $_{3951-4374,}$ as measured by fluorescence polarization assays. Two peptides also inhibited HUWE1 $_{3951-4374}$ activity in a dose dependent manner, approximately at 50 μM in an *in vitro* ubiquitination assay. Furthermore, both were able to interact with HUWE1 $_{3951-4374}$ in cell-based assays, and upregulated p53 levels, and its transcription by inhibiting HUWE1 $_{3951-4374}$ when overexpressed as YFP-ARF fusion proteins.

Though synthetic ARF peptides from both regions inhibited HUWE1 E3 ligase activity, the peptide derived from the second site, ARF $_{45-58}$, showed more prominent effects in tempering HUWE1 activity *in vitro* and *in vivo* assays. We have mapped the minimal sequence of ARF in the second site required for binding HUWE1, between ARF residues 45 and 52. An 8mer peptide, ARF $_{45-52}$ showed the strongest binding affinity compared to the other two peptides (Figure 3.2). This 8mer peptide showed the strongest inhibition to HUWE1 compared to the other two peptides in *in vitro* autoubiquitination assays. It also inhibited HUWE1-mediated p53 ubiquitination in the same dose (Figure 3.4 (A & B)). Further, this 8mer peptide induced strongest p53 activation by inhibiting HUWE1 ubiquitination in cell based assays (Figure 3.6C and 3.7B). The second ARF region (ARF $_{32-64}$) has been studied in the drug-resistant and drug-sensitive non-small cell lung adenocarcinomas (NSCLCs) (Saito et al., 2013). This region was characterized as the functional core of human ARF and found to be crucial in apoptotic response by ARF. A synthetic peptide based on this functional core suppressed the growth of drug-resistant adenocarcinomas through reducing the mitochondrial membrane potential and caspase-9 activation in *EGFR* mutated NSCLCs (Saito et al., 2013). These findings agree with our study

indicating that ARF has two binding sites for interacting with its targets (Datta et al., 2004; Lohrum et al., 2000; Saito et al., 2013; Weber et al., 1999).

We have measured the HUWE1 binding affinity of the three synthetic ARF peptides and examined their HUWE1 inhibitory effects using biochemical and cellular assays. The binding affinities (*Kd* values) were found to be in the range of 8-20 μM. These affinities fall below comparative nano-molar affinities reported for other potential peptides/peptidomimetics that showed promising roles against cancers in clinical trial studies (Zhao et al., 2015). However, we found that all three peptides inhibited HUWE1 activity and activated p53 in the cancer cells that harbor wild-type (WT) p53. The individual low affinities could be explained by the fact that ARF contains multiple HUWE1 binding sites that could create a potent inhibitory effect towards HUWE1. This type of inhibition has been observed in ARF and MDM2 interactions (Zhao et al., 2015). The combinatorial inhibition resulted from multiple binding sites, which provided a potent effect, but less affinity for individual interaction. Indeed, we see that full length and N-terminal ARF showed strong p53 activation with low levels of expression as compared to the peptide ARF $_{45\text{-}52}$ that showed inhibitory effect when overexpressed in the cell. The ARF $_{45\text{-}52}$ appeared to be more stable than ARF $_{17\text{-}30}$ and ARF $_{45\text{-}58}$. Nevertheless, ARF $_{45\text{-}52}$ peptide showed growth inhibition in colony formation assays for HCT116 and U2OS cells dependent on the presence of WT p53 indicating that ARF $_{45\text{-}52}$ functions required the presence of p53 (Figures 3.7 and 3.7S, respectively).

In line with the aforementioned interacting and inhibiting properties of these ARF peptides, HDX data further showed the interaction between ARF $_{45\text{-}52}$ and HUWE1 HECT domain collected by Mass Spectrometry (MS). The MS data showed that important hydrophobic residues including α1 helix in the N-lobe and the hinge linker of the HUWE1 HECT domain

protected HUWE1 from exchanging with deuterium upon binding to ARF $_{45-52}$ peptide (Figures 3.5 (A & B)). These results suggest that ARF $_{45-52}$ interacts with the N-lobe of HUWE1 HECT domain. The N-lobe HUWE1 residues are conserved within all known HECT domain E3 ligases and form an important α1 helix that confers the HUWE1 structural stability. The α1 helix is also known to restrict the auto-ubiquitination and house the Ub-charged E2 during ubiquitination (Huang et al., 1999; Pandya et al., 2010; Verdecia et al., 2003). Due to engaging with ARF peptide, the N-lobe of HUWE1 HECT domain would not be able to make contact with Ub-loaded E2, hence would disrupt the ubiquitination by HUWE1. Furthermore, due to engaging with the ARF peptide, the hinge linker that connects N-and C-lobes becomes affected too. This would pose the steric hindrance to keep the enzyme in one orientation only. Therefore, the enzyme lost the dynamic functional state. p53 ubiquitination by HUWE1 was also hampered for the same reason. This is what we found in *in vitro* ubiquitination assays (Figures 3.4). Together these findings suggest that ARF $_{45-52}$ interaction with HUWE1 HECT domain disrupts its function.

Although the interaction affinity between ARF peptide and HUWE1 is comparatively low, our study could be served as proof of the concept that can be used to design small molecule inhibitors targeting ARF-HUWE1 interaction in cancers where HUWE1 is overexpressed.

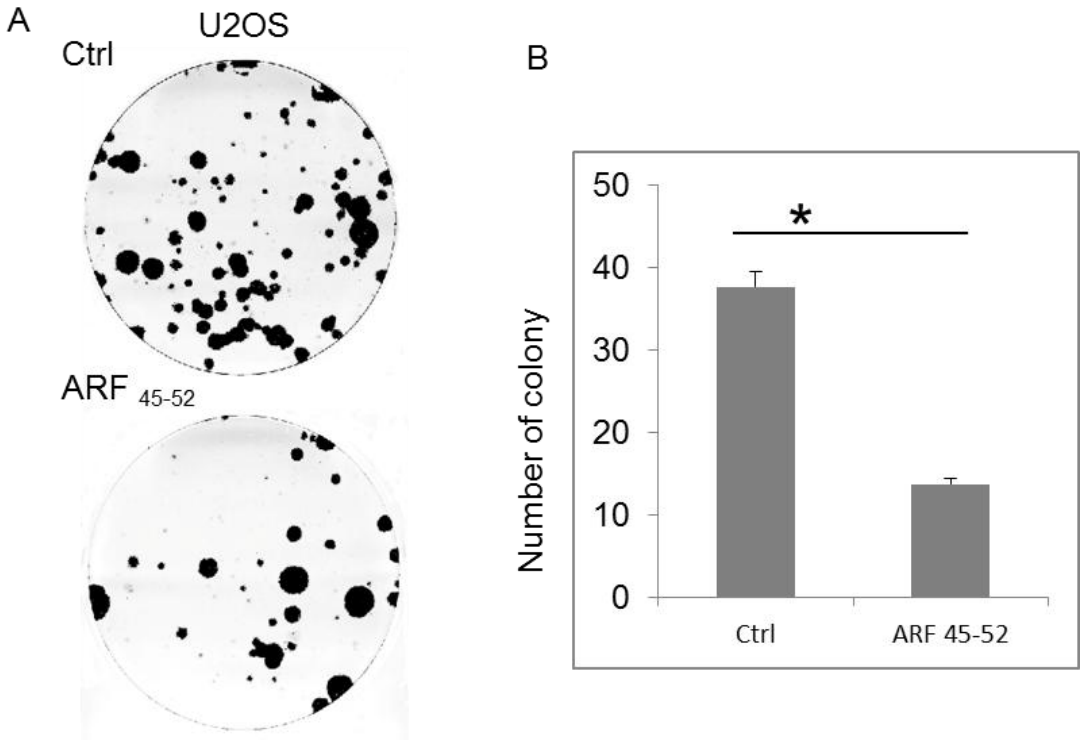

Figure 3.7S : ARF $_{45-52}$ peptide inhibits cancer cell growth in colony formation assay: (A) The colony formation assay showing cell growth inhibition of cancer cells. U2OS cells were transfected with pcDNA3.1 encoding YFP-ARF $_{45-52}$ peptide or with empty vector (YFP, Ctrl) and selected with 400µg/ml G418. 6000 cells/well were seeded as triplicate and cell growths were chased for two weeks. Plates were stained with crystal violet solution containing methanol and were scanned. (B) The number of colonies of U2OS cells , WT or p53 null cells , expressing YFP or ARF peptide from experiment A. Results are the mean ± SD (n = 3). ∗P<0.05.

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Chapter 4

E3 Ubiquitin Ligase HUWE1 Targets β-Catenin under Conditions of Hyperactive Wnt Signaling

Note: This is a collaborative project with Dr Tak Mak lab at the University of Toronto, Canada. Therefore, we presented our part of contribution in this dissertation. Data presented in Figure 4.2 was done by RK, and data presented in Figure 5 was performed by RK and YS. The published manuscript is attached in the appendix-I.

4.1 Introduction

The canonical Wnt signaling pathway (Wnt/β-catenin) regulates a diverse group of cell fate decisions involved in embryonic development, and in the maintenance of tissue homeostasis, in many adult organs. The Wnt proteins are the secretory glycoproteins which bind to the cell surface receptors in order to activate the Wnt signaling pathway and play a crucial role in stem cell self-renewal and proliferation. After activation, Wnt signals are relayed and passed to the cytoplasm and then to the nucleus through the interactions of various proteins in order to activate the transcription of Wnt target genes (Katoh and Katoh, 2007; Mao et al., 2001).

