2021

# Development of Linked-Domain Protein Inhibitors of the E2-Conjugating Enzyme Ube2D

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# DEVELOPMENT OF LINKED-DOMAIN PROTEIN INHIBITORS OF THE E2-CONJUGATING ENZYME UBE2D

By

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A Thesis Submitted to the

Graduate School

In Partial Fulfillment of the

Requirements for the Degree of

MASTER OF SCIENCE

Thomas J. Long School of Pharmacy and Health Sciences
Pharmaceutical and Chemical Sciences

University of the Pacific
Stockton, California

2021

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# DEDICATION

In loving memory of my grandfather Ton Nederkoorn (July 1936 – November 2020)

# ACKNOWLEDGEMENTS

To start, I would like to thank my advisor Dr. Joseph Harrison for his guidance and support throughout these past couple of years. He has exposed me to so many different techniques, opportunities, and other researchers to work with, all of which have provided me with a well-rounded scientific education. This work could never have been completed without his help. He is also to thank for the fact that I will never seize to view chemistry undergraduates as 'Young Chemists'.

This work is the result of a large collaborative effort, for which I have many to thank. My sincere thanks to Dr. Jose Henrique Pereira and Dr. Petrus Zwart from Lawrence Berkeley National Lab, who had a substantial impact on the preliminary UUD1.1~Ube2D1 crystallization attempts.

Secondly, my gratitude goes out to Dr. William Chan and Yujie Yang for helping me with the early cell culture work, as well as the Far Western Assays. Yujie has been a great resource and was always willing to answer my many questions about cell culture.

Thirdly, I would like to acknowledge Dr. Geoff and Joan Lin-Cereghino. Their lab was essentially my "second lab home". I want to thank them for their ever so positive spirit, their generosity and of course their extremely cute dog Peaches.

I must thank Dr. Liang Xue for letting me use his lab's equipment, especially his imaging apparatus and the ITC. I also thank Vanessa Rangel for her support in setting up the ITC experiments. Specifically, I would like to acknowledge the NSF major instrument grant (MRI-1828179) which funded the ITC experiments.

My sincere gratitude goes out to Dr. Craig Vierra for allowing me access to his dissection microscope and his Orbitrap instrument and teaching me the basics of proteomics.

I would like to acknowledge my lab mates Vinh Truong and Li Gu for their encouragement throughout my research. Vinh, I will never forget that you nominated me for secretary of AAPS in my first year, thanks for that. Li, your calming presence has really gotten me through some rough patches. I really feel like I gained a true friend in you.

My appreciation goes out to my thesis committee and their support in writing this work: Dr. Chan, Dr. Tsai and Dr. Pantouris. George thank you for all the helpful life advice, I will make sure that I start my PhD on the right foot.

Lastly, Faith and Sonya, thank you for all you do, I do not know where all of us would be without you both, honestly. That goes out to the rest of the PCSP faculty and staff as well.

Without my family, this thesis would have *definitely* never been completed. Being so far away from home for so long is only possible with an amazing support system. So, to my parents, brother, sister, and friends in the Netherlands: Bedankt voor al jullie support. De afgelopen 6 jaar zijn emotioneel (en soms fysiek) ongelooflijk zwaar geweest met veel heimwee en moeilijke momenten. Pap en mam, zonder jullie was ik überhaupt nooit naar de Verenigde Staten vertrokken, en had ik al deze waardevolle ervaringen moeten missen. From “The Lunchbox Experiment” in 2014 to “Development of Novel Protein Inhibitors of the E2-Conjugating Enzyme Ube2D” in 2021. You never realize how much you are learning until you rewind the clock and see where you were a couple years ago.

# DEVELOPMENT OF LINKED-DOMAIN PROTEIN INHIBITORS OF THE E2-CONJUGATING ENZYME UBE2D

## Abstract

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2021

In most eukaryotic organisms, the ubiquitination pathway is one of the most important and versatile signaling systems in use. It is integral to processes such as protein degradation and homeostasis, DNA repair cell cycle regulation, signaling and regulation, epigenetics, and many more.

Ubiquitin (Ub) is a short polypeptide of 8.6 kDa, 76 residues that functions as a reversible post-translation modification (PTM). It furthermore contains 7 different lysine residues (K6, K11, K27, K29, K33, K48, K63), all of which can form isopeptide linkages with one another to link individual Ub moieties to form unique polyUb chains onto substrates. The type of polyUb chain a substrate gets labeled with can determine the subsequent activity of that substrate.

Substrate ubiquitination is achieved through an enzymatic cascade. First, an E1-activating enzyme activates a free Ub moiety. Then Ub is transferred onto an E2-conjugating enzyme, and finally an E3 ligase interacts with both substrate and E2~Ub complex to facilitate Ub transfer onto a substrate. Within this scheme, the E2-enzyme acts as a master manipulator in that, it controls when, where and how a ubiquitin chain is transferred onto a substrate.

Irregular activity of E2-conjugating enzyme has been implicated in a wide variety of diseases such as cancer, neurodegenerative diseases, muscular dystrophy, genetic azoospermia

and more. While attempts have been made to inhibit other ubiquitination cascade enzymes such as E3 ligases and E1-activating enzymes, there is a strikingly small number of inhibitors specifically targeting E2 enzymes mainly due to the high degree of structural conservation that exists among members of the E2 enzyme family

In this work, we introduce 3 novel linked-domain protein inhibitors of the E2-conjugating enzyme Ube2D. We covalently attached either UHRF1 RING domain or an affinity optimized U-box domain, with UHRF1 UBL domain or UbvD1.1 (A ubiquitin variant specific for Ube2D), through a glycine-serine linker, producing 3 unique inhibitors: Ring-UBL (RU), U-box-UBL (UU), and U-box-UbvD1.1 (UUD1.1).

In this way, we attempt to specifically inhibit Ube2D for two purposes : 1) While Ube2D can interact with the largest number of E3 ligases and facilitate the largest number of polyUb chains, very little is known about cellular phenotypes specifically associated with Ube2D; 2) We want to establish whether targeting the E2 enzyme in general can be utilized as a viable therapeutic treatment for cancer.

We show that all three inhibitors are able to inhibit ubiquitin assays using Ube2D and using ITC we measured binding affinities of UUD1.1 (5 nM) > UUWT (300 nM) > RU WT (3 μM). Furthermore, we found that all inhibitors could prevent E1, E3 and backside binding domain interactions simultaneously, which single domain UBL could not. UU and RU showed specificity towards Ube2D when tested against APC/C and Cullin1 E3 ligases and their cognate E2 enzymes.

We propose that linking domains in this way, by targeting the backside binding domains of E2 enzymes, could be a strategy that can be standardized and applied to the rest of the E2 enzyme family as well. *In vivo* testing must now elucidate whether these inhibitors can provide

more information about the cellular role of Ube2D and whether it is a viable therapeutic target to treat cancer.

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| | |
|---|---|
| ~ | Protein interaction |
| Ab | Antibody |
| ACN | Acetonitrile |
| Ade | Adenine |
| Amp | Ampicillin |
| APS | Ammonium persulfate |
| C/M | Chloroform/methanol |
| DMEM | Dulbecco's modified eagle's medium |
| DMSO | Dimethyl Sulfoxide |
| DTT | Dithiothreitol |
| DUB | Deubiquitinating Enzym |
| GlyNP-40 | Glycine Nonidet P-40 |
| HECT | Homologous to E6AP C-terminus |
| HEPES | Hydroxyethylpiperazine ethane sulfonic acid |
| His | Histidine |
| IAP2 | Inhibitor of apoptosis protein 2 |
| IPTG | Isopropyl β-D-1-thiogalactopyranoside |
| ITC | Isothermal Titration Calorimetry |
| Kan | Kanamycin |
| LB | Luria Bertani |
| LC | Liquid chromatography |

| | |
|---|---|
| Leu | Leucine |
| MBP | Maltose Binding Protein |
| MG132 | Carbobenzoxyleu-leu-leucinal |
| $MgCl_2$ | Magnesium chloride |
| Ni-NTA | Nickel-nitrilotriacetic acid |
| O/N | Overnight |
| PCR | Polymerase Chain Reaction |
| PLATE solution | PEG, Lithium acetate, Tris-HCl, EDTA solution |
| polyUb | Polyubiquitin |
| PTM | Post-translational modification |
| RBR | Ring-between-ring |
| RING | Really Interesting New Gene |
| RU | Ring-Ubl |
| SDS-PAGE | Sodium dodecyl sulfate – polyacrylamide gel electrophoresis |
| SEC | Size exclusion chromatography |
| TAE | Tris-acetate-EDTA |
| TEMED | Tetramethylethylenediamine |
| TEV protease | Tobacco Etch Virus protease |
| TFA | Trifluoroacetic acid |
| Ub | Ubiquitin |
| UU | Ubox-UBL |
| UUD1.1 | Ubox-Ubv.D1.1 |
| UHRF1 | Ubiquitin-Like With PHD And Ring Finger Domains 1 |

| | |
|---|---|
| UPS | Ubiquitin-Proteasome System |
| WT | Wild type |
| Y2H | Yeast two-hybrid |
| YPD | Yeast peptone dextrose |

# CHAPTER 1: INTRODUCTION

## 1.1 The Ubiquitination Pathway

In most eukaryotic organisms, the ubiquitination pathway is one of the most important and versatile signaling systems in use. It is integral to processes such as protein degradation and homeostasis (Deng et al., 2020), DNA repair (L. Sun & Chen, 2004), membrane protein trafficking (Katzmann et al., 2002), cell cycle regulation (Schnell & Hicke, 2003; Yau, Doerner, Castellanos, Matsumoto, et al., 2017), protein kinase activation, signaling and regulation (S. Liu & Chen, 2011; L. Sun & Chen, 2004), epigenetics (Vaughan et al., 2021), and many more.

Ubiquitin (Ub) itself is a short polypeptide, 8.6 kDa, containing 76 amino acid residues. It is a reversible post-translation modification (PTM) that is a member of the UBL domain

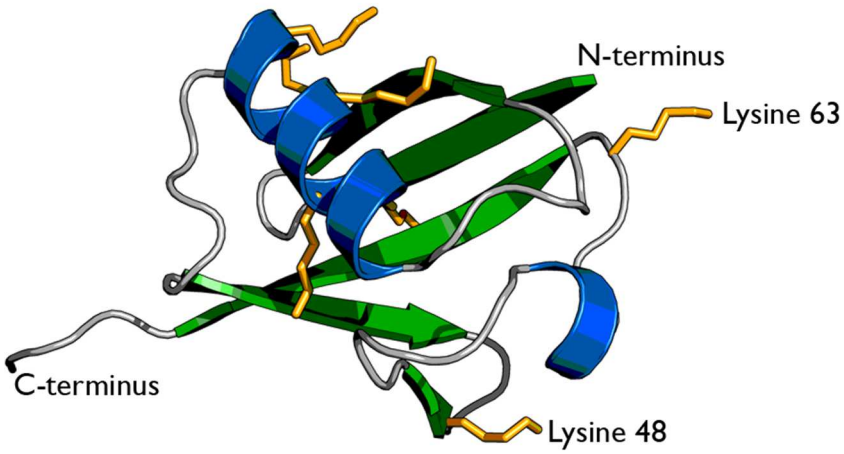



*Figure 1.* Schematic representation of the structure of ubiquitin. Beta-sheets are shown in green; alpha-helices are shown in blue; coils and turns are shown in grey. Lysine residues that facilitate polyUb chain formation are shown in yellow (PDB: 1UBI).

family. PTMs are covalent modifications of proteins after protein synthesis, that can affect protein function. Common PTMs include acetylation, phosphorylation, glycosylation, lipidation,

etc. The focus of this work will be ubiquitination. The UBL domain is characterized by a "beta-grasp" protein fold that consists of a 5-strand antiparallel beta sheet surrounding an alpha helix. As such, this "beta-grasp" protein fold also exists in Ub (*fig.1*).

Ub exerts its influence on target proteins by covalently attaching to them through an (iso)peptide bond formed between Ub's C-terminal glycine (Gly76) and most commonly the amino-group of a lysine residue in the target protein (Stone, 2016). Noncanonical covalent attachments between Ub's Gly76 and cysteine, serine, or threonine residues on target proteins have been reported (McClellan et al., 2019; X. Wang et al., 2007) and their importance has been increasingly characterized over the past decades (Cadwell & Coscoy, 2005; McDowell & Philpott, 2013; Vosper et al., 2009)

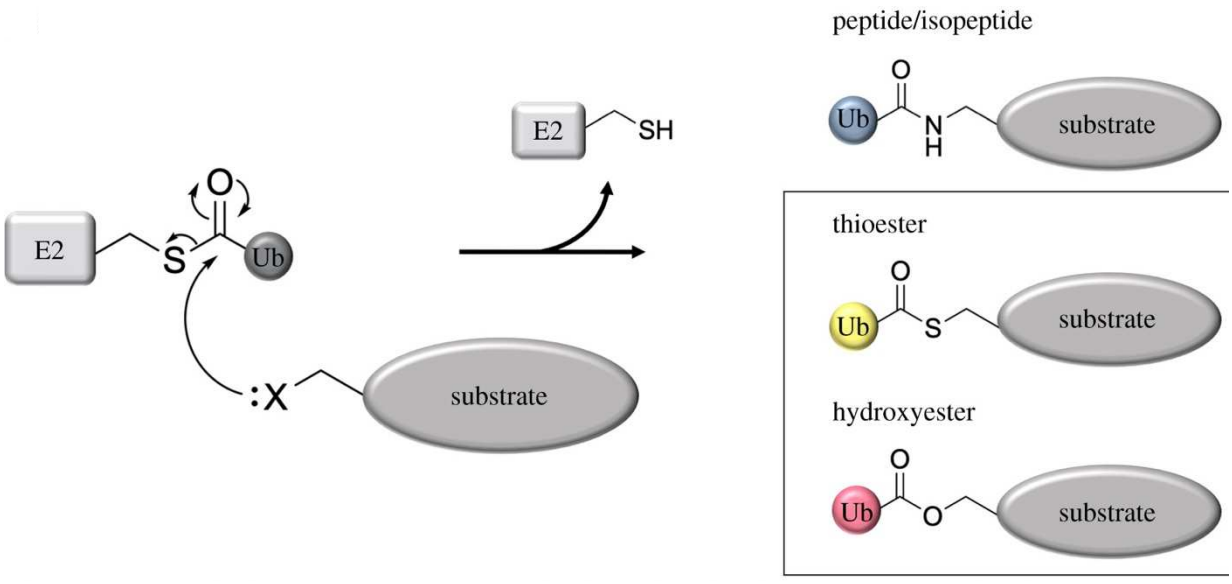



*Figure 2.* Canonical and noncanonical ubiquitination of target proteins. Adapted from (McClellan et al., 2019).

Target ubiquitination is achieved through an enzymatic cascade of events that starts with the ATP/Mg dependent activation of Ub by one of two human E1-activating enzymes (Hershko et al., 1981; Pickart, 2001). Ub forms an adenylate intermediate that is subsequently trapped in a high energy thioester bond with E1 (E1~Ub) (Kwon & Ciechanover, 2017). Ub is than passed to the active site cysteine of an E2-conjugating enzyme, an enzyme family that consists of over 30 members in the human body (Stewart et al., 2016), through a trans thiolation reaction, which forms a high energy E2~Ub conjugate (Hershko et al., 1983). Finally, an E3 Ligase (>600 human members), facilitates the final transfer of Ub from an E2 enzyme onto the substrate. Often, the ubiquitination cascade is then repeated to add more Ub moieties to the already existing Ub moiety, effectively producing a unique polyubiquitin chain signal (McDowell & Philpott, 2013).

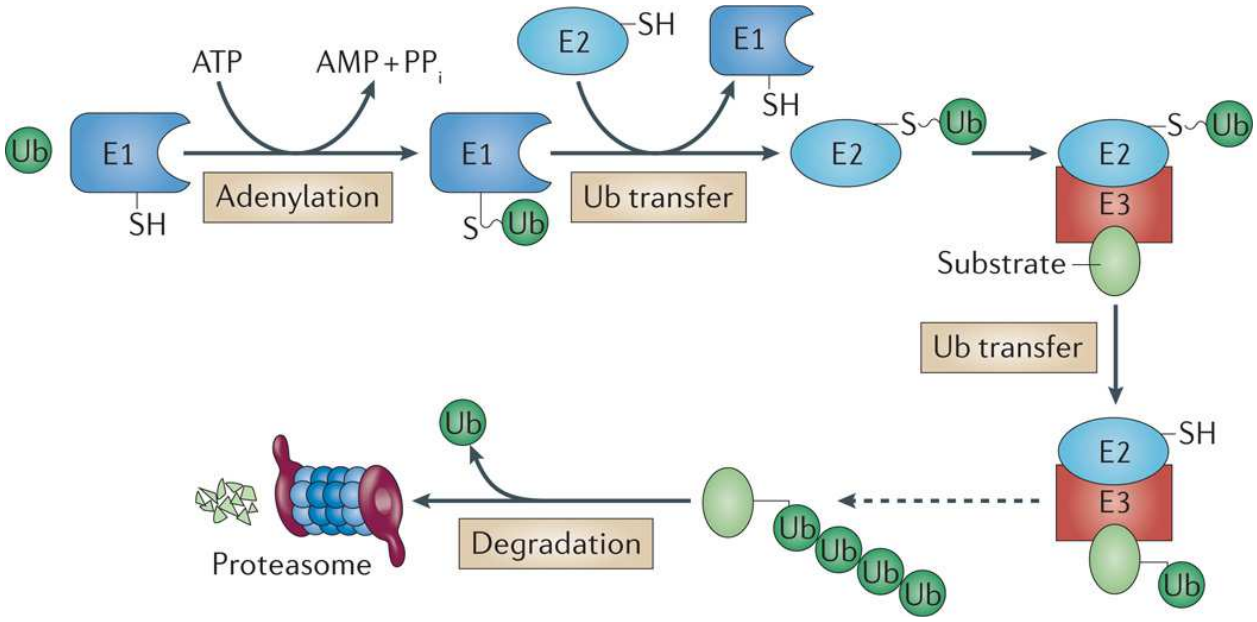



*Figure 3.* The ubiquitin proteasome system (UPS). Substrate is labelled with a unique polyubiquitin moiety that results in substrate 26s proteasomal degradation. Adapted from (Maupin-Furlow, 2012).