In molecular terms, upon binding a Wnt ligand, the cell surface receptor Frizzled (Fz) associates with co-receptor low-density lipoprotein related proteins 5 and 6 (LRP5/6). This association recruits the cytoplasmic adapter protein Dishevelled (Dvl) to the receptor complex. Dvl is a phosphoprotein that binds directly to the Wnt-bound Fz and forms the larger protein complex. This initiates the sequential phosphorylation of LRP5/6 and polymerization of Dvl, which in turn recruits the other scaffolding protein Axin2 and glycogen synthase kinase 3β (GSK-3β). Both are the components of multifunctional destruction complexes, along with adenomatous polyposis Coli (APC) (APC/Axin2/ GSK-3β-complex/CK1) (Korinek et al., 1997). APC is an important tumor suppressor, and this recruitment displaces the APC from the destruction complex that binds to the signal transduction effector protein β-catenin in order to trigger its proteasomal degradation via β-TRCP/SKP. Either constitutive activation of Wnt ligands, or mutations in APC protein, can lead to the accumulation of β-catenin in the cytoplasm. This then translocates into the nucleus and binds to the T-cell factor/lymphoid enhancing factor transcription factors (TCF/LEF) in order to turn on the transcription of a large number of Wnt target genes (Clevers, 2006; de Lau et al., 2007; Gregorieff and Clevers, 2005b; Le Guen et al.,

2015; Li et al., 2015). Deregulation of Wnt signaling has been implicated in many cancers including colon, prostrate, thyroid, breast, ovary, melanoma, and hepatocellular carcinomas. Among them, the best-studied one is colorectal cancer. Studies in primary cancer cell lines and in mouse models of colorectal tumors showed that due to mutation APC in the destruction complex, APC is unable to control β-catenin degradation in the cytoplasm. In such a case, β-catenin is accumulated, moves to the nucleus, and activates the Wnt program in an uncontrolled manner to drive cancer (Basu et al., 2016; Clevers and Nusse, 2012).

B-catenin is a 93 Kda protein, originally identified in a complex with E-cadherin, that regulates the cell-cell adhesion in mammals (McCrea et al., 1991). Later it was found that β-catenin is a critical mediator of the canonical Wnt signaling pathway, where it acts as a transcriptional coactivator of the T-cell factor/lymphoid enhancer (TCF/LEF) family of transcription factors. In response to the Wnt ligand, β-catenin accumulates in cytosol and moves to the nucleus, where it forms a complex with TCF in order to regulate its transcription. Aberrant expressions of β-catenin have also been implicated in many diseases including cancers (Burgess et al., 2011; Horst, 2012; Keerthivasan et al., 2014).

Recent research indicates that HUWE1 is involved in the regulation of the Wnt/β-catenin pathway. HUWE1 was found to regulate Dvl, a cytoplasmic scaffold that directly binds to the cytoplasmic part of the Fz, in the Wnt-bound Fz-LRP5/6 receptor complex. Dvl serves as a platform to recruit other scaffolding proteins and kinases to the complex. HUWE1 was shown to associate and ubiquitinate Dvl through the Lys63-linked polyUb chain, which does not destine Dvl for degradation. Rather, it prevents Dvl multimerization. This eventually inhibits Dvl binding to Axin2, a part of destruction complex, which hampers Wnt signaling activation.

Therefore, HUWE1 mediated Dvl ubiquitination suppresses Wnt activation; hence prevents cancer (de Groot et al., 2014).

Further, HUWE1 was found to play a crucial role in regulating Wnt/β-catenin signaling in intestinal tissue development. Studies have also shown that HUWE1 knockout in mice intestine results in constitutive activation of Wnt signaling, and intestinal cell proliferation via up-regulating the c-Myc protein. The studies also show HUWE1 regulates the proliferation of intestinal stem and progenitor cells through the control of the c-Myc function. C-Myc is a HUWE1 substrate, which is also an important Wnt target *gene* in the intestine. Together these observations suggest that HUWE1 is regulating Wnt/β-catenin signaling via controlling the c-Myc function in intestinal tissues, in order to maintain the regular physiology of intestine (Basu et al., 2016; Clevers, 2013; Dominguez-Brauer et al., 2016). Moreover, a recent study showed that c-Myc was up-regulated in *HUWE1* conditional knockout mice, not only because of the absence of HUWE1-mediated c-Myc degradation but also because of hyper activated Wnt signaling (d Groot et al.,2014). Notably, *c-Myc* is also a Wnt target gene, and its trans-activation is essential to the phenotypes of colorectal cancers (CRC) caused by presence of mutation in *APC*. As HUWE1 deletions or mutations in CRC up-regulate Wnt target genes in APC^{min} background (APC^{min} : *APC* mutation) this raises the possibility that HUWE1 may also target other proteins in the Wnt signaling pathway (Myant et al., 2017; Peter et al., 2014).

Here we described the identification and characterization of a new HUWE1 substrate, β-catenin, which is the prime canonical Wnt signal transducer in cells. We demonstrate that HUWE1 directly associates and ubiquitinates β-catenin, resulting degradation of β-catenin. HUWE1 regulates β-catenin degradation in APC^{min} background to counteract constitutive Wnt

signaling where APC function is permanently diminished due to mutation. Our results suggest that HUWE1 is a negative regulator of Wnt/ β-catenin signaling.

4.2 Experimental procedures

4.2.1 Cell lines

HEK293T cells (ATCC) were cultured in DMEM H21 (Invitrogen). HCT116 cells (ATCC) were cultured in McCoy's medium. Media were supplemented with 10% FBS (Sigma), and penicillin/streptomycin.

4.2.2 Immunoprecipitation

The cellular interaction of HUWE1 and β-catenin was carried out in HEK293T stable cell lines overexpressing FLAG-tagged HUWE1 or control vector. Total cell lysates (TCL) were prepared in lysis buffer [50 mM Tris, (pH 8.0), 150 mM NaCl, 0.6% Nonidet P-40, and 1X protease inhibitor cocktail (Roche)]. For immunoprecipitation studies, TCL were incubated at 4°C overnight with anti-FLAG M2 antibody (Sigma #F3165) in order to immunoprecipitate FLAG-tagged HUWE1, or were incubated with an anti-β-catenin antibody (CST #8480) to immunoprecipitate endogenous β-catenin. Cellular interaction of endogenous HUWE1 and β-catenin was carried out in HCT116 cell lines. TCL were incubated at 4°C overnight with anti-HUWE1 antibody (monoclone antibody 7BF12, generated by Tak Mak lab) in order to immunoprecipitate endogenous HUWE1, or were incubated with an anti-β-catenin antibody (CST #8480) in order to immunoprecipitate endogenous β-catenin. IgG was used as a negative control. In both cases the immuno complexes bound to Protein A/G resin were washed 5X in Wash buffer. Interaction complexes were detached from the agarose using SDS-PAGE loading buffer, and detected by immunoblotting using anti-FLAG (or anti- HUWE1) and anti- β-catenin antibodies.

4.2.3 *In vitro* GST pull-down assays

Cell lysates of HEK293T overexpressing HUWE1-WT or empty vector (3 mg) were mixed with 20 µl Anti-FLAG M2 agarose (Sigma #M8823) pre-cleared with 5% BSA at 4 °C in 1xPBS buffer for 2 hours. After incubation, the anti-FLAG M2 agarose resin was washed extensively with a buffer of 50 mM Tris pH 7.6, 500 mM NaCl, 10% glycerol, 0.1% Triton X-100, and protease inhibitor cocktail (Roche), and mixed with recombinant His- tagged β-catenin (20 µg) in 500 µl binding buffer containing 50 mM Tris pH7.6, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100 and protease inhibitor cocktail at 4 °C for 2 hours. After the second incubation, anti-FLAG M2 agarose was washed extensively with the same buffer, and the resulting immobilized proteins were detected using immunoblotting. GST pull-down assay was carried out by mixing equal amounts of recombinant GST tagged N-terminal truncated HUWE1 (3760-4374), and His-tagged β-catenin (20 µg) in the binding buffer at 4 °C for 2 hours. After extensive wash with the binding buffer, the immobilized proteins were eluted using reduced glutathione, and detected by immunoblotting using anti-His (Novagen #71841) or GST antibodies (Thermo #8-326). Recombinant GST tagged N-terminal truncated HUWE1-CT (HUWE1 residues 3760-4374) and His-tagged β-catenin was expressed in *E. coli* BL21 cells, and purified using standard GST-and Ni-affinity chromatography methods.

4.2.4 Protein stability-cycloheximide chase assay

Cells were cultured with cycloheximide at a concentration of100 µg/ml and incubated for various hours. Cells were harvested at the indicated time points. Lysates were prepared and subjected to immunoblotting analysis. The protein stability was determined by the % of β-catenin remaining at indicated time points compared to the initial time point. Data was plotted from three independent trials.

4.2.5 *In vivo* ubiquitination

HEK293T stable cell lines overexpressing FLAG-tagged WT HUWE1 or inactive HUWE1 C4341A were cultured as described above. Near-confluent cells were transfected with *pcDNA 3.1-HA-Ub* using PolyJet transfection reagent (SignaGen® laboratories), recovered after 6 hrs, and grown for a total of 48 hr. Cells were treated with 25 μM MG132 for 4 hrs before harvesting, and total cell lysates (TCL) were prepared in lysis buffer of 50 mM Tris, (pH 8.0), 150 mM NaCl, 0.6% Nonidet P-40, 1X protease inhibitor cocktail (Roche), with 10 mM freshly prepared N-Ethylmaleimide**.** β-catenin was immunoprecipitated from TCL by incubating it with anti-β catenin Ab (CST #8480) and Protein A/G resin. Ubiquitinated β-catenin was detected on immunoblots using anti-Ub Ab (P4G7, Covance MMS-258R) and anti-β catenin Ab (CST #8480).

4.2.6 *In vitro* ubiquitination

Recombinant His-tagged β-catenin (full-length) and GST- HUWE1-CT (HUWE1 residues 3760-4374) were expressed in the *E. coli* BL-21 DE3 strains, and purified using standard affinity purification methods. Ubiquitination was performed as previously described (22496338) by mixing 10 μM each of E1, E2, Ub, and various amounts of GST- HUWE1 as the E3 ligase, and His-tagged β-catenin as the substrate, in reaction buffer (50 mM Tris, pH 7.6, 2 mM ATP, 5 mM $MgCl_2$, 2.5 mM DTT). Reactions were carried out for 90 min at 30°C, and terminated by adding SDS-PAGE loading buffer. Ubiquitinated β-catenin was detected by immunoblotting using anti-ubiquitin (P4G7, Covance MMS-258R) and anti-β-catenin (CST #8480) antibodies.

4.2.7 siRNA knockdown

HCT116 cells were transfected with siRNA against HUWE1 (AAUUGCUAUGUCU CUGGGACA) (Chen et al., 2005) or negative control siRNA (both from Genepharma). siRNAs were transfected into the cells using Lipojet (SignaGen) according to the manufacturer's protocol, and cells were harvested at 48 hr post-transfection. Lysates were resolved by 7.5% SDS-PAGE, followed by immunoblotting as described above.

4.2.8 qRT-PCR

RNA was isolated from HEK293T stable cell lines overexpressing FLAG-tagged WT HUWE1 or inactive HUWE1 C4341A mutant (HUWE1 catalytically dead mutant, HUWE1-CA) using TRIzol (Invitrogen), according to the manufacturer's instructions. RNA was reversed to the cDNA using random primers and quantitative (q)RT-PCR analysis was performed for the relative expression levels of *Axin2* and *Cyclin D1*, respectively using Qiagen qRT-PCR kit.

4.3 Results

4.3.1 HUWE1 interacts directly with β-catenin and targets it for ubiquitination

To determine the mechanism by which loss of HUWE1 leads to an increase in β-catenin protein, we first tested whether HUWE1 and β-catenin can physically interact. We expressed FLAG-tagged human wildtype HUWE1 (HUWE1-WT) in HEK293T and carried out co-immunoprecipitation experiments. We found that anti-FLAG Ab was able to co-immunoprecipitate β-catenin just as well as anti-β-catenin was able to coimmunoprecipitate with FLAG (HUWE1) (Figure 4.1A). A similar reciprocal experiment conducted in HCT116 cells to detect the interaction between endogenous β-catenin and HUWE1 yielded the same results (Figure 4.1B). We next examined whether the interaction of HUWE1 and β-catenin is binary. We incubated M2 Agarose immobilized FLAG-tagged HUWE1 purified from HUWE1-WT cells

with recombinant His-tagged β-catenin from *E. coli*. FLAG-tagged HUWE1 was able to pull down recombinant His-tagged β-catenin, suggesting that HUWE1 directly interacts with non-modified β-catenin. Thus the interaction is not dependent on the phosphorylation sites of GSK-3β (Figure 4.1C). An *in vitro* GST pull-down assay using an N-terminal truncated GST-HUWE1 protein and recombinant His-tagged β-catenin further confirm the binary interaction between HUWE1 and β-catenin (Figure 4.1D).

Because *HUWE1 cKO Apc^{min}* organoids showed such a large accumulation of β-catenin protein, we hypothesized that HUWE1 might serve as an E3 ligase regulating its stability. To test this hypothesis, we performed an intracellular ubiquitination assay in which HUWE1-overexpressing HEK293T cells (or HEK293T cells expressing control empty vector) were transfected with plasmids expressing HA-tagged ubiquitin (Ub). We then subjected lysates of these cells to immunoprecipitation with anti- β-catenin (Ab) and showed that the endogenous β-catenin protein can be polyubiquitinated in the presence of HUWE1 (Figure 4.1E). To determine whether HUWE1 does indeed directly ubiquitinate β-catenin, we performed *in vitro* ubiquitination assays. The results confirmed that recombinant full-length β-catenin protein was readily polyubiquitinated by the E3 ligase activity of an N-terminal truncated GST-HUWE1 protein (HUWE1 residues 3760-4374) (Figure 4.1F), suggesting that β-catenin is a *bona fide* HUWE1 substrate.