Ubiquitination is a reversible process, since ~100 different deubiquitinase enzymes (DUBs) exist that can bind and remove ubiquitin from substrates, adding another level of complexity to the ubiquitination system. (Nijman et al., 2005)

### 1.1.1 Polyubiquitin Linkages and Signal Specificity

Ubiquitin can convey a large variety of signals onto target substrates. This versatility can be explained by the inherent dynamics of Ub's structure. 7 different lysine residues (Lys6, 11, 27, 29, 33, 48, 63) as well as the N-terminal methionine within Ub's structure can form isopeptide linkages to produce distinct and specific polyubiquitin chains. Polyubiquitin (polyUb) chains adopt many different conformations depending on which lysine residues are linked together (Kwon & Ciechanover, 2017; Lutz et al., 2020). Polyubiquitin chains are divided into 3 different groups: homotypic and heterotypic. Homotypic chains consist of Ub moieties linked through the same lysine residue, e.g., each Ub is linked at K48 (*Fig.4*). Heterotypic chains consist of Ub moieties linked at different lysines, e.g., K48-K33-K48-K33-etc. (*Fig. 4*). Heterotypic chains can also form branched chains, chains in which a ubiquitin moiety is linked to two or more ubiquitin molecules in the same chain (*Fig. 4*) (Meyer & Rape, 2014; Y. S. Wang et al., 2020)

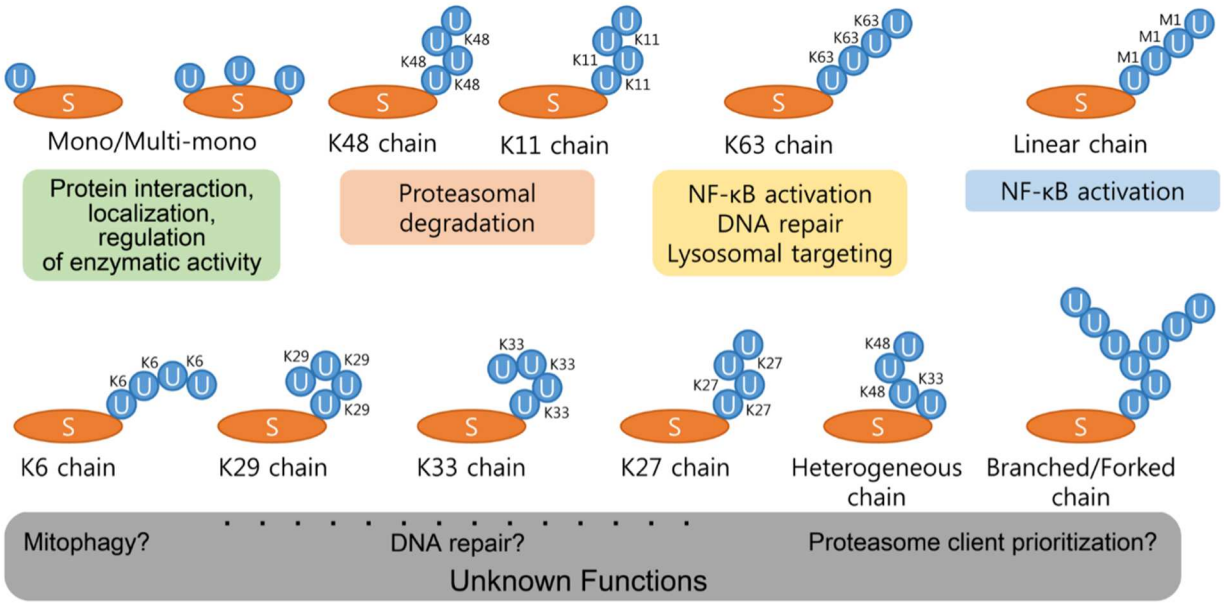



*Figure 4.* Schematic representation of different types of polyubiquitin linkages that can be formed, and what kind of signal they convey to the target protein. Adapted from (C.-W. Park & Ryu, 2014).

Different polyUb chain topologies result in different functional outcomes. The most well-understood types of polyUb chain linkages are K48 and K63-linked polyUb. K48-linked polyUb is widely recognized as a proteolytic signal (Ciechanover & Schwartz, 1998; Hochstrasser, 1996; Thrower et al., 2000). Substrates covalently attached to K48-linked polyUb at least four Ub's long (Lutz et al., 2020; Thrower et al., 2000), are marked for effective degradation by the 26S proteasome, a large multi subunit proteinase complex that degrades K48-linked polyUb into short peptides and recycles the Ub molecules (Hochstrasser, 1996). Consequently, the ubiquitination cascade can control intracellular protein levels by finetuning the level of K48-linked substrate polyubiquitination.

Even though at first K48-linked polyUb was mainly associated with 26S proteasomal degradation, later studies found that K11-linked polyUb is also able to target substrates degradation (Matsumoto et al., 2010; Song & Rape, 2011). K11-linked polyUb has been

characterized as a vital regulator of protein degradation during mitotic cell division. During mitotic cell division, the E3 ligase APC/C (Anaphase Promoting Complex/Cyclosome), controls the progression of cell division by degrading its substrates in a time-dependent manner (Chi et al., 2019; Qiao et al., 2016; J. Zhang et al., 2014). It was found that K11-linked polyUb increased significantly as these substrates were degraded (Matsumoto et al., 2010; Sullivan & Morgan, 2007). In that way, proteasomal degradation of cell cycle regulators is directly proportional to APC/C activity: APC/C inhibition leads to a steady decrease in K11-linked polyUb, whereas full activation of APC/C results in a serious increase in K11-linked polyUb (Song & Rape, 2011)

In contrast with K11-linked polyUb, whose proteolytic effect is mainly limited to mitotic cell division, canonical proteasomal degradation directed by K48-linked polyUb is constantly active and involved with a plethora of biological processes. For instance, K48-linked polyUb accumulates near sites of DNA damage (Meerang et al., 2011). Tumor Suppressor p53 Binding Protein 1 (53BP1) is necessary for non-homologous end-joining during DNA repair (Mallette & Richard, 2012). To activate 53BP1, interaction with histone 4 dimethylated lysine 20 on histone 4 (H4K20me2) in the chromatin is necessary. The E3 ligase RNF8 modulates the covalent attachment of K48-linked polyUb onto specific proteins that mask the H4K20 site in undamaged cells. K48-linked polyUb directed degradation of these masking proteins, opens up H4K20 sites for 53BP1 interaction during DNA damage repair (Mallette & Richard, 2012). Moreover, K48-linked polyUb directed protein degradation plays roles during the inflammatory response (Meng et al., 2021), IFN-1 antiviral response (Liting Zhang et al., 2021), chromatin assembly and histone 2B degradation (Y. Liu et al., 2021), and more.

K63-linked polyubiquitin chains and their functional outcomes are also very well-understood. K63-linked polyUb has known functions in NF-kappaB activation (Z. J. Chen et al., 1996), DNA repair and lysosomal targeting (C. W. Park & Ryu, 2014) as well as others.

NF-kappaB (NF-kB) transcription factors can alter gene expression as a response to e.g., cellular stress (Kovalenko & Wallach, 2006). During NF-kB signaling, an E3 ubiquitin ligase, TRAF6 can be recruited to Toll-like receptors (TLR's) which induces TRAF6 oligomerization. TRAF6 oligomerization in turn stimulates TRAF6 auto-ubiquitination ($TRAF6^{polyUb}$) as well as downstream K63-linked polyubiquitination of targets (Zhijian J. Chen, 2005). $TRAF6^{polyUb}$ is recognized by adaptor proteins TAB2 and 3 which subsequently recruit protein kinase TAK1. TAK1 auto-phosphorylates and induces IKK activation (Kanayama et al., 2004; C. Wang et al., 2001). IKK activation initiates a series of downstream steps that results in the activation of target genes (Israël, 2000). Within this scheme non-degradative K63-polyubiquitination is vital.

Furthermore, K63-linked polyUb also plays a large role in non-proteasomal protein degradation by the lysosome, or autophagy. *In vivo*, K63-linked polyUb can target misfolded protein aggregates that are generally too large to be degraded by the 26s proteasome (a common phenomenon in neurodegenerative diseases such as Parkinson's and Huntington's) to the aggresome-autophagy pathway, facilitated by the E3 Parkin (Li & Ye, 2008; Olzmann & Chin, 2008). It also occurs during the Chaperone-mediated autophagy of certain transcription factors (Ferreira et al., 2015).

Aside from K48, K63 and K11 linked-polyUb, the other types of polyUb chains that can potentially form are K6, K29, K27 and K33 linked polyUb. These are a lot less understood and the variety of functional outcomes of each of these chains has yet to be fully elucidated. Recent studies have found that K27-linked BRAF ubiquitination in melanoma cells can result in

nonreversible activation of cytokines that can maintain the MEK-ERK signaling pathway (Yin et al., 2019). K29 linked polyUb has been shown to auto-ubiquitinate the proteasome and inhibit proteasome-substrate interactions (Besche et al., 2014). Epigenetic regulation involvement as well as a role in the inflammatory response has been implied (Jin Jin et al., 2016; Swatek & Komander, 2016). Preliminary studies associated K33-linked polyubiquitination with intracellular trafficking processes near the trans-Golgi network (TGN). The CRL Cul3-KLHL E3 ligase adds K33-linked polyUb chains to coronin 7, which in turn aids in the formation of new TGN-derived transport carriers (Swatek & Komander, 2016; W. C. Yuan et al., 2014)

Most branched, heterotypic polyUb chains have yet to be assigned a function, even though they have been found *in vitro* and for some *in vivo* (French et al., 2021).

These examples provide just a small glimpse into the complexities and regulatory effects of polyubiquitination in vivo. Even though many studies over the years have enhanced our understanding of the functional outcomes of polyubiquitination, much is yet to be discovered, on noncanonical polyubiquitination in particular, as well as its role in biological processes and diseases.

### 1.1.2 E1 Ubiquitin Activating Enzymes

The first step towards substrate (poly)ubiquitination is performed by an E1 activating enzyme. There are about 10 E1 activating enzymes that can activate a variety of Ubl-moieties including SUMO, FAT10, NEDD8, and more (Schulman & Wade Harper, 2009). However, only two distinct E1 ubiquitin activating enzymes have been identified in humans so far: Ube1 and Uba6 (Handley et al., 1991; Jianping Jin et al., 2007; F. Wang & Zhao, 2019). Ube1 was thought to be the sole E1 enzyme responsible for Ub activation within humans up until recently.

It was not until the late 2000s that Uba6 was discovered (Jianping Jin et al., 2007). E1 Ub activating enzymes generally work through the following mechanism:

$$E1_{SH} + ATP + Ub + Mg^{2+} \rightleftarrows E1_{SH}^{\cdot AMP\sim Ub(A)} + PP_i \quad (1)$$

$$E1_{SH}^{\cdot AMP\sim Ub(A)} \rightleftarrows E1_{S-Ub(T)} + AMP \quad (2)$$

$$E1_{S-Ub(T)} + ATP + Ub + Mg^{2+} \rightleftarrows E1_{S-Ub(T)}^{\cdot AMP\sim Ub(A)} + PP_i \quad (3)$$

$$E1_{S-Ub(T)}^{\cdot AMP\sim Ub(A)} + E2_{SH} \rightleftarrows E1_{SH}^{\cdot AMP\sim Ub(A)} + E2_{S-Ub} \quad (4)$$

*Scheme 1*. Mechanism of Ub activation and Ub transfer onto an E2 enzyme by Ube1. Ub (A): Ub noncovalently associated at the adenylation site on Ube1. Ub (T): Ub covalently attached through a thioester linkage at the catalytic cysteine of Ube1. Adapted from (Schulman & Wade Harper, 2009).

First, $E1_{SH}$ reacts to form an E1~Ub adenylate intermediate in an ATP-dependent reaction, yielding a pyrophosphate molecule. From the E1~Ub adenylate intermediate, Ub is attached to the E1 catalytic cysteine in a high-energy thioester bond (Barghout & Schimmer, 2021). A second Ub moiety is noncovalently associated with the $E1_{S\text{-}Ub\,(T)}$ complex, producing a E1 loaded with two Ub molecules. Ub (T) is then transferred to the catalytic cysteine of an E2 enzyme through a trans thiolation reaction (Kwon & Ciechanover, 2017; Stewart et al., 2016).

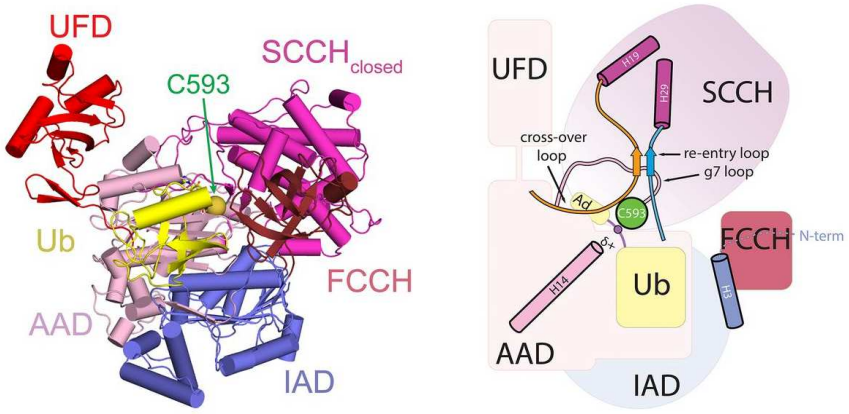



*Figure 5.* General structure of Ube1 and its domains with Ub covalently attached to the catalytic Cys593. Adapted from (Hann et al., 2019).

Ube1 contains 5 distinct domains. Active and Inactive Adenylation Domains (AAD & IAD) are responsible for recruiting Ub and facilitate the C-terminal adenylation of Ub. The catalytic cysteine (Cys593), involved with covalent thioester formation with Ub, is contained in a domain split into two halves: the First and Second Catalytic Cysteine Half domains (FCCH and SCCH). Ubiquitin Fold Domain (UFD) subsequently recruits E2 enzyme to Ube1 and facilitates the transfer of Ub from Cys593 to an E2 enzyme.(I. Lee & Schindelin, 2008; L. Yuan et al., 2021).

Uba6 was discovered in 2007 and can activate both human leukocyte antigen F-associated transcript 10 (FAT10, a Ubl-moiety) and Ub. It has about 40% sequence similarity to Ube1 (*Table* 1). Not only did this discovery add another level of complexity to ubiquitin regulation, it also showed that Ubl moieties are not necessarily regulated by merely one E1 activating enzyme (Jianping Jin et al., 2007). So far, E2 enzymes that can only accept Ub from Ube1, have been well characterized. Uba6 however, has only one identified Uba6-specific E2 enzyme (Referred to as Use1). Use1 is merely able to accept Ub and FAT10 from Uba6

(F. Wang & Zhao, 2019). Whether there are more Uba6-specific E2 enzymes, has yet to be elucidated.

Table 1
*Structural Features of Ube1 and Uba6. Adapted from (Groettrup et al., 2008)*

| | **MW (kDa)** | **Identity (%) *** | **Ubl** | **E2** |
|---|---|---|---|---|
| Ube1 | 118 | 100 | Ub | Many |
| Uba6 | 118 | 40 | FAT10<br>Ub | Use1<br>More? |

* % Identity as related to Ube1

### 1.1.3 E3 Ubiquitin Ligases and Ub Transfer Mechanisms

Within the human body, over 600 E3 Ub ligases exist. It would be futile to attempt to summarize the function of each. Therefore, only the three main classes of E3 Ub ligases will be briefly discussed here, as well as any details pertaining to E3 Ub ligases that are relevant to this body of work.

E3 Ubiquitin Ligases are classified by their catalytic mechanisms and their conserved domains. As shown in Figure 6, there are 3 main classes of E3 Ub ligases: RING (Really Interesting New Gene) domain E3 ligases, HECT (Homologous to E6AP Carboxyl Terminus) domain E3 ligases, and RBR (Ring-Between-Ring) E3 ligases (Buetow & Huang, 2016).

RING E3s by far are the most prevalent out of the 3 classes. There are over 600 different RING E3 ligases in the human body (Deshaies & Joazeiro, 2009). Within RING E3 ligases, the RING-domain is responsible for interacting with E2 enzymes. RING E3 ligases facilitate substrate ubiquitination by tethering both E2-Ub and the substrate protein in a noncovalent complex (*Fig. 6e)* (Deshaies & Joazeiro, 2009; Metzger et al., 2014). RING E3

ligases do not form covalent adducts with Ub, they merely act as facilitators to direct Ub transfer from the E2 catalytic cysteine onto a lysine residue of substrate (L. Liu et al., 2018).