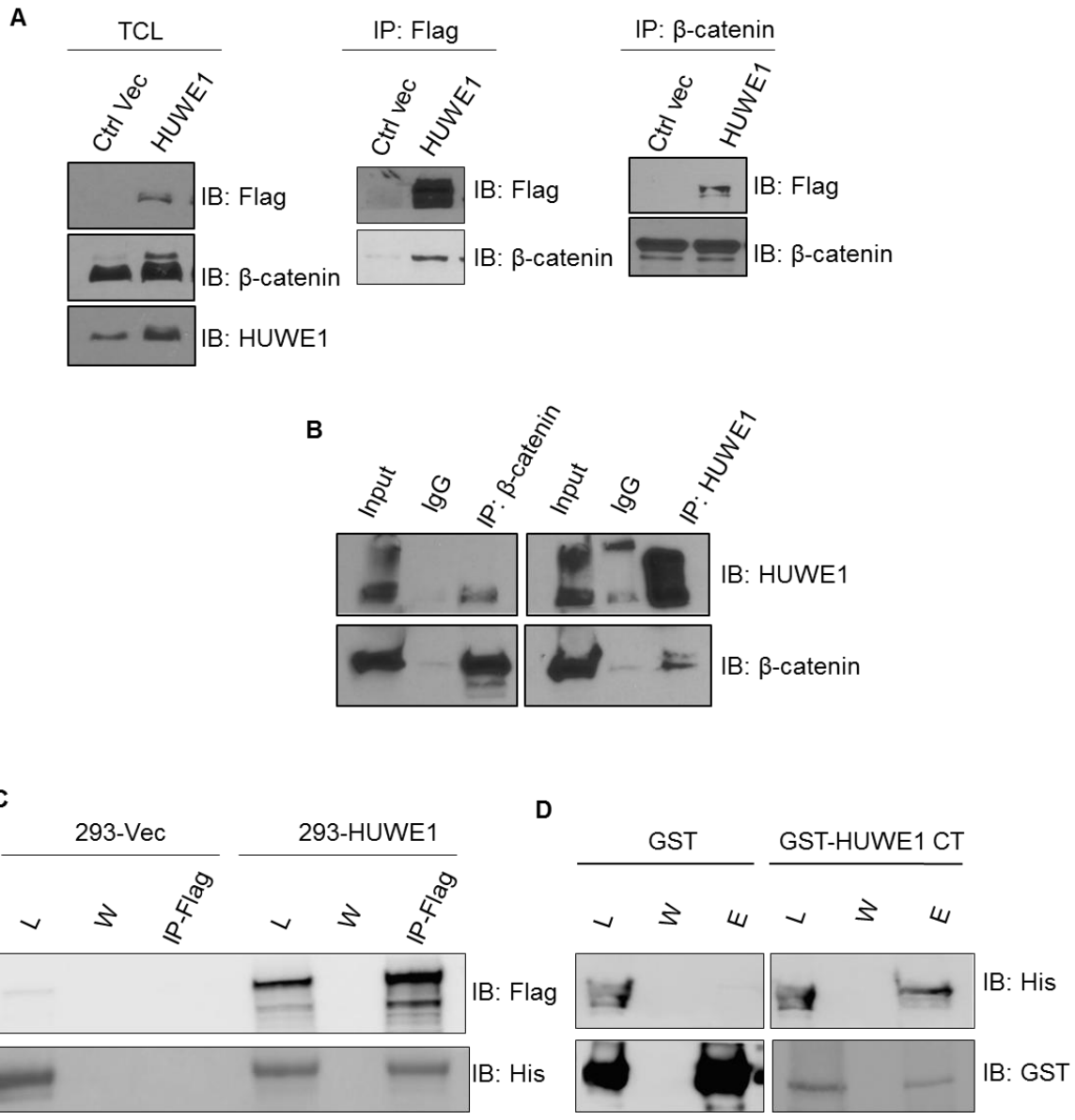


Figure 4.1 is continued in next page

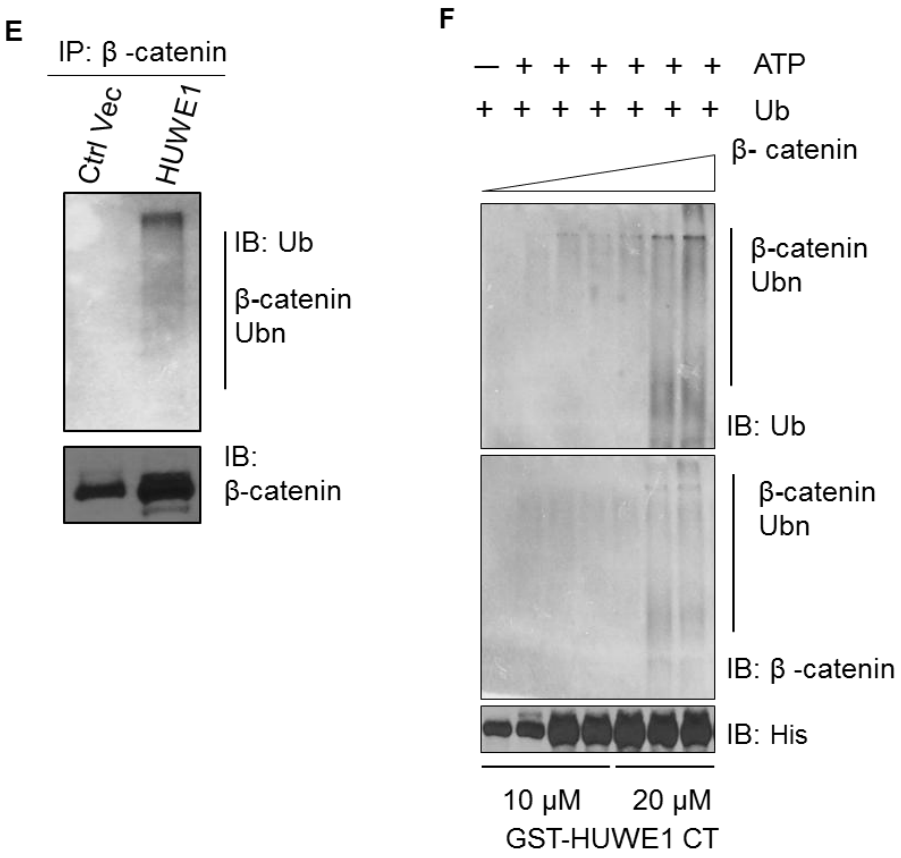


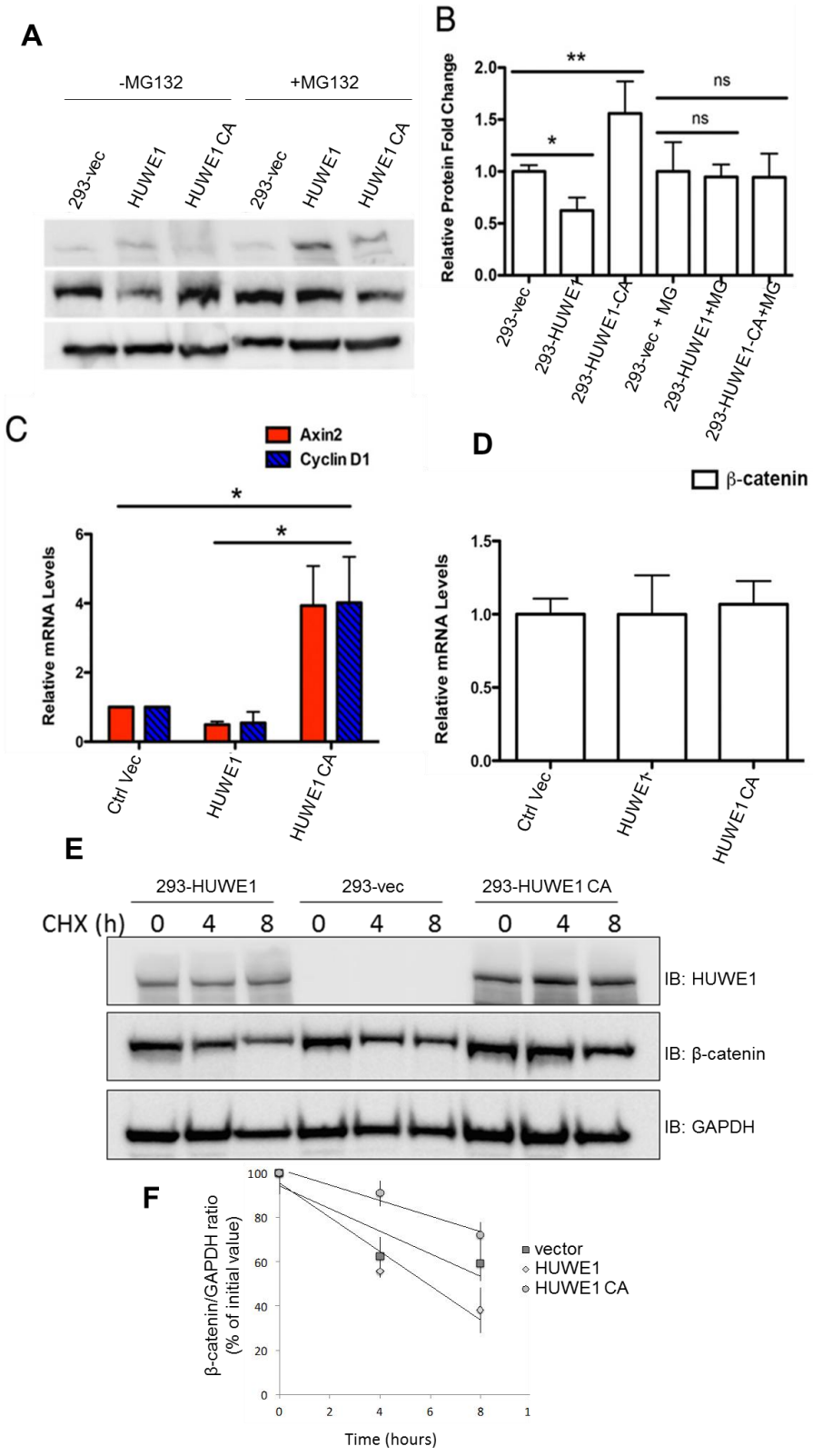
Figure 4.1: HUWE1 interacts with β-catenin *in vitro* and targets it for ubiquitination. (A) (Left) Immunoblot to detect the indicated proteins in Total cell lysates (TCL) of HEK293T cells that were stably transfected with empty vector or vector-expressing FLAG-tagged WT HUWE1 (HUWE1-WT). Immunoblot was probed with FLAG, β-catenin, and HUWE1 antibodies (Middle and Right). To detect the interaction between endogenous β-catenin and WT HUWE1, TCL was immunoprecipitated with FLAG (HUWE1) or β-catenin antibodies, and immunoblotted with FLAG (HUWE1) or β-catenin antibodies, respectively. (B) Immunoblot to detect the interaction between endogenous β-catenin and endogenous HUWE1. TCL from HCT116 cells were immunoprecipitated with β-catenin or HUWE1 antibodies, and immunoblotted with HUWE1 and β-catenin antibodies. IgG served as a control here. (C) Immunoblot to detect endogenous ubiquitinated β-catenin in HUWE1 overexpressing HEK293T cells that were transfected with vector expressing HA-Ub. Cells were treated with MG132 before harvesting to prevent β-catenin degradation and ubiquitinated β-catenin was immunoprecipitated with β-catenin, and immunoblotted with HA, and β-catenin antibodies. L and W denote for samples from input and wash, respectively. (D) *In vitro* β-catenin ubiquitination using purified His-tagged β-catenin as the substrate and N-terminal truncated GST-tagged HUWE1 (GST-HUWE1 CT) as the E3 ligase. Blot was probed with Ub, β-catenin, and His antibodies. L, W, E denote for samples from input, wash and eluate, respectively. (E) Immunoblot to detect endogenous ubiquitinated β-catenin in HUWE1 overexpressing HEK293T cells that were transfected with HA-Ub. (F) Immunoblot to detect ubiquitinated β-catenin after an in in vitro ubiquitination assay using recombinant His- β-catenin as the substrate and N-terminal truncated GST-tagged HUWE1 (GST-HUWE1-CT) as the E3 ligase. All experiments were replicated twice with similar results.

4.3.2 HUWE1 destabilizes the β-catenin protein and downregulates Wnt target gene expression

To test whether HUWE1-mediated ubiquitination of β-catenin actually destabilized it, we determined steady-state levels of β-catenin in HEK293T cells that overexpressed HUWE1-WT or HUWE1-CA and were treated (or not) with the proteasome inhibitor MG132. In line with our hypothesis, β-catenin levels were decreased in untreated cells expressing HUWE1-WT, but increased in untreated cells expressing the inactive HUWE1-CA protein (HUWE1 catalytically dead mutant) (Figure 4.2 (A and B). Conversely, MG132 treatment prevented HUWE1-mediated β-catenin destabilization, suggesting that HUWE1 can indeed target β-catenin for proteasomal degradation. Consistent with our hypothesis, HUWE1-WT-overexpressing cells showed a 2-fold downregulation of the Wnt target genes *Axin2* and *CCND1* (Cyclin D1), compared to empty vector-expressing controls; whereas these genes were upregulated 4-fold in HUWE1-CA-overexpressing cells (Figure 4.2C). The gene expression levels of β-catenin in HUWE1-WT or HUWE1-CA-overexpressing cells were similar to empty vector-expressing controls (Figure 4.2D). We next measured the β-catenin protein stability by a cycloheximide chase assay in HEK293T cells when overexpressing HUWE1-WT or the mutant. β-catenin was degraded more quickly in HUWE1-WT-overexpressing cells and became stabilized in HUWE1-CA-overexpressing cells compared to the empty vector-expressing controls (Figure 4.2 (E and F)).

We further demonstrated the ability of HUWE1 to promote the degradation of β-catenin by examining the effect of siRNA-mediated HUWE1 knockdown in HCT116 cells. HCT116 cells carry a β-catenin allele with a mutation in exon 3 that deletes the GSK-3β phosphorylation site, resulting in constitutive activation of the β-catenin protein. We found that steady-state levels of β-catenin were higher in HCT116 cells with HUWE1 knockdown compared to HCT116 cells

treated with control siRNA, but not the gene expression levels of β-catenin (Figure 4.2 (G and H)). Consistent with this result, *Axin2* and *CCND1* (CyclinD1) mRNAs were upregulated 2-fold in HUWE1 knockdown HCT116 cells compared to HUWE1-expressing controls (Figure 4.2I).



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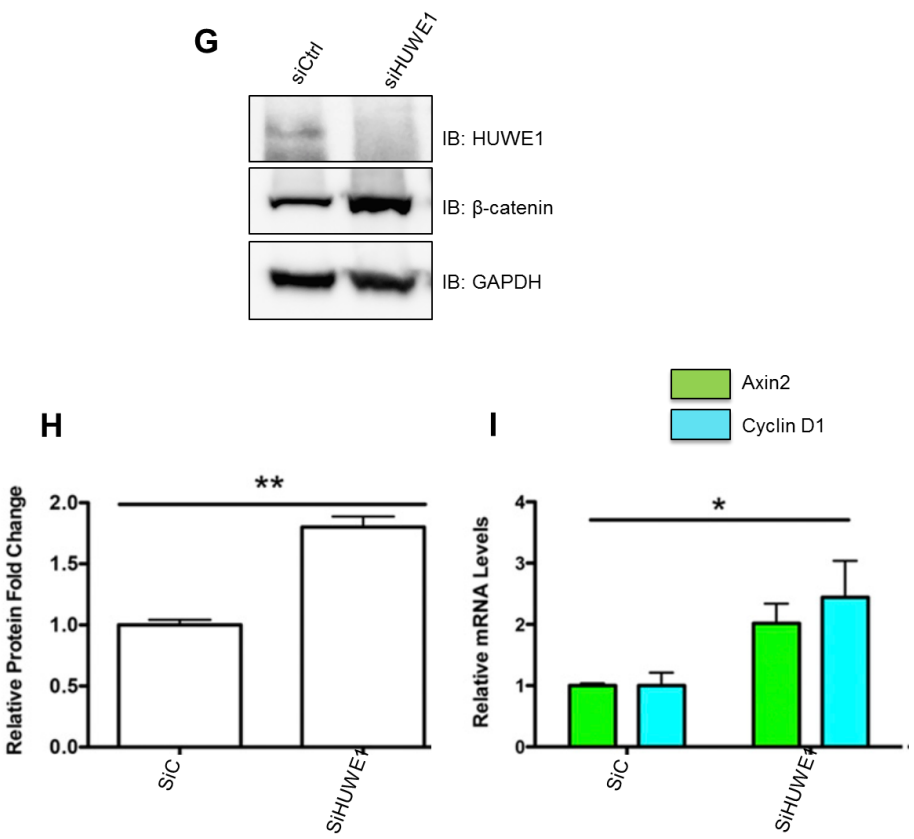

Figure 4.2: HUWE1 destabilizes β-catenin and downregulates Wnt target gene expression. (A) Immunoblot to detect the indicated proteins in total cell lysate (TCL) of HEK293T cells that were stably transfected with empty vector or vector-expressing FLAG-tagged WT HUWE1 (HUWE1-WT) or inactive HUWE1 (HUWE1-CA, catalytically HUWE1 dead mutant). Cells were treated (or not) with MG132 for 6 h before harvest. (B) Quantitative bar graph shows the relative protein fold changes of the immunoblot A. Values were determined by quantitation of bands by Image J, followed by normalization first to the loading control and then to 293T vector. (C) Quantitative (q)RT-PCR analysis of relative transcript levels of the indicated genes in HEK293T cells that were stably transfected with empty vector or vector-expressing FLAG-tagged WT HUWE1 (HUWE1-WT) or inactive HUWE1 (HUWE1-CA, catalytically HUWE1 dead mutant). Results are the mean ± SD (n = 4). (D) qRT-PCR analysis of relative transcript levels of β-catenin in HEK293T cells that were stably transfected with empty vector or vector expressing FLAG-tagged WT HUWE1 (HUWE1-WT) or inactive HUWE1 (HUWE1-CA). Results are the mean ± SD (n = 3). (E) Immunoblot shows the cycloheximide chase assay of β-catenin. (F) Shows the relative β-catenin protein levels of the immunoblot E. Values were determined by quantitation of bands by Image J, followed by normalization first to the loading control and then to (HUWE1-CA). (G) Immunoblot shows the indicated proteins in HCT116 cells upon silencing HUWE1 using HUWE1 specific siRNA or control siRNA. Immunoblot is representative of three experiments. (H) Quantitative bar graph showing relative protein fold change of the immunoblot G. (I) qRT-PCR analysis of relative transcript levels of the indicated genes in HCT116 cells that were transfected with siRNA specific for HUWE1 or control siRNA. Results are the mean ± SD (n = 3). ns, not significant. *$*P<0.05, **P<0.01$*.

4.4 Discussion

β-catenin is the main signal transducer in the canonical Wnt signaling pathway and regulates the transcription of a variety of Wnt target genes. β-catenin is predominantly a cytoplasmic protein, and is constantly degraded through phosphorylation by GSK-3β and subsequent ubiquitination by β-TRCP via ubiquitin-proteasome pathway. Ligand (Wnt) binding to the receptor drives its translocation to the nucleus where it regulate the Wnt signaling program. However, failure in controlling the hyper-activity of the β-catenin function has been implicated in a wide range of cancers and many developmental disorders. Therefore, fine tuning of the β-catenin function is essential for maintaining proper growth and development, and suppression of malignancy. In this study we identified that HUWE1, like other E3 ligases, controls β-catenin stability and directs its degradation through proteasome in constitutive Wnt signaling. Our study established that HUWE1 is a negative regulator of β-catenin in the Wnt/ β-catenin signaling pathway.

HUWE1, like many other E3 ubiquitin ligases, regulates numerous substrates. Most HUWE1 substrates have been discovered *in vitro*, and some have been found to have opposing roles in cell proliferation and tumorigenesis, such as p53 and c-Myc. For these reasons the role of HUWE1 in various tissues, whether at steady state or in disease, has been elusive. Over the past year, we and others have exploited different HUWE1 knockout mouse models to demonstrate that HUWE1 is involved in the regulation of intestinal, hematopoietic, and neural stem cells (Dominguez-Brauer et al., 2016; King et al., 2016b; Urban et al., 2016). Significantly, the developmental programs of all these tissues are driven by the Wnt pathway (Gregorieff and Clevers, 2005a; Lento et al., 2013; Lie et al., 2005). In the absence of Wnt, the protein stability of β-catenin is strictly controlled by the destruction complex, resulting in its phosphorylation and

subsequent ubiquitination by β-TRCP. In the presence of Wnt, β-catenin can accumulate and translocate into the nucleus to activate the Wnt transcriptional programme (Schuijers and Clevers, 2012). In our study, we show that, at least in a hyper proliferative setting, HUWE1 can serve as a "back up" E3 ubiquitin ligase to β-catenin to target it for degradation and thereby help to quench Wnt signaling in the intestine. Our data suggests the association between HUWE1 and β-catenin occurs under conditions where both proteins are highly expressed. We have also demonstrated that the interaction is not phosphorylation dependent (Figure 4.2D). It remains to be determined whether this regulation is tissue-specific or extends to other Wnt-driven tissues. Previously, studies have shown that ablation of HUWE1 in mice of the APC^{min} background (APC^{min} :*APC* mutation) heightens Wnt signaling to the point that intestinal crypts, which are isolated from these animals and placed in culture instantly, adopt the undifferentiated spheroid cyst-like morphology of cancer stem cell. Although this phenotype may not be entirely due to loss of HUWE1-mediated regulation of β-catenin, we can conclude that HUWE1 plays an important part in fine-tuning Wnt signaling in both the normal and diseased intestine.

These findings are significant because Wnt signaling is the main proliferative pathway in the gut that controls cell fate along the crypt/villus axis. Thus in the absence of HUWE1, constitutive Wnt signaling can drive stem cell expansion and the conversion of a normal stem cell into a cancer stem cell. In this study, through in vivo and in vitro characterization, we provide strong evidence to support the direct role of HUWE1 in the regulation of stability of β-catenin. We showed, HUWE1 overexpression could destabilize β-catenin and decrease β-catenin mediated transcription. On the other hand, silencing of HUWE1 in cancer cells, or HUWE1 knockout in APC^{min} background mouse model, results β-catenin stabilization and upregulation of Wnt target gene expressions.

HUWE1 has been shown to be overexpressed in cancers of the breast, lung, colon, liver, pancreas, thyroid, prostrate, and larynx. It is also subject to somatic mutations, homozygous deletion or amplification (http://www.cbioportal.org). In respect to colon cancer, HUWE1 is overexpressed in 50% of all cases; moreover its overexpression is higher in tumor samples compared to non-malignant tissue and increases with grade (http://cancergenome.nih.gov/publications/publicationguidelines. Mutations within HUWE1 HECT domain have been identified in patients with colon rectal adenocarcinomas (http://sanger.ac.uk). We searched available databases to specifically determine if mutations within HUWE1, whether in its functional (HECT) domain or elsewhere, would correlate differentially with expression. While we found no significant difference in expression with mutational site, no conclusion can be made given the small number of tumors reported. At this point, we favor the hypothesis that a mutation within HUWE1, regardless of location, would alter its structure in such a way that either it will not effectively bind its substrates or will render it inactive.

In conclusion, our in vitro and in vivo efforts have shown that HUWE1 regulation of the Wnt pathway extends to include its main signal transducer, β-catenin. We have demonstrated that HUWE1 plays a vital role in fine-tuning the Wnt signaling in the diseased intestine. Thus, HUWE1 has an indirect but important influence on intestinal stem cell self-renewal and cell fate.

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Chapter 5

SUMMARY & FUTURE DIRECTIONS

5.1 Summary of the project

Ubiquitin serves as an intracellular signal to regulate a multitude of cellular processes once covalently attached to the target protein. A sequential enzymatic cascade consisting of E1-Ub activating enzyme, E2-Ub conjugating enzyme, and E3-Ub ligase, attaches Ub to the substrate protein in an ATP-dependent manner. Among them, Ub E3 ligases play prominent roles in conferring the substrate specificity in this regulatory process. Thus, understanding the structure and function of E3 ligases, especially how they mediate substrate ubiquitination, would provide mechanistic insights about their roles in cellular pathways. As a functionally important Ub E3 ligase, HUWE1 modulates multiple cellular pathways through catalyzing substrate ubiquitination in order to alter substrate protein function and fate. HUWE1 is a multi-domain, large HECT domain containing E3 ligase. However, only the structure of the C-terminal HECT domain was characterized when this study started in 2011. Therefore, a multidisciplinary approach was taken to elucidate the structure and function of this important ubiquitin E3 ligase within the cell.