About one third of the RING E3 ligase family consists of a smaller subclass of RING E3 ligases: Cullin/Ring ubiquitin ligases (CRLs). CRLs are multi-subunit E3 ligases containing a small RING-finger domain, RBX, that interacts with E2 enzymes. CRL's furthermore contain a substrate receptor protein (which interacts with target proteins) and a Cullin scaffolding protein (J. Liu & Nussinov, 2009). Studies have identified 8 different mammalian Cullin proteins that

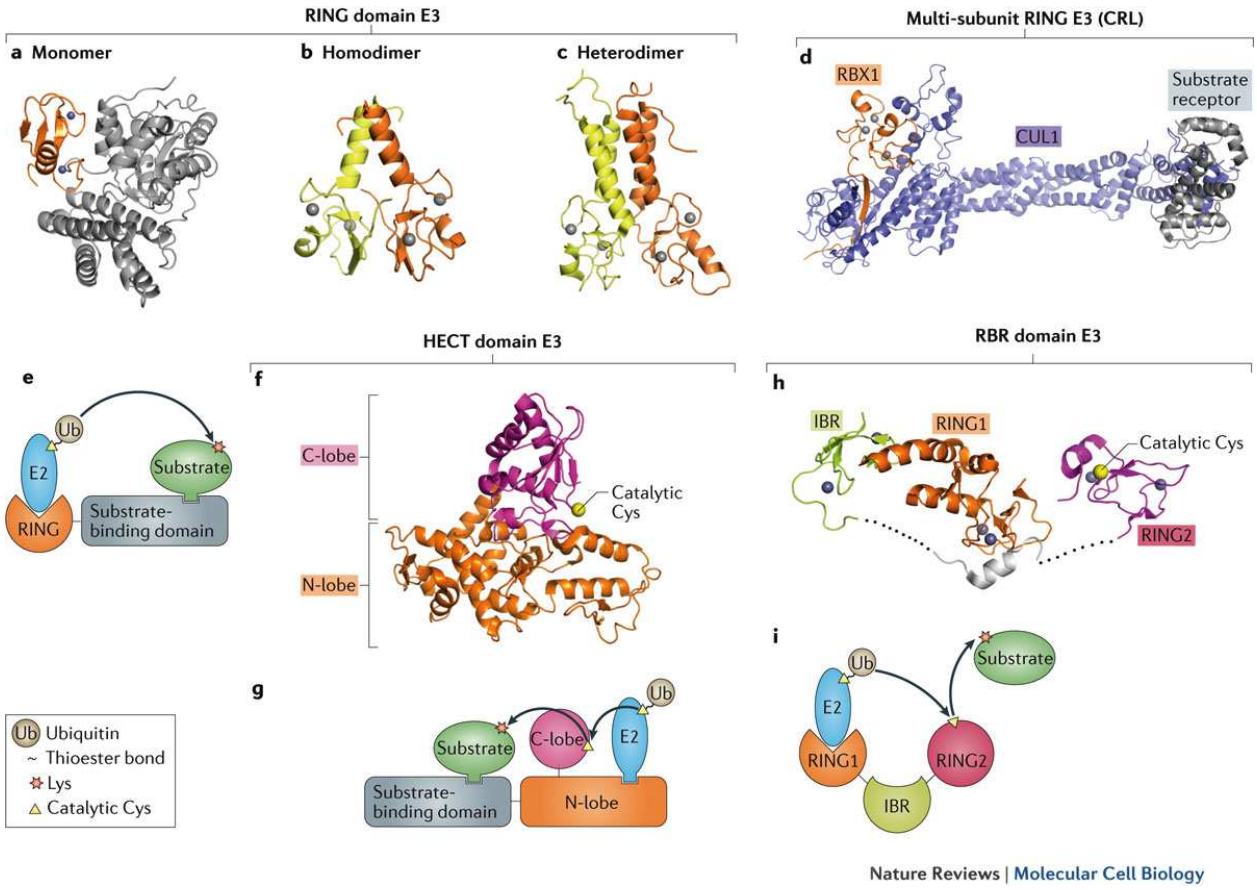



*Figure 6.* 3 different classes of E3 Ub ligases. **a-e:** RING domain E3 ligases. **f-g:** HECT E3 ligases. **h-**i: RBR E3 ligases. Adapted from (Buetow & Huang, 2016).

can act as a scaffold within a CRL (Skaar et al., 2014). SCF ubiquitin ligase is arguably the most elucidated type of CRL.

SCF (Skp1/Cullin1/F-box protein) E3 Ub ligase, has the general structure of a CRL, but contains 4 subunits: Skp1 (E2~Ub binding), RBX1 (Small Ring domain), F-box protein and Cullin1 (scaffolding protein) which binds Skp1 at the N-terminus and RBX1 at the C-terminus. F-box protein functions as an adaptor that recognizes and recruits phosphorylated substrates to the Skp1 domain (J. Xie et al., 2019). SCF E3 ligases have been shown to play important roles in protein degradation during biological processes such as cell signaling, cell cycle progression and proliferation, DNA repair, etc. (C. M. Xie et al., 2013)

The mechanism of direct transfer of Ub from E2~Ub onto a target substrate, mediated by an E3 ligase is also adopted by the RING-like family of U-box proteins. The U-box domain, another E2 binding domain, is very similar in structure to the canonical RING domain but lacks the two stabilizing $Zn^{2+}$ ions present in canonical RING. Instead, U-box contains elaborate H-bond and salt bridge networks in those locations (Ohi et al., 2003). Typically, U-box domain E3 ligases are considered part of the RING-type class of E3 ligases. U-box proteins, 70 amino acids long, were originally characterized by their ability to facilitate polyubiquitylation in the absence of either RING-finger E3s or HECT-domain E3s (Hatakeyama & Nakayama, 2003).

The second class of E3 ubiquitin ligases, HECT-domain E3s, represent a smaller percentage of the E3 ligase family. 28 HECT-domain E3s have been identified thus far (Weber et al., 2019). In contrast with RING-domain E3s, which do not directly associate with Ub, HECT-domain Ub transfer involves a two-step mechanism, in which Ub is first transferred from E2 to the catalytic cysteine of a HECT E3 through a trans thiolation reaction, and then attached to a lysine on a target substrate (Rotin & Kumar, 2009; Sluimer & Distel, 2018). Structurally,

HECT E3s are vastly different from RING-finger E3s. HECT domain consists of a large N-lobe, a C-lobe, and a substrate binding domain (*Fig. 6g)*. The N-lobe recruits the E2~Ub complex and

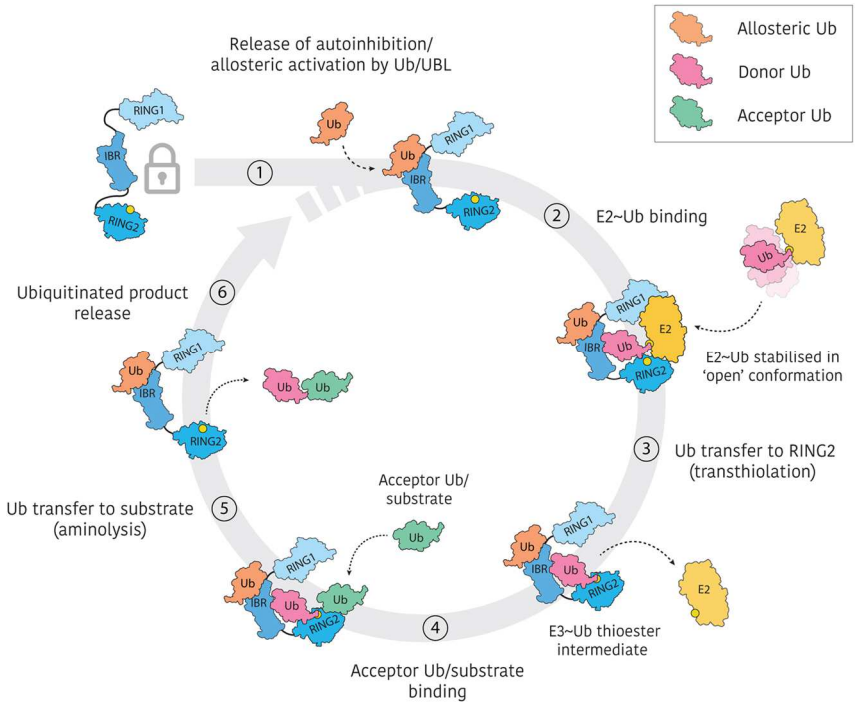



*Figure 7.* Catalytic mechanism of RBR-domain E3 ligases. Adapted from (Cotton & Lechtenberg, 2020).

brings Ub in close proximity to the catalytic cysteine, which is located on the C-lobe. The two lobes are connected by a flexible catalytic cleft, which provides the C-lobe sufficient mobility to modulate Ub transfer onto the target substrate (L. Huang et al., 1999a; Kamadurai et al., 2009; Verdecia et al., 2003).

RBR-domain E3 ligases make up the third class of E3 ligases. First described in plants and fungi in 1999 (Morett & Bork, 1999), RBR-domain E3s are the smallest class within the E3 ligase family with only 14 members (Marín et al., 2004) RBR's are generally described as a type of RING/HECT E3 Ub ligase hybrid. RBR E3s contain 3 domains, two of which are RING domains (RING2 is named after RING, but structurally bears no similarity to canonical RING

domain): RING1 and RING2. RING1 acts as the E2-binding domain and RING2 contains a catalytic cysteine. The two RING domains are interconnected by an IBR (In Between Ring) domain.

Though the presence of a RING E2 binding domain reminds of the canonical RING-finger E3 ligase, ubiquitylation is catalyzed through a more complex Ub transfer mechanism compared to both HECT and RING E3 ligases. RBRs have a more complex catalytic mechanism because the RBR requires allosteric activation of the IBR domain to lift the native "auto-inhibited" state the RBR E3 ligase exists in (Cotton & Lechtenberg, 2020). Allosteric activation unlocks RING1 domain, which subsequently recruits the E2~Ub complex (*Fig. 7*)(Cotton & Lechtenberg, 2020). After this, a two-step mechanism similar to HECT E3s occurs, where Ub is transferred onto the conserved catalytic cysteine of RING2 domain through a trans thiolation reaction first, after which substrate ubiquitylation can occur (Wenzel et al., 2011).

One notable example of an RBR E3 Ligase, is Parkin, originally determined to be an RBR-type ligase in 2011 (Wenzel et al., 2011). Many studies have focused on Parkin ever since it was discovered that certain mutations in Parkin can cause early-onset Parkinson's disease (Marín et al., 2004). Studies have shown that RBR E3 ligases are involved in processes such as translation (N. G. S. Tan et al., 2003) and immune signaling (Tokunaga et al., 2009), as well as DNA repair (Kao, 2009a, 2009b)

**1.1.4 Deubiquitinating Enzymes**

Deubiquitinating enzymes (DUBs) are specific proteases that can remove ubiquitin chains from targets by hydrolyzing Ub-substrate and Ub-Ub isopeptide bonds, disassemble free ubiquitin chains and prepare precursors of ubiquitin for chain formation (Reyes-Turcu & Wilkinson, 2009). They are also important in maintaining normal proteasome degradation

activity, partially by removing free polyUb chains that can compete with substrate linked polyUb for 26s proteasomal degradation (*Fig. 8*) (Amerik & Hochstrasser, 2004; Lam et al., 1997). There are about 100 DUBs currently characterized, some of which have been shown to cleave specific types of polyUb *in vivo*, some *in vitro*.

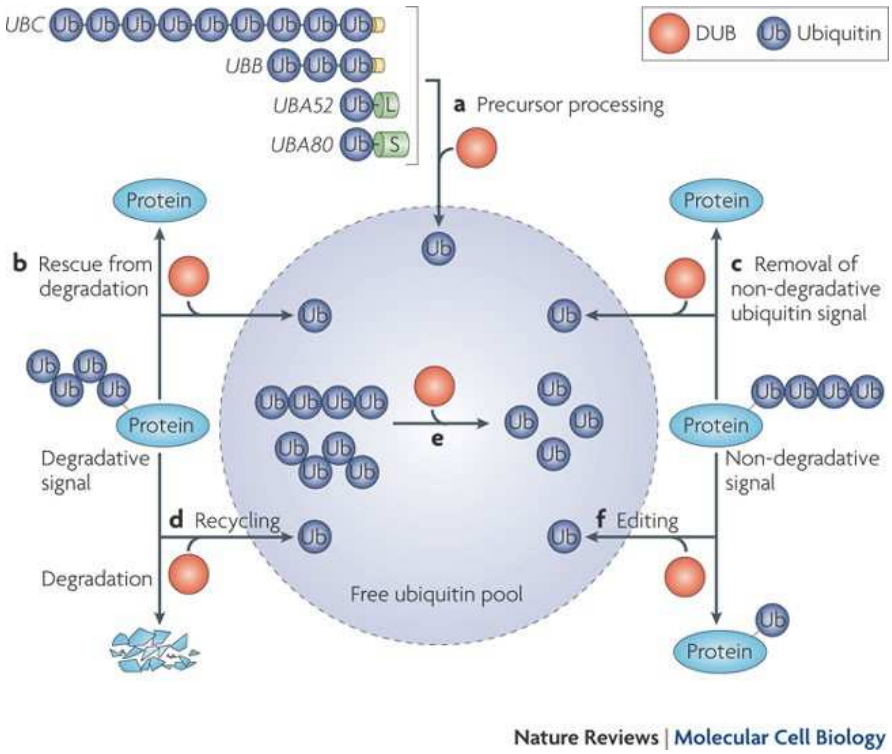



*Figure 8*. Deubiquitinating enzymes and the wide variety of functions they can perform within the cell. Adapted from (Komander et al., 2009).

A major characteristic of DUBs in general is their ability to recognize and distinguish the different types of polyUb (linked through different lysine residues in ubiquitin). A leading theory explaining this ability, credits the diverse topologies different types of polyUb can conform to. I t is speculated that conformational differences aid specific polyUb recognition by DUBs (Reyes-Turcu & Wilkinson, 2009). This theory has been supported by several studies showing DUB specificity for particular polyUb chains (Hu et al., 2005; Komander et al., 2008;

Wilkinson et al., 1995). Even though some evidence exists for DUB polyUb chain selectivity in certain cases, DUB chain selectivity research is ongoing. Using Ub-probes targeting the active sites of DUBs to discover, profile and target DUBs has been a popular strategy so far (Borodovsky et al., 2001; de Jong et al., 2012). A recent study utilized di-Ub chain probes that mimicked all 8 different polyUb chain linkages, and utilized proteomic methods to determine the DUB selectivity for these Di-Ub probes in cells (McGouran et al., 2013)

Human DUBs fall into one of 7 classes of DUBs: Ubiquitin Specific Proteases (USP/UBP), Ubiquitin C-terminal Hydrolases (UCH), Ovarian Tumor Proteases (OTU), Ataxin-3/Josephins, JAB1/MPN/MOV34 (JAMM) DUBs zinc finger with UFM1-specific peptidase domain protein protease (ZUFSP) and MINDY (Amerik & Hochstrasser, 2004; Suresh et al., 2020). Most of these are cysteine proteases, containing cysteine active sites. A so-called catalytic triad (consisting of the active site cysteine, a histidine, and an aspartate) aids in the nucleophilic attack of the carbonyl group of a Ub-target isopeptide bond and subsequent DUB~Ub intermediate formation, followed by the hydrolyzed release of Ub from the DUB protease active site (Nijman et al., 2005) The only metalloproteases out of the seven classes are JAMMs. JAMMs contain two $Zn^{2+}$ ions. These $Zn^{2+}$ ions are used to activate water molecules to attack the isopeptide bond between a substrate and Ub (Fuchs et al., 2018; Nijman et al., 2005).

The importance of DUBs counteracting influence on ubiquitination has been illustrated in a variety of studies and reviews. For instance, the DUB OTUB1 can stabilize and inhibit E2 enzymes and their interactions with Ub, e.g. the Ub~Ube2N interaction (Que et al., 2020). Ube2N dimerizes with the pseudo-E2 Ube2V1 (E2 structure lacking a catalytic cysteine). The Ube2N-Ube2V1 complex works in tandem with the E3 ligase RNF168 to produce K63-linked polyubiquitinated substrates. It was found that the allosteric association of a second Ub moiety

to the N-terminal of OTUB1 increases its affinity to the Ube2N~Ub complex (Wiener et al., 2012). By associating to the Ub~Ube2N intermediate, K63-linked polyUb formation was blocked as nucleophilic attack of a donor Ub was also blocked. Since Ube2N~Ube2V1 forms K63-linked polyUb at sides of DNA damage to recruit repair proteins, OTUB1 can be seen as a DNA repair regulator inhibition of E2 enzymes (Pasupala et al., 2018; Que et al., 2020; Wiener et al., 2012, 2013).

The DUB USP10 has well-documented roles in protein aggregation, cell apoptosis and the DNA damage response and also acts as a tumor suppressor ((Bhattacharya et al., 2020; Takahashi et al., 2018; J. Yuan et al., 2010) USP10 is able to stabilize the tumor suppressor transcription factor p53, which is vital for its tumor suppressor function. USP10 is also able to deubiquitinate p53, which prevents its degradation and improves its intracellular life-time (J. Yuan et al., 2010). USP10 doesn't just interact with the transcription factor p53, it also interacts with p62. In cells, an excess of ubiquitinated proteins can induce cell apoptosis. USP10 counteracts apoptosis by associating with the ubiquitin receptor p62, which can induce protein aggregation and aggresome formation (Takahashi et al., 2018). Aggresomes seize to be cytotoxic, as they can be cleared through autophagy mediated protein degradation. In this way USP10 plays an important role in protein degradation cell apoptosis (Takahashi et al., 2018; Wong et al., 2008).

It is clear that DUB's play an essential role in the regulation of the ubiquitination cascade. Mutations and irregularities in their function could have catastrophic effects for cellular activity, in the worst case resulting in cell death. They are involved in a wide variety of biological processes and regulate the half-life of many oncogenes and tumor-suppressors, which is why they have been deemed plausible targets for drug therapies (Komander et al., 2009).

## 1.2 Anatomy of the E2 Conjugating Enzyme

The last main enzyme within the ubiquitination cascade, is the E2-conjugating enzyme. There are ~40 human E2 conjugating enzymes currently identified that can interact with either Ub or Ub-like structures such as FAT10, SUMO and NEDD8. The E2 enzyme originally has been viewed as merely a middle man, an enzyme that connects the Ub activation by the E1 enzyme, and the covalent attachment of Ub by an E3 ligase (Stewart et al., 2016). In recent years however, it has become more clear that the E2 enzyme plays a vital role in deciding when, where and how ubiquitin gets attached to a target protein (Wijk & Timmers, 2010; Ye & Rape, 2009). More than a decade of studies have now determined that E2 enzymes are in charge of: 1) switching from chain initiation to elongation; 2) processivity of ubiquitin chain formation and 3) selecting polyUb linkage, essentially making them the masterminds behind the ubiquitination cascade (Ye & Rape, 2009).

The E2 enzyme is characterized by a highly conserved ~ 150 amino acid-long UBC (Ubiquitin-Conjugating) core structure that generally consists of 4 α-helices and one β-sheet consisting of 4 anti-parallel β-strands (*Fig. 9*) (Gundogdu & Walden, 2019). Within the UBC core, certain sections have assigned functions. For example, an E2 enzyme recognizes one of the two human E1 enzymes (Jianping Jin et al., 2007; Schulman & Wade Harper, 2009) charged with two Ub molecules, through α-helix1, which contains the E1-binding site (Haas et al., 1988; Williams et al., 2019). While E1~Ub binding to a cognate E2 enzyme is mainly catalyzed through the E1 binding motif in α-helix1, some E2 enzymes contain N-terminal extensions that also interact with the E1~Ub complex and can improve the affinity of the E1~E2 interaction and simultaneously destabilize the E1~Ub interaction. Other factors such as E1 conformational changes also play a part during the E1~E2 interaction (Olsen & Lima, 2013). These types of

interactions facilitate the transfer of Ub onto the catalytic cysteine of a cognate E2 enzyme, but also might provide specificity in E1~E2 recognition mechanisms (Durfee et al., 2008; D. T. Huang et al., 2004, 2008; Summers et al., 2008a)

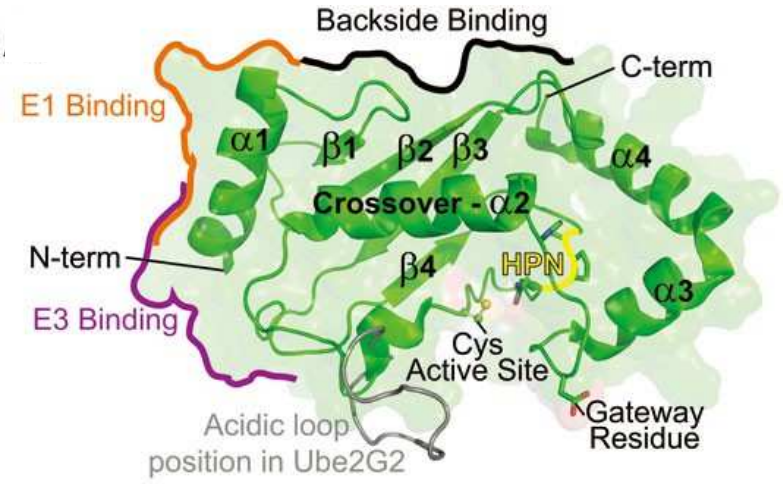



*Figure 9.* General structure of the E2 conjugating enzyme. Top: Important interaction domains are labelled onto a representative UBC domain (Ube2D3, PDBID: 2FUH). Bottom: Adapted from (Stewart et al., 2016).

The N-terminal side of α-helix1 and loop4 and loop7 (L4 and L7) make up the E3 ligase binding motif in all the E2 enzymes identified so far (Brzovic et al., 2003a). Notably, the E3 ligase binding domain overlaps with the N-terminal side of the E1 binding site. While a canonical E3 ligase binding motif does exist, structural modeling studies have identified key residues in both L4 and L7 that either convey specificity or are important for E3 binding. For instance, the 6$^{th}$ residue of L4 is used by most E2 enzymes for interaction. Furthermore, all E2 enzymes are able to bind both HECT and RING E3s, but only the 2$^{nd}$ residue of L4 interacts with HECT E3s not RING E3s (Kar et al., 2012; Stewart et al., 2016). E2~E3 interactions can only occur after an E1 transfers Ub to the cysteine active site and the E1 is discharged from the E1 binding motif (Eletr et al., 2005). Studies have shown that allosteric effects, including accessory

E3 binding regions, can enhance and stabilize the E2~E3 binding interaction to facilitate increased processivity of Ub transfer onto a substrate (Das et al., 2013; L. M. Duncan et al., 2010). So far, the E3 binding motif is the object of many studies attempting to identify E2~E3 interactions to shed light on how E3 ligases know which E2 enzymes to utilize and vice versa.

E2~Ub intermediates can exist in either a "closed" or "open" conformation. For some RING-E3 ligases, Ub transfer and E3 interactions can only occur in the closed E2~Ub state. For many other RING domains however, this is not the case. Ub contains a key residue, I44, located in a hydrophobic patch. I44 has been shown to mediate many key interactions. On the E2 enzyme, the α-helix2 is widely recognized as the "cross-over helix". E3 binding, as well as cross-over helix-I44 Ub interactions, play a vital part in inducing the closed state of the E2~Ub intermediate, which is the state that promotes nucleophilic attack of the thioester linkage to induce Ub aminolysis, resulting in substrate ubiquitination for some RING mediated Ub transfers (Branigan et al., 2020; Brzovic et al., 2006a; Dove et al., 2016; Lips et al., 2020; Saha et al., 2011; Stewart et al., 2016).

Nearly all E2 structures contain a conserved cysteine active site in a small section formed by a short loop connecting the cross-over helix with α-helix3 and a long loop near the active site (*Fig*. 9) (Olsen & Lima, 2013; Tolbert et al., 2005; L. Yuan et al., 2017). The catalytic cysteine enables both trans thiolation reactions (E1~Ub to E2 transfer, HECT-mediated E2~Ub onto E3 transfer) and aminolysis reactions (E2~Ub transfer onto lysine of target substrate). While most E2 structures contain this catalytic cysteine, some pseudo-E2's exist that are made of a conserved UBC domain, do not. These nonenzymatic pseudo-E2s are named UEVs (ubiquitin conjugating E2 enzyme variant) and are mostly deemed cofactors for the E2-conjugating enzyme

Ube2N to aid in polyUb chain formation (Alpi et al., 2016; Andersen et al., 2005; Eddins et al., 2006; Ghilarducci et al., 2021; McKenna et al., 2003; Tsui et al., 2005).

Loop7 (L7) (part of the E3 ligase binding domain) colloquially is named the 'Acidic Loop'. Reason for this being that the acidic loop is an insertion only occurring in a few E2 enzymes that contains a combination of hydrophobic and 4 acidic residues. Research involving the acidic loop has focused mainly on its presence in Ube2R1, although a select few other E2s also include acidic loop insertions (Arrigoni et al., 2014; Choi et al., 2015; Papaleo et al., 2011; Saha et al., 2011; Ziemba et al., 2013). The acidic loop has been implicated in several functions. In Ube2R1, substrate phosphorylation stimulates the acidic loop to conform to an open conformation, which in turn allows donor ubiquitin to interact with the cognate E2 enzyme (Arrigoni et al., 2014). In both Ube2G1 and Ube2R1, specific acidic loop residues (two tyrosine's) facilitate enhanced K48-linked polyubiquitination activity, independent from an E3 ligase (Choi et al., 2015). Other studies found that the inherent properties of the acidic loop insertion contributes to improved catalysis by not only Ube2R1, but its cognate SCF ligase (Kleiger et al., 2009; Mathias et al., 1998; Wu et al., 2002; Ziemba et al., 2013).

α-helix4 and α-helix5 (HTH) are included in the UBC domains of nearly all E2 enzymes and in most present themselves structurally in a typical V-shape (*Fig.9, 10*). Very little is currently known about the functionality of this HTH, even though some reports have indicated E3 interactions with the HTH to activate E2~Ub transfer(Brown et al., 2016; Yamaguchi et al., 2016). Specifically, for the E3 ligase complex APC/C, which utilizes both Ube2S (chain elongation; formation of branched K11-K48 linked polyUb chains) and Ube2C (substrate Ub priming) these interactions have been shown (Aristarkhov et al., 1996; Meyer & Rape, 2014; Wild et al., 2016; Williamson et al., 2009). Helices of APC2, a subunit of APC/C interacts with

the HTH of Ube2S to facilitate Ub transfer (Brown et al., 2016; Yamaguchi et al., 2016). More studies will have to be done to elucidate the exact mechanism by which the HTH influences ubiquitination activity for certain E2 enzymes.

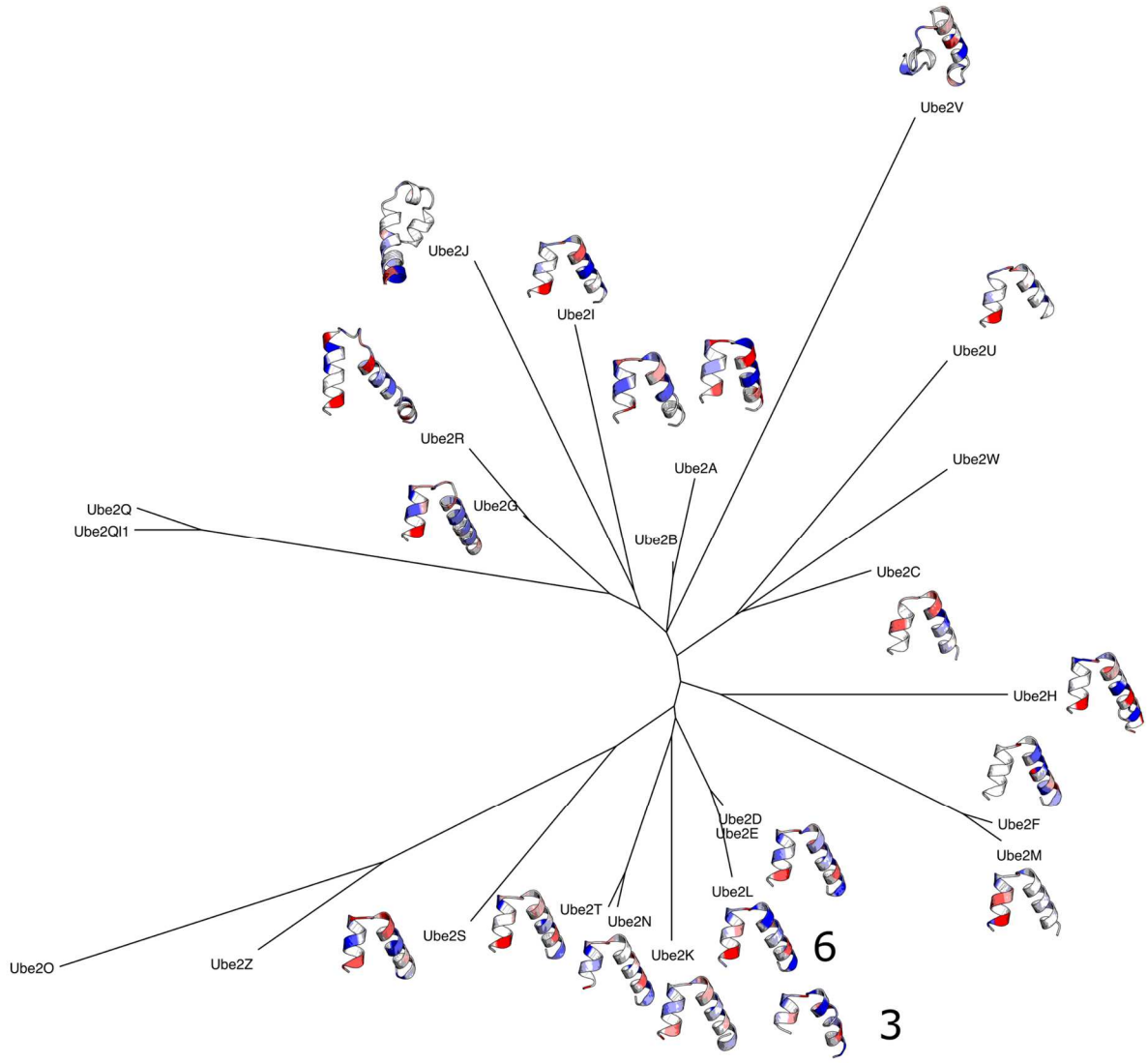



*Figure 10*. Family tree of the α-helix4-turn-α-helix5 (HTH) of most E2 enzymes. HTH structures are colored by charge. Ube2Q, Ube2QL1, Ube2O and Ube2Z do not have assigned HTH structures, as their structures are very dissimilar to the rest of the E2 enzymes and do not have identifiable HTHs.

The last domain to be discussed, the domain of most relevance to this body of work is the backside binding domain (BBD). The backside binding domain is present on most E2 enzymes and much of what is known about its function has only been elucidated in the past 15 years. The backside binding domain is located on the opposite of the cross-over helix and the catalytic cysteine, making up most of the antiparallel β-sheet UBC residues, the C-terminal end of α-helix1 and possibly some of α-helix4 (Stewart et al., 2016). The BBD is essentially a hotspot for noncovalent, allosteric regulatory events. The most well-known BBD interaction is that of allosteric Ub. For example, in 2006 the first model of Ube2D3 in complex with noncovalent Ub through Ub's hydrophobic patch residues was determined (Brzovic et al., 2006b), and subsequent studies informed of Ub's function in polyUb chain formation and aiding in polyUb chain building processivity by E3 ligases first for Ube2D3 (Brzovic et al., 2006b; Page et al., 2012; Sakata et al., 2010; Stewart et al., 2016), but later for other E2s as well such as the Ube2A homolog RAD6 (Hibbert et al., 2011; Kumar et al., 2015) in which backside binding also determines what kind of Ub signal is transferred onto the substrate and Ube2I (generally known as a SUMO-specific E2) (Cappadocia & Lima, 2018; Garg et al., 2020; Knipscheer et al., 2007).

A notable example of Ub-BBD interactions is the Ube2N~Ube2V2 dimer that catalyzes K63 polyUb chain formation. Ube2V2 is a pseudo-E2 and does not have a conserved catalytic cysteine. It does, however, contain a BBD. This BBD can bind an acceptor Ub and position it in such a way so that the K63 of the acceptor Ub is facing the catalytic cysteine of the other half of the heterodimer, Ube2N. In this way Ube2V2 facilitates the formation of K63-linked polyUb (Eddins et al., 2006; Tsui et al., 2005; Wright et al., 2016)

Furthermore the BBD has been shown to interact with E3 ligase domains such as RING to stabilize E2~E3 interactions and activate subsequent thioester linked Ub transfer (Patel et al.,

2019). For instance, it was discovered that the cullin subunit of APC/C, APC2 interacts with the backside binding domain of Ube2C and plays a vital role in recruiting Ube2C to the RING domain of APC/C (Brown et al., 2015). Another example of this includes the E2 enzyme Ube2G2 and a cognate E3 gp78. Gp78 contains a domain, G2BR, that allosterically binds the BBD of Ube2G2 and stabilizes the Ube2G2~gp78 interaction thus positively affecting ubiquitylation by gp78 (Das et al., 2009). For the E3 ligase UHRF1, whose activity is associated with the epigenetic maintenance of DNA, not its RING domain, but its UBL domain has recently been shown to bind to the BBD of Ube2D with high affinity. The UBL domain in this case, does not function to stabilize the E2~E3 interactions and enhance Ub aminolysis to ubiquitinate substrates, instead it plays an important role in E2 recruitment as well as targeted ubiquitination of histone 3 (DaRosa et al., 2018a).

While most BBD interactions have some type of positive effect either on E2 recruitment, E2~E3 stabilization, polyUb assembly and processivity, etc., in some cases E2 BBD interactions can result in no or negative allosteric effects. This is the case for Ube2E, in several studies it was found that Ub binding to the BBD of Ube2E, in tandem with the effects of the N-terminal extension that exists for Ube2E, severely limits Ube2E's ability to form polyUb chains (Nguyen et al., 2014; Schumacher et al., 2013a). Upon mutation of several residues of the BBD, Ube2E3 was able to synthesize multiple types of polyUb, suggesting a negative allosteric effect of Ub~BBD interactions on Ube2E3 (Nguyen et al., 2014).

A wide variety of BBD interactions have been recorded for over a quarter of E2 enzymes, and no doubt more will be discovered in the future. What is clear though, is that the backside binding domain is an important allosteric regulatory mechanism of E2 function as well as Ub transfer onto substrates or E3s.

Overall, the majority of E2 conjugating enzymes contain a conserved UBC core domain. However, as mentioned in this chapter, several contain either sequence insertions, N-terminal extensions, or C-terminal extensions (*Fig. 11*). These adjustments have been deemed to bestow specific functions onto each of these E2s, and perhaps the key to understanding how E3s choose E2s to collaborate with and vice versa, lies in these small changes amongst E2s.

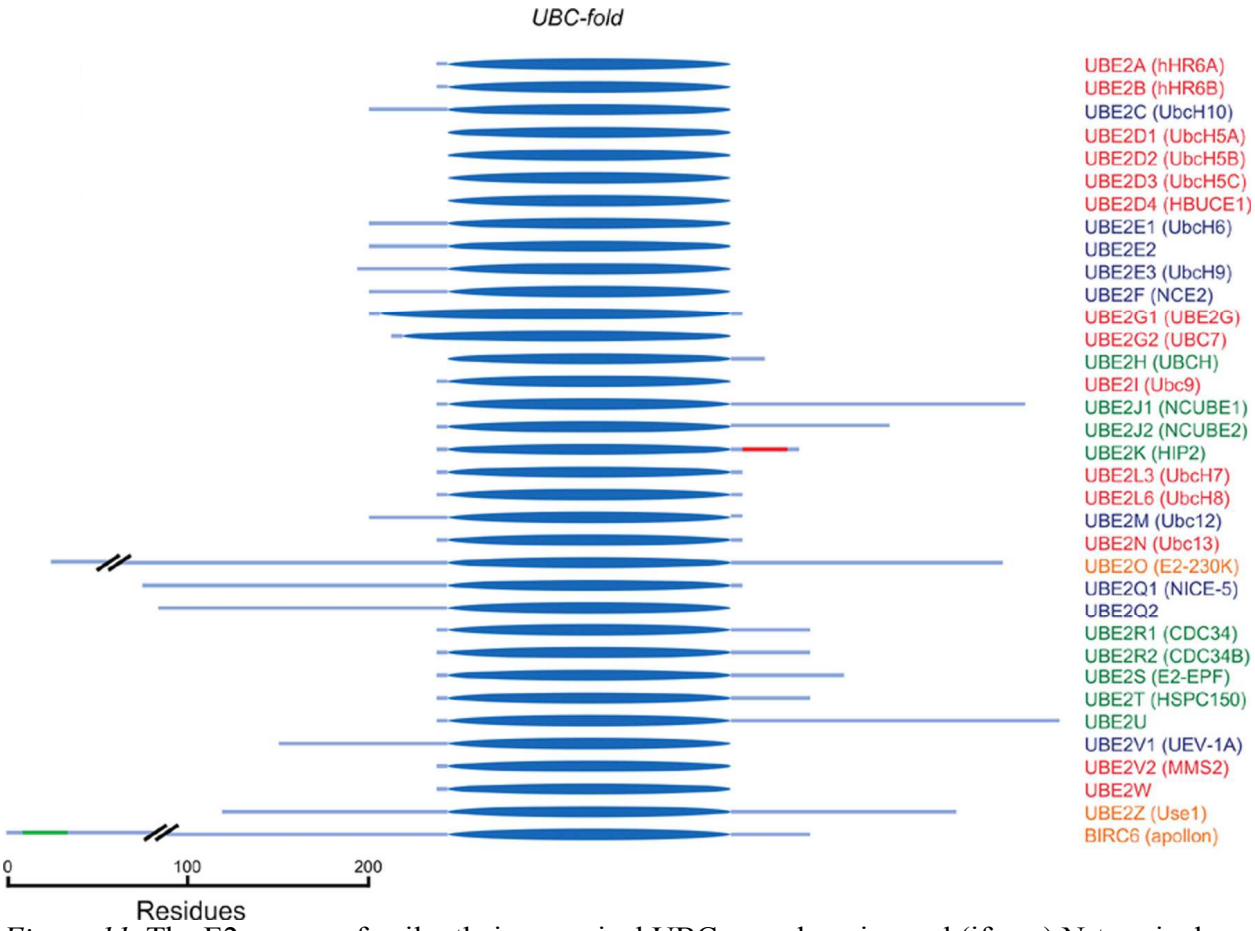



*Figure 11*. The E2 enzyme family, their canonical UBC core domains and (if any) N-terminal or C-terminal extensions. E2s colored red are made up of merely a UBC core domain. E2s colored blue contain N-terminal extensions. E2s colored red contain C-terminal extensions. E2s colored orange contain both N-terminal and C-terminal extensions. Adapted from (Wijk & Timmers, 2010).