In this dissertation, we focused on characterization of new functional domains of HUWE1 and reported a new tandem Ub-binding motif of HUWE1. We investigated the structure and function of this motif and examined how this motif modulates HUWE1 activity. Our study, for the first time, demonstrates that HUWE1 contains this novel Ub binding motif, consisting of three independently folded Ub-binding units which enhance HUWE1-mediated polyubiquitination. Most significantly, we discovered that the inherited X-linked mental retardation mutation, R2981H that lies within the first motif of tandem UBMs is a structural mutant, which attenuates HUWE1-mediated polyubiquitination.

In this study, we also investigated the mechanism of HUWE1 inhibition by ARF. We identified the region of ARF that is responsible for the inhibition of HUWE1 function. We also discovered an ARF peptide derived from the HUWE1 binding region that could suppress cancer cell growth by inhibition of HUWE1 function and activation of p53 transcription.

To advance our current understanding of HUWE1 function, we collaborated with Dr Tak Mak at Campbell Family Institute for Breast Cancer Research to discover new HUWE1 cellular substrate proteins by employing a combined genetic and biochemical approach. We found that HUWE1 directly binds and ubiquitinates β-catenin, a master signal transducer in the canonical Wnt signalling pathway. This study has significant implications in understanding the role of HUWE1 in tumorigenesis in colorectal cancers.

In these final remarks, we summarize the most important findings from this study and focus on the challenges encountered during the investigation, as well as future directions for studying HUWE1 function.

5.2 Discovery of a tandem Ub-binding motif and its role in HUWE1-mediated polyubiquitination highlights a new role for the Ub-binding domain

In chapter 2, we reported that HUWE1 contains a tandem ubiquitin binding motif consisting of three independently folded Ub-binding domains. It could act as a *cis*-element to modulate HECT domain containing E3 ligase activity. Our biochemical and biophysical data confirmed that each motif in tandem binds mono-Ub independently. Similarly, with the majority of UBDs reported so far (Husnjak and Dikic, 2012), each motif within the tandem UBM of HUWE1 interact with Ub in a similar mode. Our 2D HSQC NMR data confirmed that all three UBMs make contact with Ub through the canonical hydrophobic surface patch formed by the residues Leu8, Ile44 and Val70 on Ub. The same surfaces were also recognized by the

polymerases ι ubiquitin-binding motif (Bomar et al., 2010; Burschowsky et al., 2011) (Ub-binding domains of Translesion polymerases were referred to as UBMs). As per our knowledge, HUWE1 is the first and only HECT Ub E3 ligase that contains a tandem Ub-binding motif. No other E3 ligase had been shown to possess tandem Ub-binding domain previously. This study uncovered two important aspects of the presence of tandem UBDs in HUWE1. One is the preferential binding of tandem UBM to di-and tri-Ub linkages, and the other is tandem UBM induced enhancement of the poly-ubiquitination chain synthesis by HUWE1 HECT domain.

This class of Ub-binding domains was reported previously in Translesion DNA polymerases that bind mono-ubiquitinated PCNA. These were essential in localizing the polymerases to the replication foci during DNA damage signals (Bomar et al., 2010; Burschowsky et al., 2011). Though a recent study identified the PCNA binding motif in HUWE1 and demonstrated that HUWE1 modulates cell survival during DNA damage response, the mechanism of how HUWE1 is targeted to the stalled replication foci is unknown (Choe et al., 2016). Therefore, it would be worthwhile to investigate further whether the HUWE1 tandem motif is involved in localizing it in replication foci.

Tandem UBDs in Ub receptors (UBD containing proteins, not E3s) were often found to perform diverse functions. These include recognition of specific Ub linkages, cooperation within multiple UBDs to achieve stronger binding affinity, sensing the Ub chain length and protecting Ub chains from elongation/degradation. Moreover, the tandem UBDs in Ub receptors were often found to influence the host protein functions (Hawryluk et al., 2006; Ikeda et al., 2010; Laplantine et al., 2009; Nicastro et al., 2009; Nicastro et al., 2010; Reyes-Turcu et al., 2008; Sato et al., 2009a; Sims and Cohen, 2009; Sims et al., 2009; Swanson et al., 2003; Tyrrell et al., 2010). The biophysical data from this study illustrates that as a tandem, Ub-binding motif of

HUWE1 interacts with di- and tri-Ub-linked chains 10 times more strongly compared to the mono-Ub, suggesting that individual Ub-binding motifs in tandem cooperate with each other to increase the Ub-binding affinity. It also showed preferences to bind linear and Lys63-linked chains over Lys48-linked chains. The ability of HUWE1 tandem motif to interact with different linkages suggests that HUWE1 interactions with differentially ubiquitinated proteins may allow it to regulate multiple cellular pathways. Previous studies of cIAPs (a RING domain E3) show that UBA domains provide a platform to bind with polyubiquitinated partners, (such as NEMO, RIPI or itself in the signaling pathway). Thereby, c-IAP1 interacts with polyubiquitinated NEMO (by interacting both with Lys63- and Met1-linked chains attached in NEMO) in activating the NF-κB signaling pathway (Blankenship et al., 2009; Silke and Meier, 2013). HUWE1 may also utilize its tandem motif to preferentially recognize different polyubiquitinated proteins and thereby regulate specific cellular signalling pathways.

5.3 The molecular basis of XLMR mutation of HUWE1, R2981H, underscores the role of a disease related point mutation in HUWE1 function

Another important finding of this study is uncovering the molecular basis of the R2981H mutation of HUWE1. This mutation was discovered in affected males of a severe to profound X-linked mentally retarded (XLMR) family, along with two other mutations through genomic sequencing (Friez et al., 2016; Froyen et al., 2008). The R2981 residue of HUWE1is highly conserved throughout evolution and resides within the UBM1 motif. The R2981H is the only one out of three familial mental retardation mutations that is located outside of the HECT domain. The other two are located in the HECT domain. This emphasizes the important function of this R2981H residue. This mutation co-segregated with mental retardation phenotypes in the family but was absent in the control group. Therefore, it was hypothesized that this mutation is

detrimental to HUWE1 function and is associated with the XLMR phenotype of the affected individuals. Our structural and biophysical data established that the R2981H mutation is a structural mutant. UBM1 structural data illustrates that R2981 is important for stabilizing the C-terminal helix of the UBM1 motif and the overall structural integrity of the motif (Figure 4C & 4D).Therefore the substitution of R2981 to R2981H diminished the structural fold by destabilizing the C-terminal helix within the structure. Consequently, R2981H disrupted the binding and affinity of UBM1 to Ub in *in vitro* biochemical and biophysical assays and interrupted the HUWE1 auto-and p53 ubiquitination by HUWE1. Our results established the importance of structural stability of the HUWE1 tandem Ub-binding motif and its essential role in modulating HUWE1 activity and function. It also highlights the significance of a disease related mutation in HUWE1-dependent cellular processes. The mechanistic insights from this study may be useful in designing innovative therapy to control XLMR related diseases in near future. However, more research needs to be done to validate our findings in neuronal cell lines and in animal modelling.

5.3.1 The potential roles of tandem Ub-binding motif in different cellular pathways regulated by HUWE1

Although current studies have identified a previously uncharacterized tandem Ub-binding motif and its role in HUWE1 function, further analyses are required to confirm its role in the context of full length HUWE1 protein. For example, our study showed that tandem Ub-binding motif enhanced HUWE1 HECT domain-mediated polyubiquitination both *in vitro* and *in vivo* assays. However, in the current study, all cell based and *in vitro* assays were performed using truncated HUWE1 lacking the N-terminal 2950 residues. Due to technical limitation and difficulty, we were not able to generate the 500 kDa HUWE1 full length protein and the UBM

mutant; however, we did test the UBM function in isolation i.e. UBM alone as well as UBM together with HECT domain. We feel our current study characterized the role of the isolated UBM in binding Ub and its effect on HECT domain mediated ubiquitination. Therefore, the first and foremost task ahead is to clone a HUWE1 full length mutant construct lacking the tandem Ub-binding motif and investigate the function of tandem UBM in the context of the full length HUWE1 protein.

The tandem Ub-binding motif of HUWE1 binds both mono and polyUb chains. However, the binding assays were performed using the isolated tandem motif of HUWE1 and mono-Ub/Ub-linkages. Therefore, it is important to know the cellular context of its Ub binding preferences, as some UBDs have shown variable preferences in *in vitro* studies versus *in vivo* complex formation with Ub linkages. For example, though the structural studies of UBAN domain of NEMO with di-Ub linear chains suggested that parallel UBAN dimer contacts all four Ubs in two linear di-Ub chains, cellular studies found complexes of NEMO both with Lys63- and Lys11-linked linkages (Bianchi and Meier, 2009; Dynek et al., 2010; Lo et al., 2009; Rahighi et al., 2009; Wertz et al., 2004)(UBD of NEMO is referred to as UBAN domain). Therefore, further experiments should include the pull-down of HUWE1, and utilize LCMS to identify the specific linkages attached on the tandem motif of HUWE1 and the relevant substrates involved once HUWE1 protein contains tandem Ub-binding motif. That will pinpoint the distinct role of tandem Ub-binding motif as to whether it aids the oligomerization of HUWE1, or it provides the specific binding surfaces that are required to recruit HUWE1 in sub-cellular organelles in order to regulate specific substrates.

Recently HUWE1 has been found to bind PCNA during replication stress. However, how HUWE1 is recruited to the stalled foci has not been studied yet. UBMs of Translesion

polymerases were found to recruit polymerases to the stalled replication foci. Further experiments could be done to examine whether the HUWE1 tandem Ub-binding motif performs a similar function. Previous studies also showed that Ub-UBD interaction is required for host protein function and substrate regulation. For example, the interaction between Ub and the UBA domain of Cbl-b (a RING E3 ligase that regulates the fate of receptor tyrosine kinases (RTK) via ubiquitination) aids the dimerization of Cbl-b. This dimerization event allows efficient phosphorylation of Cbl-b which in turns recruits the Cbl-b to the activated RTKs. Disruption of the Ub-UBA interaction through mutation impaired the regulation of RTKs by Cbl-b (Peschard et al., 2007; Schiering et al., 2000; Shih et al., 2003). UBA domains of other receptors also have been reported to play a role in localization. For example, recognition of Lys63-linked chains on histones by the tandem UIM of RAP80 protein are essential in recruiting BRCA1/BRAD1 E3 ligase complexes to the foci to start DNA repair (Berke et al., 2005; Sims and Cohen, 2009) (two UBDs of receptor-associated protein 80; UBDs of RAP80 are known as UIM). HUWE1 also regulates the stability and functioning of approximately 15 proteins that are involved in DNA repair, development, apoptosis and tumorigenesis. Therefore further studies should address specific biological pathways and examine the involvement of the tandem Ub-binding motif in these pathways.

We solved the solution structure of the UBM1 motif. Our NMR mapping experiments suggested all three UBMs bind Ub through the canonical hydrophobic patch formed by the residues Leu8, Ile44 and Val70 on Ub. To validate these results the complex structure of Ub-UBM1 could be explored further. A similar strategy will also be useful for Ub-UBM2 and Ub-UBM3 complexes to complete our understanding of Ub interaction modes of HUWE1 UBMs. Alternatively, crystallography could be another approach in achieving the high resolution atomic

details of tandem Ub-binding motif, alone and in complex with linear- and Lys63-linked tri-Ub chains, as it showed stronger affinities to these linkages.

Our study revealed that R2981H mutation disrupts the UBM1 structure, which in turn, impairs UBM1 binding to Ub. To investigate the function of tandem UBM in the context of the HUWE1 HECT domain-mediated E3 ligase activity, we found that tandem UBM stabilized and enhanced auto-and p53 ubiquitination by HUWE1. However, HUWE1 R2981H appeared to be less stable and showed less enhancement effect compared to the WT protein. Therefore, it would be important to investigate the effect of this mutation on other HUWE1 substrates, and address their relevant biological pathways. HUWE1 was found to be involved in regulating Myc family proteins in mouse developing brain. As R2981H was discovered in neuropathic patients, further experiments should be carried out in neuronal cell lines and brain specific tissues to investigate its effect on HUWE1 substrates in these cells. Finally, the effects of this mutation should be investigated in model animals by creating a R2981H mutant mouse. This model would allow for examining whether the R2981H mutation causes similar cognitive impairment symptoms as found in patients, and also in findings of HUWE1 specific substrates related to this disease. All together these may help in designing new therapy in the near future to control the neuropathological disorders related to the HUWE1 mutation, R2981H.

5.4 Molecular mechanisms of ARF peptide-induced cancer cell growth suppression by inhibiting HUWE1-mediated p53 ubiquitination

Protein-protein interactions are crucial in regulating key signaling pathways, and therefore have serious consequences once deregulated and often lead to fatal diseases like cancers. Therefore, development of the right tools, such as targeted drug-like molecules in perturbing protein-protein interaction, has gained significant attention in the research community. For

example, several small molecules targeting p53-MDM2 interactions have advanced to preclinical and clinical phase trials in treating caners retaining wild-type p53 (Chene, 2003; Midgley et al., 2000; Pellegrino et al., 2015; Teveroni et al., 2016; Zhang et al., 2015; Zhao et al., 2015). However, the initial research was launched based on the optimization of small region/essential residues involved in p53-MDM2 interaction and the final co-crystal structure (Kussie et al., 1996; Pazgier et al., 2009; Uhrinova et al., 2005). Similarly, the essential ARF residues that interact with MDM2 were also optimized before developing small molecules (Bertwistle et al., 2004; Clark et al., 2002).

Previous work has shown that the tumor suppressor ARF inhibits HUWE1 functioning and activates p53 transcription by inhibiting HUWE1-mediated p53 ubiquitination (Chen et al., 2005). In this project, as reported in chapter 3, we investigated the mechanisms of how ARF could inhibit HUWE1 function and how ARF could liberate p53 by inhibiting HUWE1-mediated p53 ubiquitination. Herein, through biochemical and biophysical approaches, we provide evidence showing that ARF modulates the activity of HUWE1 through interacting with the N-terminus of the HUWE1 HECT domain. Our results demonstrate that three ARF peptides derived from the N-terminus of human ARF can inhibit HUWE1-mediated auto- and p53 ubiquitination *in vitro*. Our results also demonstrated that the minimal HUWE1 region required for p53 ubiquitination is between residues 3951-4374. ARF peptides also interacted with the same region of HUWE1, suggesting that HUWE1 utilizes the same area to interact both with p53 and ARF. Most significantly, all three peptides were able to bind HUWE1 and p53 *in vivo* once overexpressed as YFP-fusion proteins, indicating ARF has multiple binding sites to interact both with HUWE1 and p53. However, our results confirmed that the 8mer ARF peptide showed strongest inhibition of HUWE1 activity, which induces prominent p53 activation in cancer cells

(Figure 3.7B). This peptide also inhibits cancer cell growth in the p53-dependent manner once overexpressed as a YFP-fusion protein. It is very important that this 8mer peptide does not overlap with the MDM2 binding site of ARF, which resides within the first 20 residues in human ARF (Midgley et al., 2000; Wang et al., 2017).

Our study identified a new ARF peptide that showed potential to suppress cancer cell growth through inhibiting HUWE1 activity in cells that retain wild-type p53. We also identified the mechanism using HDX (Hydrogen to Deuterium exchange) Mass Spectrometry that showed the ARF peptide directly binds to the conserved α1 helix and the loop of the N-lobe of the HUWE1 HECT domain. This binding also affects the peptide hinge that connects the two lobes of the HECT domain, which in turn affects the conformational state of the enzyme and keeps the enzyme in an inactive state. Crystal structures of HECT domains (E6-AP, WWP1 and NEDD4L bound to E2 enzymes) and the model of HUWE1 HECT domain with NEDD4L/E2~Ub complex, show that this α1 helix/loop and adjacent β strand forms the concave binding surface for E2~Ub thioester during Ub catalysis by HECT domains (Huang et al., 1999; Kamadurai et al., 2009; Pandya et al., 2010; Verdecia et al., 2003). Recently, Mund *et al* (2014) showed that bicyclic peptides (developed by phage display) target the E2 binding sites of several HECT E3s including HUWE1, and were capable of blocking their E3 ligase activity in *in vitro* assays (Heinis et al., 2009; Mund et al., 2014). These findings reemphasize that HECT domains are the potential druggable targets, and therefore demand more research in this field.

In the Ub-proteasome system, targeting E3 ligases is the desired approach as they provide substrate specificity. Though several small peptides and synthetic molecules targeting p53-MDM2 interaction are being tested in preclinical trials, nothing is known about p53-HUWE1 interaction. Our study clearly demonstrates that the ARF peptide directly impairs HUWE E3

ligase activity allosterically through interaction with the α1 helix and hinge linker of the HUWE1 HECT domain. It also affects enzyme conformational flexibility to keep the enzyme in an inactive state. Therefore, the identified ARF peptide presents therapeutic potential as a HUWE1 specific E3 ligase inhibitor.

5.4.1 Future directions for ARF mediated HUWE1 inhibition.

Although the ARF peptide inhibits cancer cell growth, the binding affinity is low compared to the reported nano-molar affinities of other potential peptides/peptidomimetic candidates (Zhao et al., 2015). To optimize the HUWE1 binding sequence, further mapping and mutational study could be pursued. We could mutate every amino acid of the 8mer peptide and examine the effect of the mutation on the binding affinities of HUWE1. Most importantly, co-crystallization of the ARF peptide and HUWE1 would be the best approach to get atomic resolution information about ARF and HUWE1 interaction. Finally, as the YFP-ARF peptide inhibits HCT116 cell growth, further *in vivo* experiments should be pursued in xenograft models to validate the efficiency and potency of the ARF peptide in tumor suppression.

5.5 HUWE1/HUWE1 directly binds and targets β-catenin for proteasomal degradation in the canonical Wnt signaling pathway

HUWE1 regulates multiple substrates that are involved in diverse cellular pathways. Thus, understanding the network of cellular substrates regulated by HUWE1 would further strengthen our knowledge of the functioning of HUWE1 within the cell. The current list of HUWE1 substrates is far from complete, which limits our understanding of the role of HUWE1 in physiology and diseases. Our goal is to identify the cellular substrates of HUWE1. The majority of the HUWE1 substrates were discovered using *in vitro* assays. Recently, several studies using conditional knockout mouse models demonstrated that HUWE1 is involved in the regulation of

tissue homeostasis in intestine, hematopoietic and neural stem cells (Dominguez-Brauer et al., 2016; Fatehullah et al., 2013; Hao et al., 2012; King et al., 2016a; Zhao et al., 2009). In this study, we used intestine specific knock out mice or ex vivo organoids to identify new substrates (Dominguez-Brauer et al., 2016). We also developed a pair of cell lines which stably express HUWE1 WT and HUWE1 CA mutant (catalytic dead mutant of HUWE1). This is a very powerful technique that directly evaluates the function of a protein in a specific organ. Using this approach, we identified one new substrate, β-catenin, which is the main signal transducer in Wnt signaling pathways, and very often is implicated in various diseases once it is deregulated. We found that HUWE1 directly binds and ubiquitinates it for proteasomal degradation in order to control excessive β-catenin levels in cells.