## 1.3 E2 Enzymes, Disease and Ubiquitination Cascade Drug Targeting

### 1.3.1 Diseases Associated with the E2 Enzyme Family

To illustrate the importance of targeting the ubiquitination cascade, the E2 conjugating enzyme specifically, this chapter is dedicated to providing short summaries on known diseases that are associated with irregularities in some E2 enzymes.

*Ube2A*

Ube2A is mostly known for its involvement causing X-linked Intellectual Disability (XLID) type Nascimento. Specific mutations and deletions (e.g. Q93E) in the locus for the Ube2A gene cause severe intellectual impairment (Arslan Satılmış et al., 2021; De Leeuw et al., 2010; de Oliveira et al., 2019; Nascimento et al., 2006; Tzschach et al., 2015; Wolańska et al., 2021). It has also been associated with chronic myeloid leukemia progression (Magistroni et al., 2019; Nayak & Cancelas, 2019). Ube2A upregulation has been found in ovarian tumors, inducing higher degrees of resistance to DNA damage as well as providing chemoresistance (Somasagara et al., 2017; R. Zou et al., 2021)

*Ube2C*

Ube2C is a hub mediator of several types of cancer including cervical (Ube2C overexpression and mTOR/PI3K/AKT pathway activation), gastric (Ube2C mRNA overexpression), prostate cancer (Chiang et al., 2020; Yan Wang et al., 2021; Ying Wang et al., 2021) and is upregulated in ovarian cancer cells (R. Zou et al., 2021). Ube2C overexpression is also found in colon cancers (Fujita et al., 2009). Lastly, Ube2C together with APC/C and Ube2S ensures the time-dependent degradation of mitotic cell markers such as cyclin B and securin. Studies have found that the upregulation of Ube2C disrupts the mitotic timeline, causing lagging chromosomes, and aneuploidy resulting in tumors (Van Ree et al., 2010).

*Ube2D*

Some studies found that the cadmium (Cd) induced downregulation of Ube2D2 and Ube2D4 enhanced cell apoptosis after p53 accumulation lead to Cd induced renal toxicity (Fujiwara et al., 2012; J.-Y. Lee et al., 2016; Tokumoto et al., 2011). Ube2D also seems to play a role in PARKIN activation and regulation. PINK1 and PARKIN perform mitochondrial quality control where PINK1 accumulates on damaged mitochondria and in response, PARKIN translocates to the site of damage and induces mitochondrial autophagy. Ube2D knockdown resulted in a significant decrease of PARKIN translocation, hinting towards a role of Ube2D in the progression of Parkinson's disease (Bayne & Trempe, 2019; Fiesel et al., 2014; Pickrell & Youle, 2015). Another study showed that upon the usage of a Ube2D1 bearing a point mutation, LDL (low density lipoprotein) receptor (LDLR) ubiquitination and subsequent degradation was inhibited. LDLR cell build up is associated with elevated LDL cholesterol plasma levels and accelerated onset of atherosclerosis (Li Zhang et al., 2011). Ube2D1 is upregulated in hereditary hemochromatosis (Gehrke et al., 2003). Furthermore, Ube2D3~Ub forms a complex with an effector protein from the pathogen *Shigella* and as a consequence suppress the inflammatory response during the pathogenesis of *Shigella* (Pruneda et al., 2014).

*Ube2F*

Ube2F is involved exclusively in the NEDDylation pathway. Ube2F is overexpressed in Non-Small-Cell-Lung-Cancer (NSCLC) patients and neddylates cul5 which induces the K11-linked polyubiquitination and subsequent degradation of NOXA, which results in increased proliferation of NSCLC cells (W. Zhou et al., 2017). Secondly, a recent study has shown enhanced NOXA degradation by Ube2F-Cul5 activation proliferates colorectal cancer (Xu et al., 2021)

*Ube2H*

A 2003 study showed a small association with genetic mutations in the Ube2H encoding region of the genome and the neurodevelopmental disorder autism. It was postulated that Ube2H mutations could be a susceptibility marker for autism (Vourc'h et al., 2003). A recent study from 2020 found that significant amounts of free Ube2H mRNA transcripts circulate in the blood in Alzheimer's disease (AD) patients when compared to non-AD patients. Based on this, the researchers speculated that cell-free Ube2H could potentially be used as a diagnostic for AD (Lim & Joo, 2020). Even more recently, elevated expression of Ube2H decreased survival expectancy for patients with lung adenocarcinomas (Yen et al., 2021)

*Ube2I*

Ube2I is involved exclusively in the SUMOylation pathway. Ube2I mediated upregulation of KAP1 protein SUMOylation has been observed to support the phenotypes established by oncogenic GTPase KRAS mutations, which result in colorectal cancer (B. Yu et al., 2015). Ube2I upregulation was furthermore established in bladder cancer, where it induced increased lymph angiogenesis and tumor metastasis (Xiaoliang Huang et al., 2020; M. Tan et al., 2015)

*Ube2Z*

Upregulation of Ube2Z has been observed in hepatocellular carcinoma (Shi et al., 2020) and a specific mutation in the Ube2Z furthermore has shown to increase the risk of coronary artery disease in Chinese diabetic patients (Lu et al., 2017)

To be clear, in some of the cases outlined in this chapter, a causal mechanism has not been established between the E2 enzyme and the disease progression. In some cases, E2

enzymes have merely been observed to function irregularly during these diseases and are deemed markers of the disease progression.

As evidenced by the widespread involvement of E2 conjugating enzymes in a plethora of diseases, through either upregulation, downregulation or genetic insertions or deletions, E2 enzymes pose viable druggable targets for a variety of diseases. More than that, there is a dire need for molecules that can specifically and successfully target E2 conjugating enzymes *in vivo* to regulate their activity

### 1.3.2 Evaluation of Druggable Targets in the UPS

In chapter 1.3.1 a wide variety of diseases were shown to be associated with the dysregulation of E2 conjugating enzymes. The E2 enzyme is merely a single player in the ubiquitination cascade. Dysregulation of the other acting enzymes is also associated with a plethora of diseases (Amer-Sarsour et al., 2021; Chakraborty & Ziviani, 2020; Chaugule & Walden, 2016; George et al., 2018; Humphreys et al., 2021). Consequently, much research has been dedicated to developing small molecules and other types of drugs to specifically target and inhibit the ubiquitination cascade, inspired by the clinical success of the drug Bortezomib.

Bortezomib (dipeptidyl boronic acid derivative), a 26s proteasome inhibitor that is FDA-approved to treat multiple myeloma, is commonly used as the hallmark example showing that inhibiting the ubiquitination cascade can be clinically relevant (Accardi et al., 2015; Field-Smith et al., 2006; Laubach et al., 2011; Richardson et al., 2007; W. Zhang & Sidhu, 2014a). Bortezomib works by reversibly binding and inhibiting the 20s core subunit of the 26s proteasome. Since Bortezomib targets a very general entity within the cell, the 26s proteasome, logically it would follow that Bortezomib functions nonspecifically. Interestingly however, Bortezomib can actually specifically induce apoptosis in tumor cells, simply because cancer cells

generally show significantly increased proteasomal degradation activity compared to normal cells (Accardi et al., 2015; W. Zhang & Sidhu, 2014b). Therefore, while Bortezomib in fact, can affect both tumor and normal cells, cancer cells will suffer much faster, much more, and subsequently undergo apoptosis much earlier compared to non-cancerous cells.

Apart from the 26s proteasome, there are 4 other druggable entities present in the ubiquitination cascade: E1-activating enzymes, E2-conjugating enzymes, E3 ubiquitin ligases and DUBs We will discuss the "druggability" of the E2 conjugating enzyme later in this body of work.

Naturally, as was the case for Bortezomib, specificity is a major factor when formulating an inhibitor. For instance, only 2 human E1 activating enzymes have been identified so far, Ube1 and Uba6. Inhibiting Ube1, would affect nearly all E2 conjugating enzymes, E3 ubiquitin ligases, as well as the pathways they control. Developing effective inhibitors for the E1 conjugating enzyme would therefore produce a **highly nonspecific drug response**. However, several attempts have been made to develop cell permeable inhibitors targeting the E1 activating enzyme. Pyr-41 (4[4-(5-nitro-furan-2-ylmethylene)-3,5-dioxo-pyrazolidin-1-yl]-benzoic acid ethyl ester), was the first of these attempts (Y. Yang et al., 2007) and has since proven to be a valuable research tool to study Ube1 interactions. Pyr-41 was postulated to form a selective covalent bond with the active cysteine of Ube1. Utilizing Pyr-41, E1s role has been identified in the suppression of Herpes Simplex Virus-1 replication (Ikeda et al., 2020) and it has also been shown that Pyr-41 can induce p53-mediated apoptosis (Ungermannova et al., 2012). Furthermore, PYR-41 has been found to attenuate organ damage after injury in mice models (Matsuo, Chaung, et al., 2018; Matsuo, Sharma, et al., 2018). Pyr-41 never made it to clinical trials as a viable therapeutic in humans. Other E1 inhibitors that never made it to clinical trials

include: NSC624206 (Lv et al., 2017; Ungermannova et al., 2012), JS-K (Kaur et al., 2015; Kitagaki et al., 2009; Maciag et al., 2012; Xudong Zhao et al., 2019) and Ub mimicking peptides (B. Jin et al., 2018; B. Zhao et al., 2012).

MLN4924 is a selective inhibitor of NEDD8 Activating Enzyme (NAE1) and results in a buildup of cullin E3 ligase in cells and stabilization of cellular p27. MLN4924 has been found to attenuate several types of cancer, including endometrial carcinoma(H. Liu et al., 2021), glioblastoma (Hua et al., 2015) and retinoblastoma (Aubry et al., 2020) and is posed as a potential treatment in pulmonary cancers (Norton et al., 2021). Currently MLN4924 has entered clinical trials for the treatment of acute myeloid leukemia (Faessel et al., 2019; Swords et al., 2015) and patients with nonhematologic malignancies. Aside from MLN4924, just one other Ube1 inhibitor has entered clinical trials, namely TAK-243. TAK-243 has shown great promise as it was able to induce cellular distress and subsequent cell apoptosis in diffuse large B-cell lymphoma (Hyer et al., 2018)

In terms of specificity, E3 ligases are much more attractive targets for inhibition. Over 600 different E3 ligases exist, which means that inhibition of specific E3s could selectively shut down specific biological pathways. However, most E3 ligases contain RING domains and function through noncovalent interactions with E2-conjugating enzymes and substrates. An approach to inhibiting E3s could include either trapping noncovalent E2~E3 interactions or disrupting the protein-protein interface between E2 and E3. For either of these strategies to work for specifically inhibiting E3 ligases, RING E3s would need to show significant sequence divergence in their protein binding domains (Petroski, 2008). The question therefore is, do RING E3s contain enough sequence divergence to design specific inhibitors for each?

HECT and RBR ligases on the other hand, are both functionally divergent and contain catalytic cysteines. Since not every HECT or RBR ligase has the same protein substrates or possesses the same polyubiquitination activity, conformational differences must exist that can facilitate these differences. If that is true, HECT and RBR ligases might be more agreeable targets (Petroski, 2008).

In any case, several efforts to design drugs that can inhibit E3 ligases have entered clinical trials. One notable example includes the class of drugs known as Nutlins. Nutlin3a is recognized to specifically disrupt the interaction between E3 ligase MDM2 and the tumor suppressor p53 (Walter et al., 2018). p53 is heavily involved in cell proliferation, DNA repair and can induce cell apoptosis (Zilfou & Lowe, 2009). It is generally known as a tumor suppressor but in tumor cells it is often under expressed or entirely knocked out, which is why p53 is usually considered oncogenic in cancer (Blagosklonny, 2000; Petroski, 2008; Sabapathy, 2015; Shetzer et al., 2016). In tumor cells where p53 is under expressed, primary cause is the overexpression of MDM2 which forms a complex with p53, resulting in excess proteasomal degradation of p53. Nutlin3a specifically binds in the exact pocket where p53 binds, and in that way, achieves MDM2 inhibition and prevents p53 degradation (Puszynski et al., 2014). Other MDM2 inhibitors are currently being evaluated in phase I clinical trials: CGM097 (Bauer et al., 2021) and APG-115 (Aguilar et al., 2017).

### 1.4 Inhibiting the E2-Conjugating Enzyme and Associated Challenges

There are significantly less E2 conjugating enzymes (~40) compared to E3 ubiquitin ligases or even DUBs. However, the importance of the E2 enzyme in the ubiquitination cascade cannot be understated. To reiterate previous statements: E2 enzymes control processivity of ubiquitin chain formation and more importantly, they select the type of polyUb linkage that gets

formed. They are the true puppeteers of the ubiquitination cascade (Ye & Rape, 2009). Combined with the fact that every E2 enzyme is associated with at least one and often 2 or more invasive diseases (see *section 1.3.1*), it is surprising that so few inhibitors have been developed that specifically target E2 enzymes, none of which have shown clinical promise (*Fig. 12*) (Wertz & Wang, 2019).

Since E2 enzymes are in control of polyUb selection and chain building processivity, by selectively inhibiting single E2 enzymes, it might be possible to shut down specific biological pathways associated with certain types of polyUb chains. Applied clinically, that could have incredible promise.

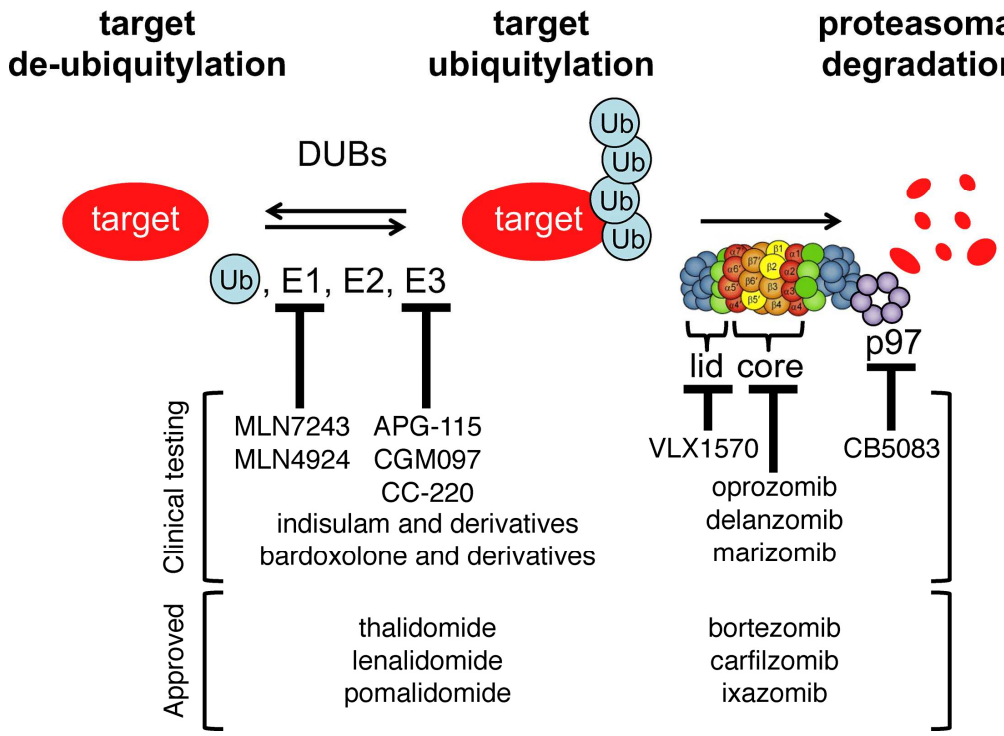



*Figure 12*. Targeting the ubiquitination cascade. Molecules are shown that can specifically inhibit certain enzymes in the UPS. Molecules featured are either undergoing clinical testing or are FDA approved drugs. Adapted from (Wertz & Wang, 2019).

Why have few E2 enzyme inhibitors been developed? The main issue lies within the general structure of E2 enzymes and mechanism of action (see *section 1.2*). All E2 enzymes contain a conserved UBC core domain (Hann et al., 2019; Randles & Walters, 2012; Schumacher et al., 2013b; Stewart et al., 2016; Wijk & Timmers, 2010). All E2 enzymes (except for pseudo-E2s Ube2V1 and Ube2V2) contain a highly conserved catalytic cysteine that is relatively shallow and easily accessible (Garg et al., 2020; Hann et al., 2019; Olsen & Lima, 2013; Papaleo et al., 2011; Randles & Walters, 2012; Wijk & Timmers, 2010). All E2s go through a version of the same general mechanism of Ub transfer (trans thiolation or aminolysis reactions). Some E2 enzymes contain small UBC domain sequence insertions, other E2 enzymes contain N-terminal extensions (Ube2C, Ube2E, Ube2F, Ube2M, Ube2Q and Ube2V1). A handful of E2 enzymes contain C-terminal extensions (Ube2H, Ube2J, Ube2K, Ube2R, Ube2S, Ube2T and Ube2U) and only three E2 enzymes contain both (Ube2O, Ube2Z and BIRC6) (Wertz & Wang, 2019; Wijk & Timmers, 2010). As mentioned previously, these changes are deemed to aid in E2 functional specificity and for some, unique additional sequences can participate in the regulation of E2 activity (Kleiger et al., 2009; Mathias et al., 1998; Wu et al., 2002; Ziemba et al., 2013). In summary, due to the high level of structural and mechanistic conservation associated with the E2 enzyme, inhibitor design faces a huge issue of specificity. There seems to be no obvious, all-inclusive, and effective approach to targeting E2 conjugating enzymes.

### 1.4.1 Some Notable Examples of Efforts to Inhibit the E2 Enzyme

While E2-targeted inhibition faces specificity issues, there are a select few examples of successful molecules that can inhibit E2 enzymes. 3 examples will be outlined in this chapter.