Wnt signaling plays a central role in the maintenance of tissue homeostasis in adult tissues including intestine, and controls organogenesis during embryonic development. Therefore, deregulation in Wnt pathways can culminate in many diseases, including cancer. Cells have evolved several precautionary mechanisms to acieve proper development and the evasion of cancer. Our study showed that HUWE1 regulates β-catenin levels in constitutively active Wnt signals. APC is a component of the destruction complex and the main negative regulator of the Wnt pathway in intestine. Previous studies showed that double mutant mice (*HUWE1 cKO APC* ^{min}) developed intestinal adenoma and spheroid like cysts faster compared to the *APC* ^{mni} *mice* alone, which correlates with enhanced Wnt gene expression. Further studies also showed that HUWE1 loss in this background promotes intestinal stem cell proliferation and expansion (Dominguez-Brauer et al., 2016). These results suggest that HUWE1 may regulate Wnt signalling downstream of APC in intestine.

Therefore along with genetic studies which were performed in the Tak Mak lab, we employed cellular and biochemical techniques to define the direct role of HUWE1in regulating β-catenin stability and its fate in cells. We used stable HEK293T cells expressing HUWE1 WT and HUWE1 catalytic mutant (HUWE1 CA mutant) that allow comparison of the catalytic activity of HUWE1 directly. We performed cellular ubiquitination assays through immunoprecipitating the endogenous β-catenin, and showed that β-catenin can be polyubiquitinated by HUWE1 in the cell (Figure 4.2E). We also performed *in vitro* ubiquitination assays using recombinant full-length β-catenin and N-terminally truncated GST-HUWE1 protein. These assays confirmed that β-catenin was readily polyubiquitinated by HUWE1 ((Figure 4.2F), suggesting β-catenin is a bona fide HUWE1 substrate. We further provide evidence that β-catenin and HUWE1 interact *in vivo* by reciprocal co-immunoprecipitation. The binary interaction between β-catenin and HUWE1 was also confirmed in 293T and HCT116 cells. Once the phosphorylation site of β-catenin is mutated, it escapes regulation by the APC in the intestine, which in turn drives tumor formation. We have demonstrated that siRNA-mediated silencing of HUWE1 in HCT116 cells upregulates β-catenin, which results in transcriptional activation of its downstream targets *Axin2* and *CCND1*.

Although our study clearly demonstrated that β-catenin is the bona fide substrate of HUWE1 in the intestine, it remains to be answered what other components of Wnt signaling are regulated by HUWE1. It would be important to study whether this regulation is tissue specific or extends to other Wnt targeted tissues, and its role in tumorigenesis. HUWE1 is the second most mutated protein after APC in intestinal cancer. Therefore it is important to further investigate the mechanisms of interaction between HUWE1 and β-catenin. These studies may provide useful information in designing rational therapy to treat colon cancer caused by HUWE1 mutation.

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Appendix-A

E3 Ubiquitin Ligase HUWE1 Targets β-Catenin under Conditions of Hyperactive Wnt Signaling

Carmen Dominguez-Brauer[a], Rahima Khatun[b], Andrew J. Elia[a], Kelsie L. Thu[a], Parameswaran Ramachandran[a], Shakiba P. Baniasadi[a], Zhenyue Hao[a], Lisa D. Jones[a], Jillian Haight[a], Yi Sheng[b,1] and Tak W. Mak[a,1]

[a]The Campbell Family Institute for Breast Cancer Research, Ontario Cancer Institute, University Health Network, Toronto, Ontario M5G 2C1, Canada

[b]Department of Biology, York University, Toronto, Ontario M3J 1P3, Canada

Appendix-B

To Whom It May Concern

This is RAHIMA KHATUN, a PhD candidate in the Biology Department, York University, Toronto, Canada. At 5th year, I was awarded a full-time trainee position at Sanofi Pasteur, Toronto site, Canada (a contract research position funded by Sanofi-Mitacs Accelerate Award for one year (Nov, 2015 to Sep 2016)).

During this period, I successfully developed the NMR (Nuclear Magnetic Resonance Spectroscopy) based quantitative analytical methods to analyze impurities for a new patentable cancer vaccine candidate (first project). For this project, I wrote one technical report and published one journal article (copy attached). I also developed NMR based analytical methods to quantitate Aluminum based adjuvants in intermediate drug substances, and final vaccine products, and wrote a technical report (second project). Currently, I am writing the journal article for the second project and York University/Sanofi gave me one more year contract for further development of the project.

Additionally, I worked for two more projects: one in our lab that is published in JBC (copy attached) and the other one with Dr Derik Wilson's group, Chemistry, York University. This project also published in Biochemistry (copy attached).

Sincerely,

Rahima Khatun
PhD candidate
Biology Department, York University, Toronto, Canada

Appendix-C

Table 3.1: List of HUWE1 proteins and the corresponding primer sequences, generated for this study.

Protein name	Vectors	Primer sequence
HUWE1 UBM1 (2951-3003)	pET15b	Nde1 For: 5`GCGGCGGCCCATATG ACAAGCAGTGAAGAAGAAGAT3` BamH1 Rev: 5`GCGCGGATCCTTAGCTATTTGTGGAGGGGGCAGTC3`
HUWE1 UBM1 (3004-3052)	pET15b	Nde1 For: 5`GCGGCGGCCCATATGTCAGCGCCTGCAGTGGTGGGGA3` BamH1 Rev: 5`GCGCGGATCCTTATGAGCTGGCATTCTGTGCTAG3`
HUWE1 UBM1 (3053-3123)	pET15b	Nde1 For: 5`GCGGCGGCCCATATGGACACCCCTATGGACCCTCTG3` BamH1Rev:5`GCGCGGATCCTTAAGCAGAGAGTGCGGAGGT GCTAC3`
HUWE1 UBM1 (2951-3123)	pET15b	Nde1 For: 5`GCGGCGGCCCATATGACAAGCAGT GAAGAAGAAGAT3` BamH1Rev:5`GCGCGGATCCTTAAGCAGAGAGTGCGGAGGT GCTAC3`
HUWE1 R2981H (2951-3123)	pET15b	For: 5`CTGCCTGTAGACATCCATCGGGAAGTTCTACAG3` Rev: 5`CTGTAGAACTTCCCGATGGATGTCATCAGGCAG3`
tUBM-CT (2951-4374)	pcDNA3.1	BamH1 For: 5`GCGCGGATCCAACAAGCAGT GAAGAAGAAGAT3` Xba1 Rev: 5` GCGCTCTAGATTAGGCCAGCCCAAAGCCTTCAGAG3`
ΔUBM-CT (3123-4374)	pcDNA3.1	BamH1 For: 5`GCGCGGATCCAATTCTCCGAAGCCCGGCTTTC3` Xba1 Rev: 5` GCGCTCTAGATTAGGCCAGCCCAAAGCCTTCAGAG3
R2981H-CT (2951-4374)	pcDNA3.1	For: 5`CTGCCTGTAGACATCCATCGGGAAGTTCTACAG3` Rev: 5`CTGTAGAACTTCCCGATGGATGTCATCAGGCAG3`
HECT domain (3760-4374)	pcDNA3.1	BamH1 For: 5`GCGCGGATCCAA AGCATCCAGGCAGCTGTTCGG3` Xba1 Rev: 5` GCGCTCTAGATTAGGCCAGCCCAAAGCCTTCAGAG3`
tUBM-HECT	pcDNA3.1	BamH1 For: 5`GCGCGGATCCAACAAGCAGT GAAGAAGAAGAT3` Xba1 Rev: 5` GCGCTCTAGATTAGGCCAGCCCAAAGCCTTCAGAG3`

R2981H-HECT	pcDNA3.1	For: 5`CTGCCTGTAGACATCCATCGGGAAGTTCTACAG3` Rev: 5`CTGTAGAACTTCCCGATGGATGTCATCAGGCAG3`
tUBM-HECT	pET28a	Nde1 For: 5`GCGGCGGCCCATATG ACAAGCAGTGAAGAAGAAGAT3` BamH1Rev:5`GCGCGGATCCTTA GGCCAGCCCAAAGCCTTCAGAG3`
R2981H-HECT	pET28a	Nde1 For: 5`CTGCCTGTAGACATCCATCGGGAAGTTCTACAG3` Bamh1 Rev: 5`CTGTAGAACTTCCCGATGGATGTCATCAGGCAG3`
HECT domain (3951-4374)	pcDNA3.1	BamH1 For: 5`GCGCGGATCCAAGACACACAGAAGTTCCTTCCC5` Xba1 Rev: 5` GCGCTCTAGATTAGGCCAGCCCAAAGCCTTCAGAG3`
ARF FL 1-393	pcDNA3.1	ARF1 For: 5` CGCCGCAAGCTTATGGTGCGC AGGTTCTTGGTG 3` ARF 393 Rev: 5`CGCCGAATTCTTAGCCAGGTCCACGGGCAGACGG 3`
ARF N 1-64	pcDNA3.1	ARF1 For: 5` CGCCGCAAGCTTATGGTGCGCAGGTTCTTGGTG 3` ARF64 Rev: 5` CGCCGAATTCTTATGGTCTTCTAGGAAGCGGCTG 3`
ARF 17-30	pcDNA3.1	ARF17 For: 5`TATGCCGCCGCGAGTGAGGGTTTTCGTGGTTCACATCCCG CGGCTCT 3` Rev: 5`GATCCTCAGAGCCGCGGGATGTGAACCACGAAAACCCTC ACTCGCGGCGGTG 3`
ARF 45-58	pcDNA3.1	ARF 45 For: 5`TATGCTCGTGCTGATGCTACTGAGGAGCCAGCGTCTAGG GCAGCAGT3` Rev: 5`GATCCTCACTGCTGCCCTAGACGCTGGCTCCTCAGTAGCA TCAGCACGAGTG 3`
ARF 45-52	pcDNA3.1	ARF 45 For: 5`TATGCTCGTGCTGATGCTACTGAGGAGCCAGCGTCTAGG GCAGCAGT3` Rev: 5`GATCC TCAGCTCCTCAGTAGCATCAGCACGAGTG 3`