For the past decade, nearly every paper featuring E2 inhibitors has brought up the molecule CC0651. CC0651 works as a specific inhibitor of the E2 enzyme Ube2R1 (or Cdc34). A specific class of RING-domain E3 ligases, the CRLs, in yeast solely utilizes Ube2R1 to facilitate substrate ubiquitination (Hill et al., 2019). In humans CRLs make use of Ube2G, Ube2R1 and a handful of other E2 enzymes (Hill et al., 2019). Ube2R1 contains an acidic tail (see *section 1.2*) that supports the processivity and formation of K48-linked polyUb (Kleiger et al., 2009; Pierce et al., 2009). Ube2R1 overexpression has been found in several types of cancer, such as lung cancer, hepatocellular carcinomas and familial breast cancer (Price et al., 2006; Tanaka et al., 2001; S. Zhang & Sun, 2020).

First discovered in 2011, Ceccarelli et al. performed a small molecule screen to find potential inhibitors of the SCF-dependent ubiquitination of $p27^{kip1}$, a cyclin-dependent inhibitor. They found that CC0651 binds with an $IC_{50}$ value of 2 μM, in a cryptic pocket away from the catalytic site and in an allosteric manner selectively inhibits Ub aminolysis from Ube2R1 onto substrates. *In vivo*, CC0651 was able to reduce $p27^{Kip1}$ ubiquitination, which increased the half-life of $p27^{Kip1}$ and attenuated cell proliferation (Ceccarelli et al., 2011). Later, the mechanism of inhibition was elucidated in slightly more detail by Ceccarelli et al. They found that upon binding of CC0651 in the cryptic pocket of Ube2R1, the E2~Ub thioester (which has a weaker interaction compared to other E2~Ub thioester linkages) was stabilized and subsequently trapped (Ceccarelli et al., 2011; H. Huang et al., 2014). They furthermore found that cognate SCF ligases had a higher affinity for the stabilized Ube2R1~Ub complex, compared to Ube2R1 on its own (Ceccarelli et al., 2011; H. Huang et al., 2014).

While it is evident that CC0651 stabilizes the Ube2R1~Ub interaction, the mechanism by which CC0651 achieves this, is still under speculation. CC0651 posed the first specific E2

inhibitor with encouraging pre-clinical data however has not been improved to the clinical development stage. Due to the inherent chemical properties of CC0651, it was thought that the structure could not be optimized to improve the potency into $IC_{50}$ values below the μM range. (Xiaodong Huang & Dixit, 2016; Kotz, 2011)

Another promising effort was done by Pulvino et al. in 2012. Pulvino et al. developed a cell-based assay to screen small molecule compound libraries for PKCβ signaling (Pulvino et al., 2012). PKCβ signaling is associated with the progression of DLBCL (diffuse large B-cell lymphoma) as it is a part of the NF-kB signaling pathway, a pathway that in DLBCL is constantly active (Bognar et al., 2016; Cardona Gloria et al., 2021; Chan et al., 2013; Compagno et al., 2009; Pulvino et al., 2012; Sasaki & Iwai, 2015; Staudt, 2010). An important mediator of the NF-kB signaling pathway is the heterodimer E2 pair Ube2N~Ube2V1. Ube2N~Ube2V1 support the formation of K63-linked polyUb, which in turn activates the NF-kB pathway (Branigan et al., 2015; Garg et al., 2020; Wiener et al., 2012). Through their cell-based assays, Pulvino et al. identified a small molecule, NSC697923, that was able to inhibit the formation of K63-linked polyUb by Ube2N~Ube2V1 in a concentration-dependent manner. It was furthermore found that NSC697923 was able to do this by blocking the covalent attachment of a donor Ub at the catalytic cysteine of Ube2N (Pulvino et al., 2012). Since its discovery, multiple studies have revealed the anticancer properties of NSC697923 as it was found to reduce cell proliferation in melanoma (Dikshit et al., 2018) and neuroblastoma cells (Cheng et al., 2014).

The most recent advancement in the development of specific E2-targeted small molecules, is the discovery of UbV's or Ubiquitin Variants that can target the backside binding domains of E2 conjugating enzymes. Since it has been shown that noncovalent interactions with the backside binding domain can modulate the activity of E2 enzymes (see *section 1.2*), and most

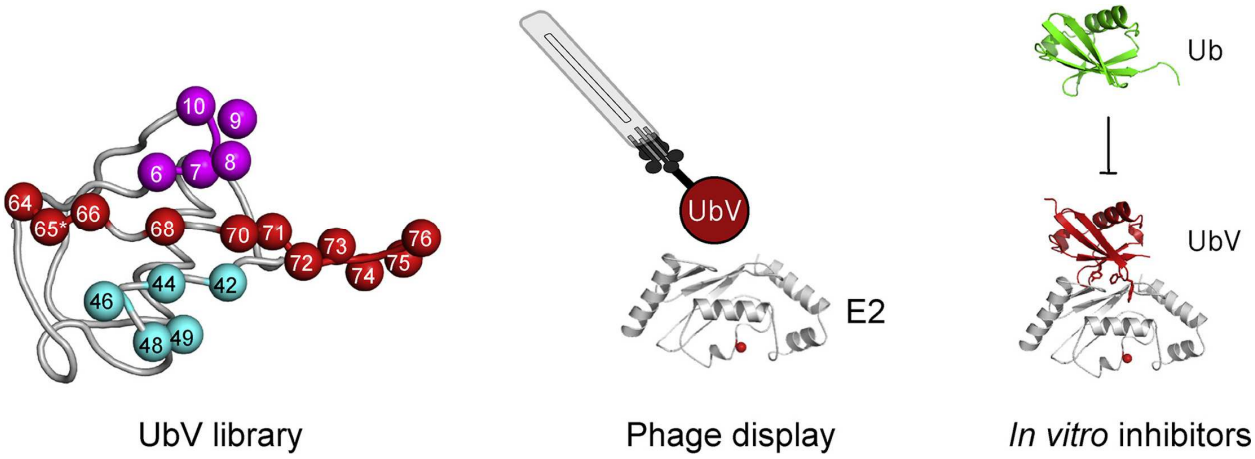



*Figure 13*. Workflow for the development of E2-targeted UbV molecules using phage display. Adapted from (Garg et al., 2020).

BBD interactions with Ub are of weak affinity (Brzovic et al., 2006b; Stewart et al., 2016), Garg et al. hypothesized that the development of high affinity and high specificity ubiquitin variants could result in selective E2 inhibitors. Multiple unique binders for Ube2D1, Ube2V1, Ube2G1 and Ube2G2 were identified in a phage-displayed library search of UbV molecules (*Fig. 13*). Subsequent Competitive ELISA based binding assays revealed that UbvD1.1 (Ube2D1 specific UbV molecule) was able to selectively bind the BBD of Ube2D1, not Ube2D2-4. UbV's developed for Ube2G did not select for Ube2G isoforms but showed no affinities for other E2s. Similarly, UbV.V1.1 (UbV specific for Ube2V1) only bound the two Ube2V isoforms (Garg et al., 2020).

Furthermore, Garg et al. performed several ubiquitination assays in the presence of UbV molecules and found that over all E2 activity was only mildly inhibited. E2~Ub thioester linkage formation was not blocked, but the catalysis of polyUb chains was disrupted in the cases of Ube2V and Ube2D (Garg et al., 2020).

These three examples show that it is possible to selectively inhibit E2 conjugating enzymes by targeting E2 structures at sites other than the catalytic cysteine.

### 1.4.2 Ube2D and Its Isoforms

Ube2D (4 isoforms) sets itself apart from its E2 enzyme family members because of its non-specific nature. This is firstly because Ube2D isoforms can facilitate the formation of nearly all types of (poly)Ub labels: K11,-K48,- K6,- K27,- K29,- and K63-linked polyUb and monoubiquitin (Christensen & Klevit, 2009; David et al., 2010; Guo et al., 2017; Huett et al., 2012; Hyoung et al., 2007; Mishra et al., 2019; Tracz & Bialek, 2021; Ye & Rape, 2009). More importantly, within the entire E2 enzyme family, Ube2D is able to interact with the most E3 ligases (Ben-Eliezer et al., 2015; Christensen & Klevit, 2009; DaRosa et al., 2018a; Hasson et al., 2013; Hill et al., 2019; Kar et al., 2012; Li Zhang et al., 2013). Ube2Ds incredible flexibility and multifunctionality clearly indicates a vital role within the ubiquitination cascade. Therefore, it is remarkable how relatively little there is known about 1) cellular phenotypes associated specifically with Ube2D and 2) the biological consequences of specific Ube2D inhibition/knock out. Is it even possible to survive without Ube2D? To answer these questions, there is a need for molecules that can reversibly achieve near complete inhibition of Ube2D and bind with sufficient affinity to show efficacy in a mammalian cell environment.

### 1.4.3 E2 Enzyme Family Sequence Conservation Analysis

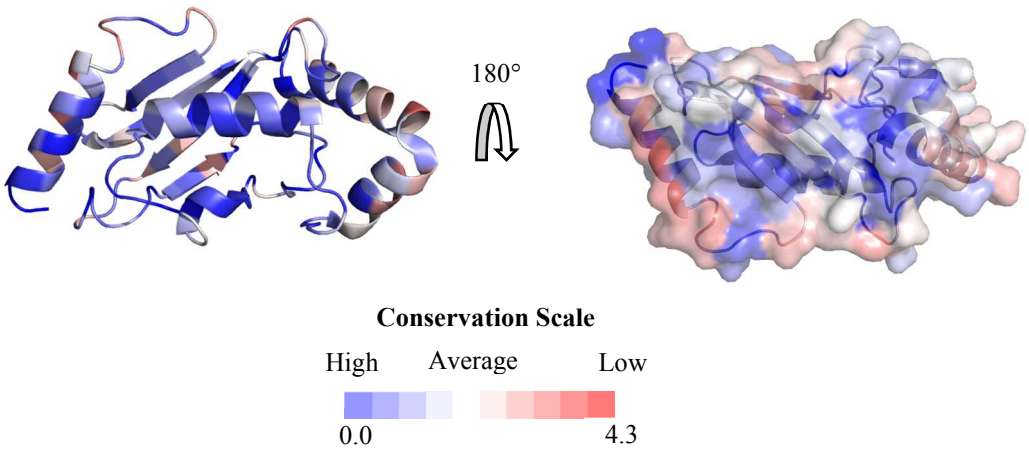
As indicated at the beginning of *section 1.4*, the main issue with designing specific E2-targeted inhibitors, is the high degree of conservation within the general structure of the E2 enzyme. The goal is not only to design a specific Ube2D inhibitor, but in doing so, develop a design that can be “personalized” to other E2 enzymes as well. To achieve this, we must

establish which area of the general E2 enzyme structure is least conserved amongst E2 enzymes so that we can exploit it to design specific E2 inhibitors.

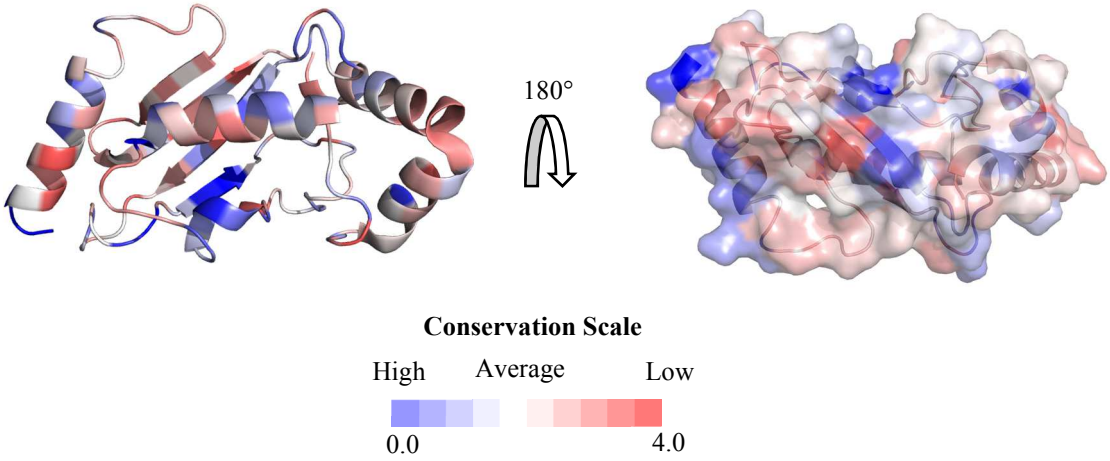
Consequently, we performed a large-scale E2 enzyme family sequence conservation analysis. We collected protein query sequences for all human E2 enzymes and performed NCBI protein BLAST runs. For each resulting data set we manually established cut offs and removed all sequences that did not correspond to the E2 enzyme of interest. Sequences for E2 isoforms were grouped together. We utilized several computational tools to remove redundant sequences and calculate multiple sequence alignments (MSA) for each E2 enzyme (23 in total). From each E2 enzyme MSA, we extracted consensus sequences. We then aligned the consensus sequences of every E2 enzyme, to produce an MSA through which we could directly compare each E2 enzyme.

To calculate the degree of sequence conservation for the E2 enzyme family in general, we calculated the Shannon entropy values for each position in the consensus sequence MSA. Shannon Entropy is a measure of sequence conservation. Relatively high Shannon entropy values represent high amino acid variability, low Shannon entropy values represent high amino acid conservation. As expected, the active site cysteine was highly conserved across all E2 enzymes, with a relatively low Shannon entropy value (*Fig. 14C*). Both the E1 binding domain and the E3 binding domain displayed relatively low Shannon entropy values (except for a few stand-out residues), indicating a relatively high degree of conservation across E2 enzyme family members. Interestingly, the highest concentration of residues with relatively high Shannon entropy values, was located in the anti-parallel β-sheet that makes up most of the backside

(A)


180°
Conservation Scale
High
Average
Low
0.0
4.3

(B)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Conservation Scale
High
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Low
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4.0

(C)

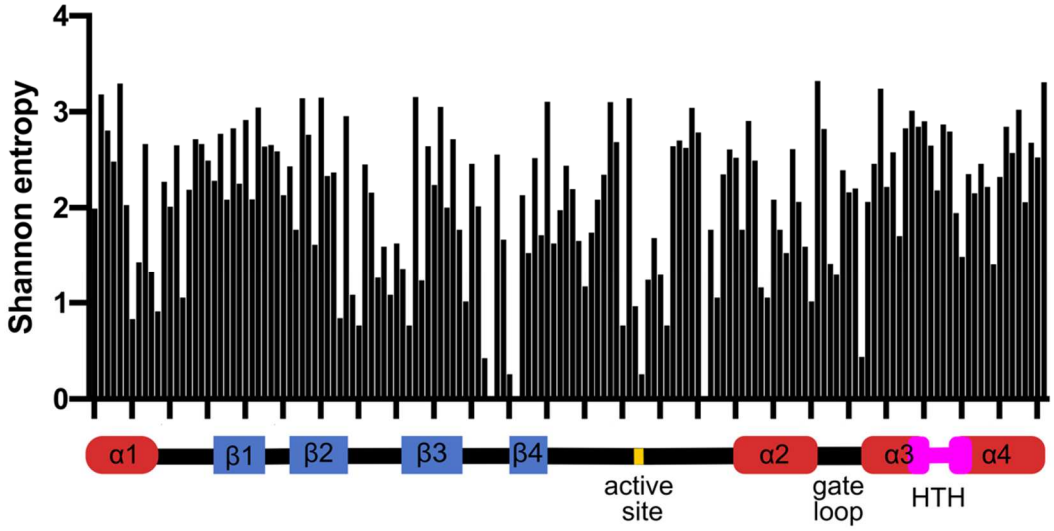

Shannon entropy
0
1
2
3
4
α1
β1
β2
β3
β4
α2
α3
α4
active site
gate loop
HTH

*Figure 14.* Ube2D colored by Shannon entropy. Shannon Entropy is a measure of sequence conservation. Relatively high Shannon entropy values represent high amino acid variability, low Shannon entropy values represent high amino acid conservation. (A) Ube2D colored by Shannon entropy values calculated from Ube2D sequence alignment. (B) Ube2D colored by Shannon entropy values calculated from E2 enzyme family consensus sequences alignment. Ube2D acts as E2 enzyme family representative. (C) E2 enzyme family sequence conservation. Shannon entropy values for every position in the alignment in relation to E2 secondary structures. Ube2D PDB: 2C4P. 2C4P images were created using the PyMOL Molecular Graphics System (v2.4.1).

binding domain of many E2 enzymes. This indicates a reasonable degree of divergence within that region.

To establish whether this level of divergence exists in the BBD region amongst different Ube2D isoforms, we also calculated the Shannon entropy values for each position in the MSA for the Ube2D enzyme alone. Whilst differences in Shannon entropy were not as robust (Ube2D isoforms are a lot more similar than individual E2 enzymes are), a similar trend was observed when compared to the MSA for E2 enzyme consensus sequences. The side opposite to the catalytic site, showed a relatively high concentration of sequence positions with higher Shannon entropy values, indicating a higher degree of sequence divergence in the general area that makes up the BBD of Ube2D.

Due to the relatively high Shannon entropy values for a number of residues located in the backside binding domain in our E2 enzyme family sequence conservation analysis, we conclude that the backside binding domain can potentially be utilized as a means to achieve specificity in E2-targeted inhibitor designs.

The fact that we also observed relatively little sequence conservation in the backside binding domain for the Ube2D MSA, suggests that we could potentially even design inhibitors that can target individual Ube2D isoforms. This observation corroborates the findings of Garg et al. (Garg et al., 2020)

## 1.5 The Merits of Fusion Proteins

One inhibitor design strategy is the construction of fusion proteins: proteins consisting of 2 or more otherwise unconnected domains/proteins with different functions, producing a multifunctional, multidomain entity. The goal of this chapter is to illustrate the viability of this strategy by means of 2 examples.

In a 2020 study, enzymatic degradation of plastics was investigated. Plastic pollution is a major issue threatening the environment currently, illustrated by phenomena such as the Great Pacific Garbage Patch (Cózar et al., 2014; Jambeck et al., 2015; Lamb et al., 2018; Law et al., 2010). Historically, organisms have evolved elaborate enzymatic systems to degrade higher polymeric substances that normally have relatively long half-lives (e.g. cellulose, chitin, etc.) (Baty, Eastburn, Diwu, et al., 2000; Baty, Eastburn, Techkarnjanaruk, et al., 2000; Beier & Bertilsson, 2013). A major component of plastic is polyethylene terephthalate (PET). In a remarkable progression of events, certain lifeforms have adapted to the increase in plastic pollution by evolving enzymatic mechanisms that can degrade plastic into soluble components that can be utilized as nutrients (Bombelli et al., 2017; Brunner et al., 2018; Knott et al., 2020; Wei & Zimmermann, 2017; J. Yang et al., 2014; Yoshida et al., 2016).

In a study by Knott et al., one such mechanism was investigated. A specific strain of bacteria, *Ideonella sakaiensis,* was recently discovered to have developed the ability to degrade polyethylene terephthalate in a two-enzyme biocatalytic process (da Costa et al., 2021; Knott et al., 2020; Son et al., 2020). Knott et al. found that *I. sakaiensis* achieves PET degradation by first utilizing a PETase enzyme to hydrolyze PET polymer into mono(2-hydroxyethyl) terephthalic acid (MHET), bis(2-hydroxyethyl)-TPA (BHET), and terephthalic acid (TPA). A

second enzyme, MHETase, is then introduced that can hydrolyze MHET into terephthalic acid and ethylene glycol (da Costa et al., 2021; Knott et al., 2020; Son et al., 2020).

To explore the potential of repurposing *I. sakaiensis'* two step PET degradation mechanism for environmental cleanup, Knott et al. designed a chimeric protein by covalently linking the N-terminal of PETase through a glycine-serine linker to the C-terminal of MHETase, in hopes that proximity would improve the already existing synergetic relationship between PETase and MHETase. The researchers hypothesized that proximity would speed up the catalysis of PET degradation (Knott et al., 2020). They found that PETase~MHETase sped up PET catalysis significantly compared to PETase alone and an equimolar mixture of unlinked PETase and MHETase (Knott et al., 2020).

Another excellent example of moieties that are covalently connected through a linker to produce a bifunctional molecule, is PROTAC (PROeolysis TArgeting Chimera) (Sakamoto, 2005). PROTACs are chimeric proteins that are utilized to induce targeted degradation of specific substrates. PROTACs consist of three parts: a ligand that specifically binds the substrate, a linker (usually a PEG-based linker) and a ligand that can bind an E3 ligase. By bridging the distance between a target substrate and an E3 ligase, PROTAC facilitates substrate polyubiquitination and subsequent proteasomal degradation (Alabi & Crews, 2021; Gadd et al., 2017; Gao et al., 2020; Y. Zou et al., 2019).

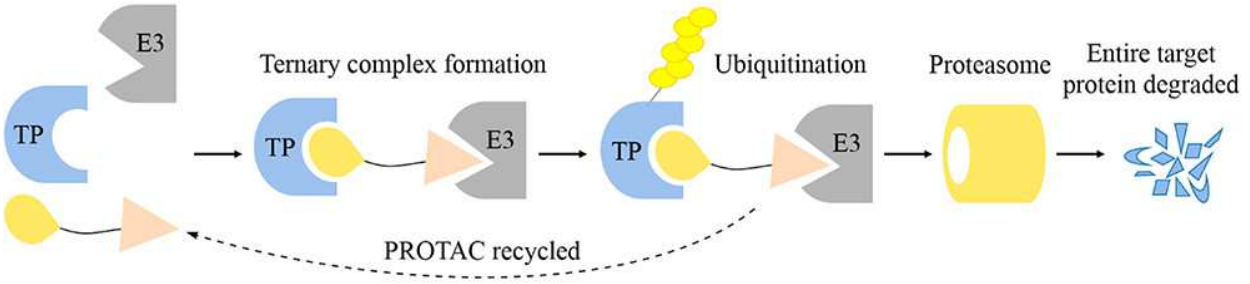



*Figure 15.* Mechanism of PROTACs induced targeted substrate degradation. Adapted from (Gao et al., 2020).

Pharmaceutical companies viewed PROTAC as a potential technique to develop novel drug therapies. As a result, a large amount of studies have since applied PROTACs to target a variety of proteins involved a plethora of diseases (Bassi et al., 2018; Bondeson et al., 2015; McCoull et al., 2018; Nunes et al., 2019; Sakamoto et al., 2003; Salami et al., 2018; Y. Sun et al., 2019)

These two examples illustrate how covalently linking molecules can enhance already existing functions or create entirely novel functions that could potentially aid in environmental cleanup, treat diseases and much more.

### 1.6 Rational Design of Novel Ube2D Protein Inhibitors

To design Ube2D inhibitors, we wanted to utilize a linked-domain approach (examples in *section 1.5*) to create a bifunctional hyper potent inhibitor molecule that makes use of the already existing properties of Ube2D.As outlined in *section 1.4.3* the backside binding domain of Ube2D seems most promising as an inhibitor target to introduce inhibitor specificity.

We hypothesized that if we could create a linked-domain inhibitor that consists of three parts - 1) a moiety that specifically targets the BBD of Ube2D; 2) a moiety that binds with high affinity to the E3 binding domain and 3) a flexible linker that covalently links both moieties - we could block E3 interactions, BBD interactions and E1 interactions all at once. While this

inhibitor would not directly interact at the E1 binding domain on Ube2D, as indicated in *section 1.2*, the E1 and E3 binding site on Ube2D overlap at α-helix1. This could mean that a linked-domain inhibitor could create a type of "blanket" effect and by default also block the E1 binding domain.

To determine the structure of the linked-domain inhibitor we utilized the characteristics of the E3 ligase UHRF1. UHRF1, up until this point, has only been determined to interact with Ube2D (DaRosa et al., 2018a; Foster et al., 2018). Since UHRF1 shows specificity towards Ube2D, we hypothesized that each UHRF1 domain would also be specific towards Ube2D.

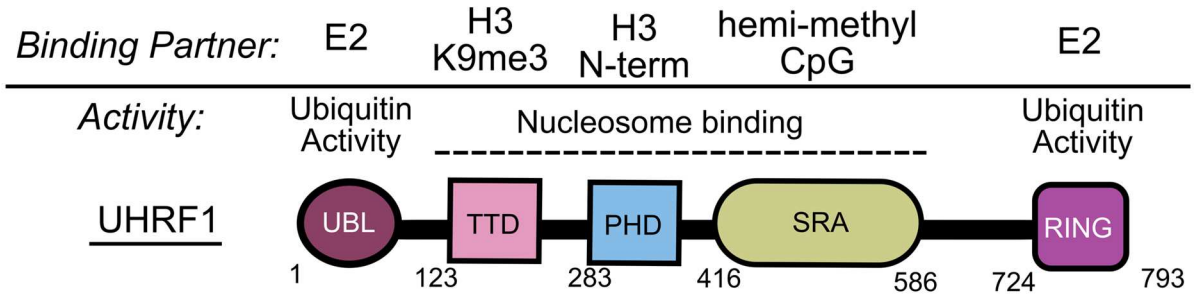
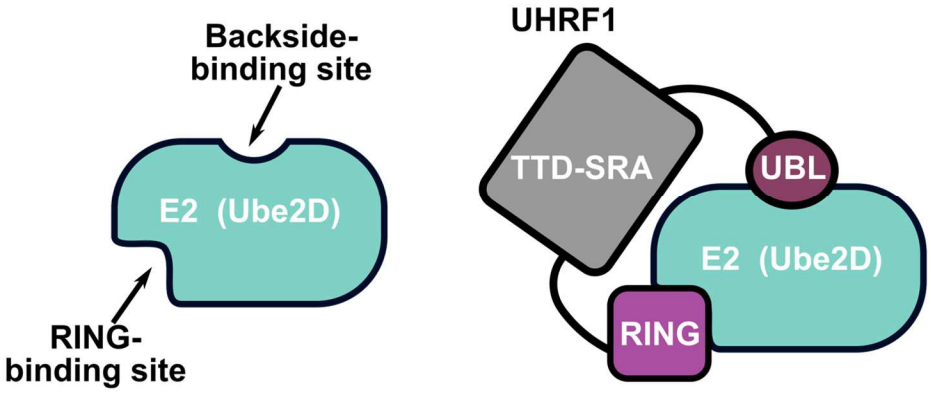



*Figure 16*. All domains of the E3 ligase UHRF1, assigned activities and binding partners.

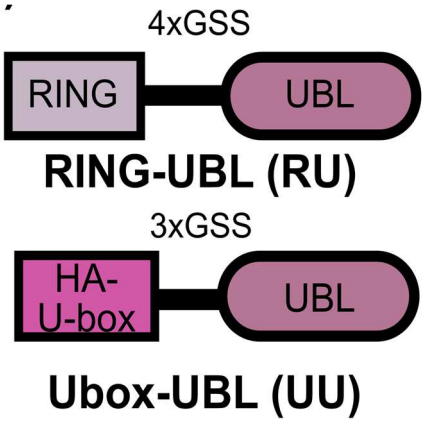
We therefore decided to covalently link UHRF1's RING domain and UBL domain. UBL domain is known to bind the backside binding domain with a $K_d$ of ~15 μM (DaRosa et al., 2018a), which should outcompete any Ube2D~Ub interactions (Ub has an affinity with Ube2D of about 200 μM (DaRosa et al., 2018a)). We also created a second-generation design where we substituted the RING-domain for an affinity optimized U-box domain (see *section 1.1.3*) as featured in Starita et al. (Starita et al., 2013).

We opted for a commonly used flexible glycine-serine linker (X. Chen et al., 2013; Reddy Chichili et al., 2013) and estimated the optimal linker length to be ~3-4 x GSS repeats.

(A)


Backside-
binding site
E2 (Ube2D)
RING-
binding site
UHRF1
TTD-SRA
UBL
E2 (Ube2D)
RING

(B)


4xGSS
RING
UBL
RING-UBL (RU)
3xGSS
HA-
U-box
UBL
Ubox-UBL (UU)

(C)

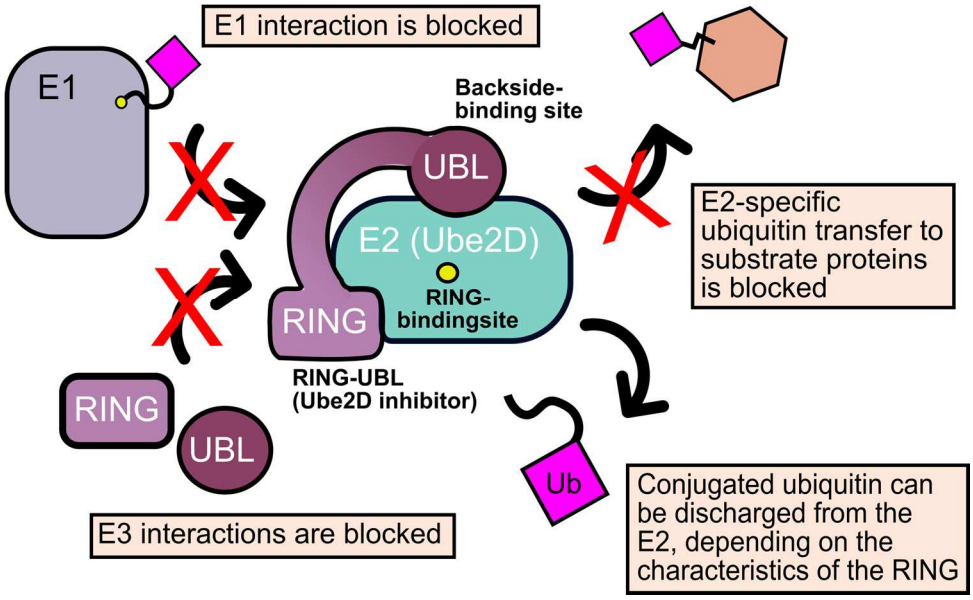

E1 interaction is blocked
E1
Backside-
binding site
UBL
E2 (Ube2D)
RING-
bindingsite
RING
RING-UBL
(Ube2D inhibitor)
E2-specific
ubiquitin transfer to
substrate proteins
is blocked
RING
UBL
Ub
Conjugated ubiquitin can
be discharged from the
E2, depending on the
characteristics of the RING
E3 interactions are blocked

*Figure 17.* Design of novel 1st and 2nd generation linked domain Ube2D inhibitors. (A) UHRF1 RING domain binds Ube2D at the E3-binding domain on Ube2D. UHRF1 UBL domain can noncovalently associate with the BBD of Ube2D. (B) Novel designs of linked-domain protein inhibitors of Ube2D, domains extracted from the structure of UHRF1. Domains are linked through a flexible glycine-serine linker. (C) Projected mode of inhibition of linked-domain inhibitors. We expect they can block both E1 and E3 interactions simultaneously.

We then arrived at inhibitor designs as shown in *figure 17B:* Ring-4xGSS-UBL (RU) and HA-U-box-3xGSS-UBL (UU).

# CHAPTER 2: RESEARCH GOALS

Based on the information available in the literature on the E2 enzyme, its role in the ubiquitination cascade and in the cell in general, this study will pursue the following set of goals:

**Firstly, we will aim to design and successfully purify selective high-affinity Ube2D inhibitors that utilize the backside binding domain of Ube2D.** The backside binding domain of the Ube2D can bind certain protein structures such as Ub and our sequence conservation analysis showed a high degree of sequence divergence within that region. Therefore, we propose to design inhibitors that take the backside binding capabilities of Ube2D into account. It is possible to utilize a structure that has a better binding affinity with the Ube2D back side binding domain than Ub, e.g. UBL domain. In the design, we will aim to make use of the inherent binding domains present on Ube2D, including the E3 binding domain. We hypothesize that linking two domains together, such as RING and UBL domain will produce an inhibitor with significantly enhanced binding affinity to Ube2D than of a single domain alone.

**Secondly, we will test the efficacy of the newly designed inhibitor *in vitro*. We will also determine the thermodynamic properties of the inhibitors-Ube2D1 complex.** To determine whether the design is functional, we will have to perform multiple tests. We will perform ubiquitination assays to establish their ability to prevent E3 ligase ubiquitination activity. We will also perform isothermal titration calorimetry experiments to determine their binding affinities towards the Ube2D1 target.

**Thirdly, we will evaluate the effects of Ube2D1 inhibition *in vivo*.** There have been multiple efforts to inhibit the Ube2D enzyme and several of these efforts have been successful. However, not a lot of information is available on the usage (or efficacy) of these inhibitors *in*

*vivo*. Ube2D is known as the "non-specific" E2, meaning it is the most abundant and most versatile out of all the different E2 enzymes. Its abundance and versatility implicate a very important role inside the cell. It is therefore striking how little there is known about the cellular effects of Ube2D1 inhibition. This work will seek to elucidate the effect of Ube2D1 inhibition in vivo.

**Lastly, we will evaluate Ube2D as a potential therapeutic target for diseases such as cancer.** Based on all the gathered evidence, we will end with an evaluation on the merits of Ube2D as a potential therapeutic target for e.g., cancer treatments.

# CHAPTER 3: RESULTS

## 3.1 IAP2 Inhibition Assays UU and RU

RU and UU, first and second generation Ube2D inhibitors, were tested by performing IAP2 ubiquitination inhibition assays. IAP2 (Inhibitor of Apoptosis 2) is an E3 Ligase binding partner of Ube2D and is able to auto ubiquitinate (Bertrand et al., 2008; Cheung et al., 2008; Y. Yang et al., 2000). Hypothetically, if Ube2D is inhibited, IAP2 will not be able to auto ubiquitinate. We can therefore use IAP2 auto-ubiquitination activity as an indirect measure of Ube2D inhibition. We performed ubiquitination assays in the presence of several concentrations of either RU or UU, ranging from 0 to 100 μM.

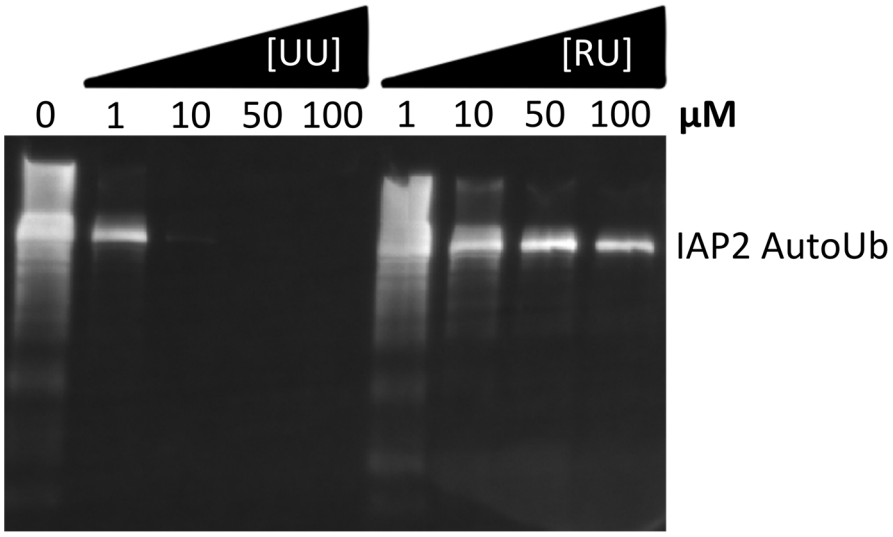



*Figure 18.* IAP2 ubiquitination inhibition assays using UU and RU. Inhibitors were added to the reactions in concentrations of 0, 1, 10, 50 and 100 μM. IAP2 auto-ubiquitination was monitored with fluorescein-labelled ubiquitin.

We found that in the presence of 10, 50 and 100 μM of UU, IAP2 auto-ubiquitination was blocked completely. At 1 μM of UU, IAP2 auto-ubiquitination was still moderately

inhibited. RU did not show a robust inhibitory response towards Ube2D compared to UU. At higher concentrations of RU, IAP2 activity was moderately inhibited and at 10 and 1 μM, IAP2 ubiquitination activity was completely restored. Based on these results, we confirmed that these linked-domain inhibitors act as concentration dependent inhibitors: at lower concentrations of inhibitor, more abundant proteins such as E3 ligase can outcompete the inhibitor and block inhibitor binding. From these experiments, we also conclude UU to be a relatively more potent inhibitor than RU.

### 3.2 Isothermal Titration Calorimetry (ITC) and $K_d$ Determination RU and UU

IAP2 inhibition assays can only provide qualitative information as to the binding affinity of UU and RU with respect to Ube2D. To acquire quantitative binding affinity data, we performed isothermal titration calorimetry studies on both UU and RU by determining $K_d$ (equilibrium dissociation constant) values for each. Higher $K_d$ values imply smaller binding strength and inversely lower $K_d$ values imply greater binding strength. During ITC, a ligand is titrated/injected into a target cell. If a binding event between ligand and target occurs, a single injection is associated with a heat change (endo- or exothermic). These injection-associated heat changes are then compared to a reference cell, whose temperature is held constant. Based on the area under the curve for each injection, $K_d$ values can be determined.

We fit our data to an independent model (single-binding site, no allostery), and determined the $K_d$ value of UU to be 300 nM, and the $K_d$ value of RU to be ~3 μM. These values support the conclusions that were drawn from earlier IAP2 auto-ubiquitination inhibition assays, as UU's lower $K_d$ value compared to RU, is in accordance with the observations that UU shows more robust IAP2 inhibition compared to RU.

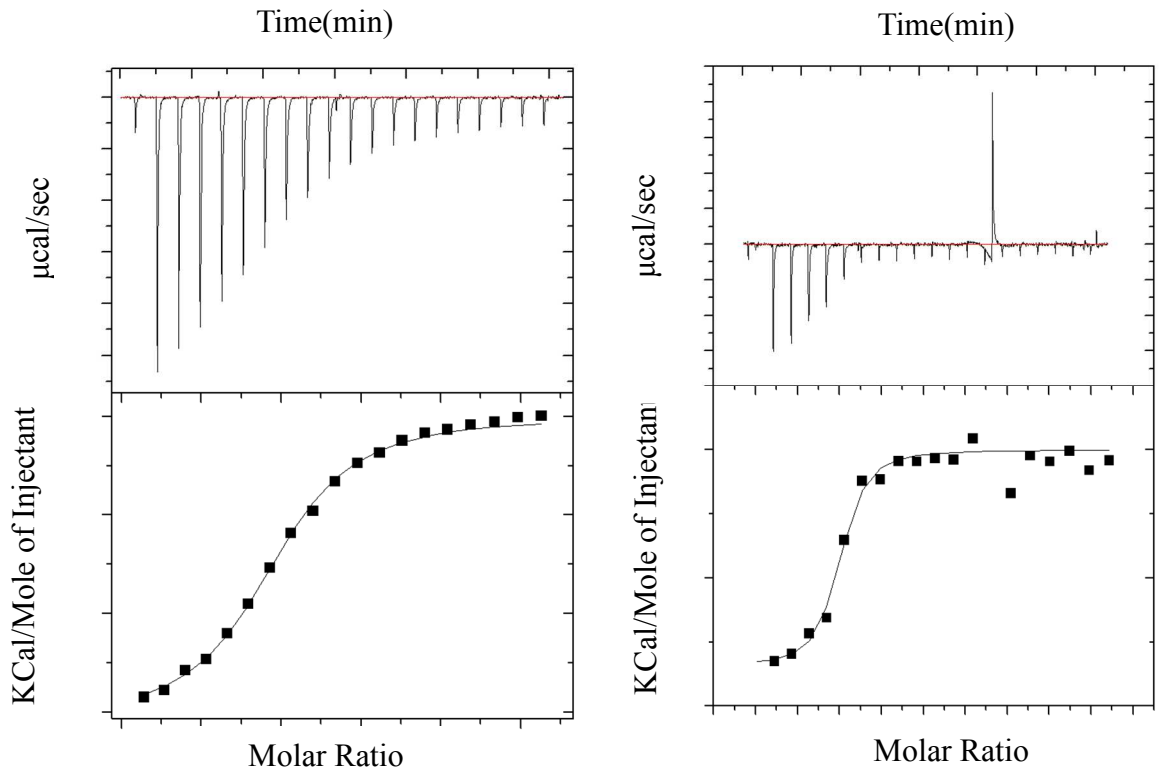



*Figure 19.* Determining the binding affinity of RU and UU. Top left: Raw heat burst curve RU~Ube2D1 in µcal/sec. Bottom left: Heat release per mole of injectant, data was fit to an independent model. Top right: Raw heat burst curve UU~Ube2D1 in µcal/sec. Bottom right: Heat release per mole of injectant, data was fit to an independent model.

### 3.3 Preliminary Inhibitor Specificity Ubiquitination Assays

Both domains that make up RU are directly derived from the E3 ligase UHRF1. So far, Ube2D has been the only identified UHRF1 binding partner in humans (DaRosa et al., 2018b; Foster et al., 2018). Since UHRF1 shows such specific affinity for Ube2D, and both RING and UBL domain are UHRF1 domains, by extrapolation it follows that RU (and UU) should be specific Ube2D inhibitors. To test this hypothesis, we sought to perform specificity ubiquitination assays, testing two different systems.

We first performed ubiquitination assays using the E3 ligase complex APC/C (Anaphase Promoting Complex/Cyclosome). APC/C is vital in controlling the separation of sister

chromatids during mitotic cell division in eukaryotic cells and is activated by the substrate adaptor CDC20 (cell division cycle 20).(Qiao et al., 2016; Yamano, 2019). APC/C not only initiates anaphase, but it also controls the end of mitosis by mediating the time-dependent

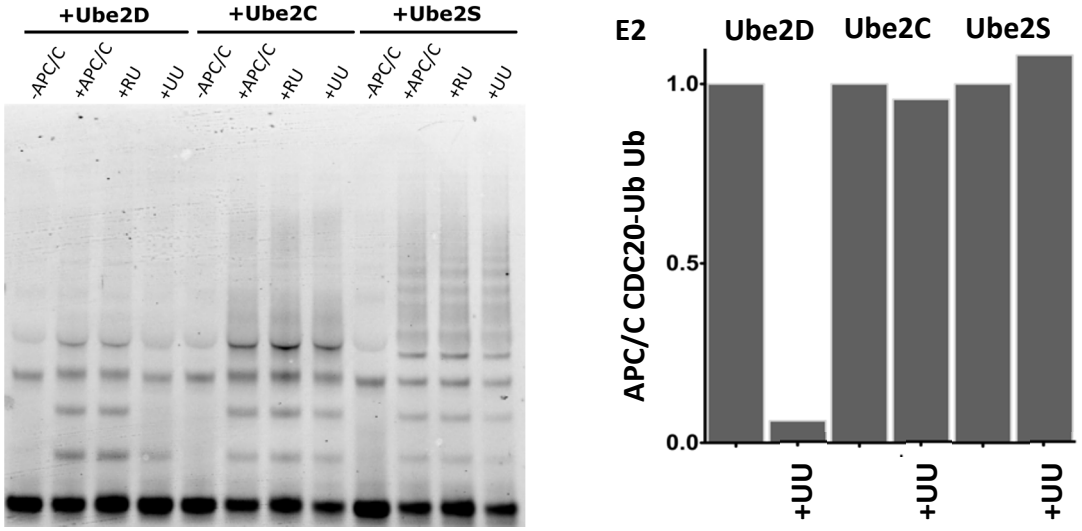



*Figure 20.* APC/C ubiquitination assays to test UU inhibitor specificity towards Ube2D. Ubiquitination assays were performed utilizing the E3 ligase APC/C. APC/C was combined with either Ube2D, Ube2C or Ube2S. Reactions were performed in either the absence or presence of UU or RU inhibitor. APC/C protein ubiquitination was quantified for each reaction.

ubiquitination and subsequent degradation of cyclin B and securin (Chi et al., 2019; J. Zhang et al., 2014; Z. Zhou et al., 2016). APC/C ubiquitylates cyclin B and securin by recognizing certain sequences, namely Destruction box and KEN box (Qiao et al., 2016). However, to detect these sequences APC/C needs to associate with the adaptor protein CDC20. CDC20 binds the target substrate as well as APC/C and allows APC/C to ubiquitylate these substrates. By fluorescently labelling ubiquitin we can measure APC/C activity indirectly, through detecting APC/C dependent protein ubiquitination (Jarvis et al., 2016). APC/C is able to monoubiquitinate substrates using two different E2 enzymes: Ube2C (initial priming ubiquitylation) and Ube2S (chain extension to form K11-linked polyubiquitin). (Aristarkhov et al., 1996; Kirkpatrick et al.,

2006; Meyer & Rape, 2014; H. Yu et al., 1996). In the absence of Ube2C, studies have shown Ube2D is also able to associate with APC/C to support initial substrate ubiquitination in tandem with Ube2S (Summers et al., 2008b; Wild et al., 2016) .

In our ubiquitination assays, APC/C was combined with either Ube2D, Ube2C or Ube2S. Reactions were then performed in either the absence or presence of UU inhibitor. APC/C dependent protein ubiquitylation was then measured and quantified. Our results indicate that in the presence of Ube2S and Ube2C, APC/C dependent substrate ubiquitylation is unaffected by UU inhibitor. However, when Ube2D was utilized, APC/C dependent substrate ubiquitylation decreased significantly, suggesting that UU was only able to affect and inhibit Ube2D, not Ube2S or Ube2C.

The second system we tested utilized a different E3 ligase domain called Cullin1. Cullin1 is part of the S phase kinase-associated protein 1 (Skp1)-Cullin1-F-box protein (SCF) E3 ligase, which is a type of RING finger E3 ligase that consists of a scaffolding protein (Cullin1) an adaptor protein that binds the substrate (e.g. F-box protein) and an RBX domain which acts as the E3 ubiquitin ligase (L. Liu et al., 2018). Previous studies have shown that SCF E3 ligase is involved in the regulation and homeostasis of β-catenin levels in the Wnt signaling pathway. (Ci et al., 2018). Targeted phosphorylation of β-catenin can result in its recognition and ubiquitination by SCF E3 ligase, specifically an SCF containing the F-box protein β-TrCP2 (Ci et al., 2018; Hart et al., 1999; Tauriello & Maurice, 2010).

Ubiquitination assays were performed utilizing Skp1/Cullin1/β-TrCP2 E3 ligase ($SCF^{\beta\text{-}TrCP2}$). The RBX1 Ring domain in SCF is able to associate with both Ube2R and Ube2D (Baek et al., 2020; Ceccarelli et al., 2011; H. Huang et al., 2014). $SCF^{\beta\text{-}TrCP2}$ was combined with either Ube2D or Ube2R and reactions were performed in either the absence or presence of UU

inhibitor. UU inhibitor efficacy was determined by monitoring β-catenin ubiquitination ($SCF^{\beta\text{-}TrCP2}$ substrate).

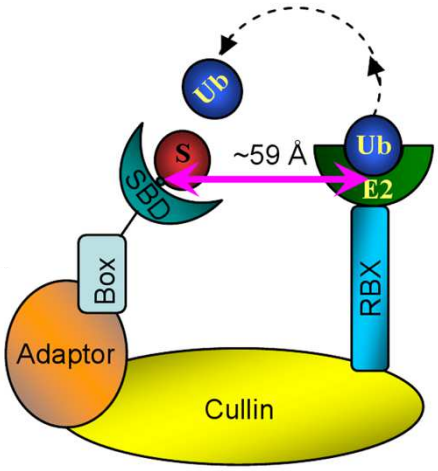



*Figure 21*. General structure of an SCF E3 Ubiquitin Ligase. Adapted from (J. Liu & Nussinov, 2009).

The results showed that in the presence of Ube2R, β-catenin ubiquitination was not significantly reduced, indicating that Ube2R activity was not impaired by the presence of UU inhibitor. In contrast, β-catenin ubiquitination activity was almost completely blocked when $SCF^{\beta\text{-}TrCP2}$ was added to Ube2D and UU inhibitor. These results show that UU interferes with Ube2D activity, while leaving Ube2R unaffected.

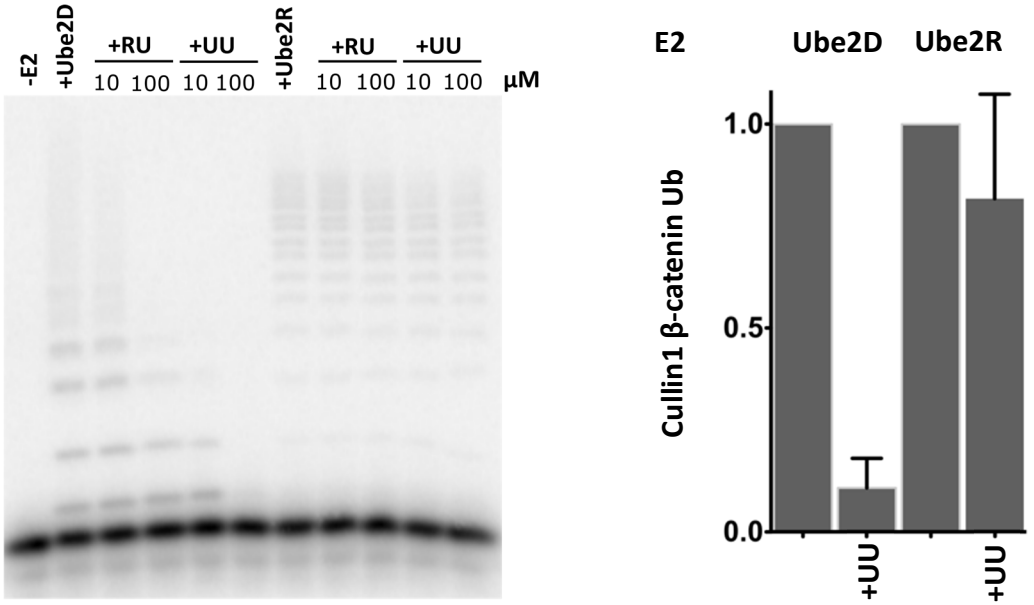



*Figure* 22. Cullin1 ubiquitination assays to test UU inhibitor specificity towards Ube2D. Cullin1 was combined with either Ube2D or Ube2R. Reactions were performed in either the absence or presence of UU or RU inhibitor. We used β-catenin ubiquitination as an indirect measure of E2 inhibition. Assays were performed in collaboration with Dr. Gary Kleiger and Dr. Spencer Hill (University of Nevada, Las Vegas).

### 3.4 E2 Loading Assays Using UU, RU and UBL Domain

Originally, we hypothesized that the linked-domain inhibitor design should be able to block both E1 and E3 interactions with Ube2D simultaneously. Since it is known that both RING and U-box domain bind Ube2D directly at the E3-binding site, it follows that E3 interactions are blocked. However, neither one of the two domains in our first- and second-generation inhibitors specifically targets the E1 binding domain on Ube2D. To test whether UU and RU would be able to block E1 interactions and prevent ubiquitin from being loaded onto Ube2D by E1, E2 loading assays were performed. During these E2 loading assays, all components necessary to go through the ubiquitination cascade are added except for the E3 Ub ligase. Where applicable, the appropriate inhibitor was also added. We fluorescently labelled

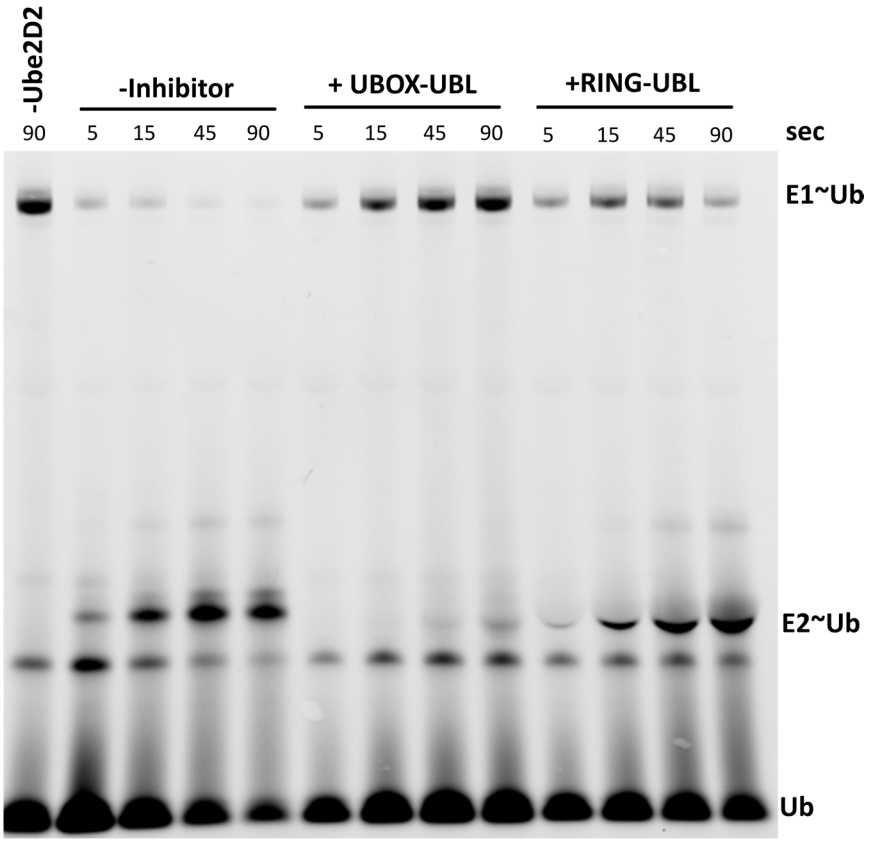



*Figure 23*. E2 loading assays using UU and RU. 55 μM inhibitor (if applicable), 2 μM Ube2D2 and 4 μM ubiquitin were mixed at RT for 5 minutes. Time points were taken at 5, 15, 45 and 90 seconds. One reaction was performed in the absence of Ube2D2 as a negative control. Ub was fluorescently labelled and imaged with the purpose of monitoring all Ub-protein conjugates within each reaction. Assays were performed in collaboration with Derek Bolhuis and Dr. Nicholas Brown (University of North Carolina, Chapel Hill).

ubiquitin and performed ubiquitination assays in the presence of either Ubox-UBL and RING-UBL. After mixing Ube2D2, ubiquitin, and inhibitor, timepoints were taken after 5, 15, 45 and 90 seconds and samples were subsequently imaged to visualize Ub-protein conjugates. A control reaction was performed in the absence of Ube2D2 to mimic E2 obstruction resulting in the lack of Ub loading onto Ube2D2. It was found that in the absence of inhibitor, E1 was able to successfully transfer Ub onto Ube2D2 to form an E2~Ub conjugate (*Fig. 23*). In the presence of Ubox-UBL, Ub was not able to be discharged from E1 and loaded onto Ube2D2, indicating that Ube2D2 was blocked by UU resulting Ub sequestration onto E1. When RU was added, E2

loading was observed, however Ub transfer from E1 onto Ube2D2 was not very efficient as some E1~Ub sequestration was still observed, whereas in the absence of inhibitor, virtually no E1~Ub conjugate was observed.

Ubox-UBL is a linked domain inhibitor that binds both the backside binding domain on Ube2D as well as the E3-binding domain simultaneously. UBL domain by itself binds the

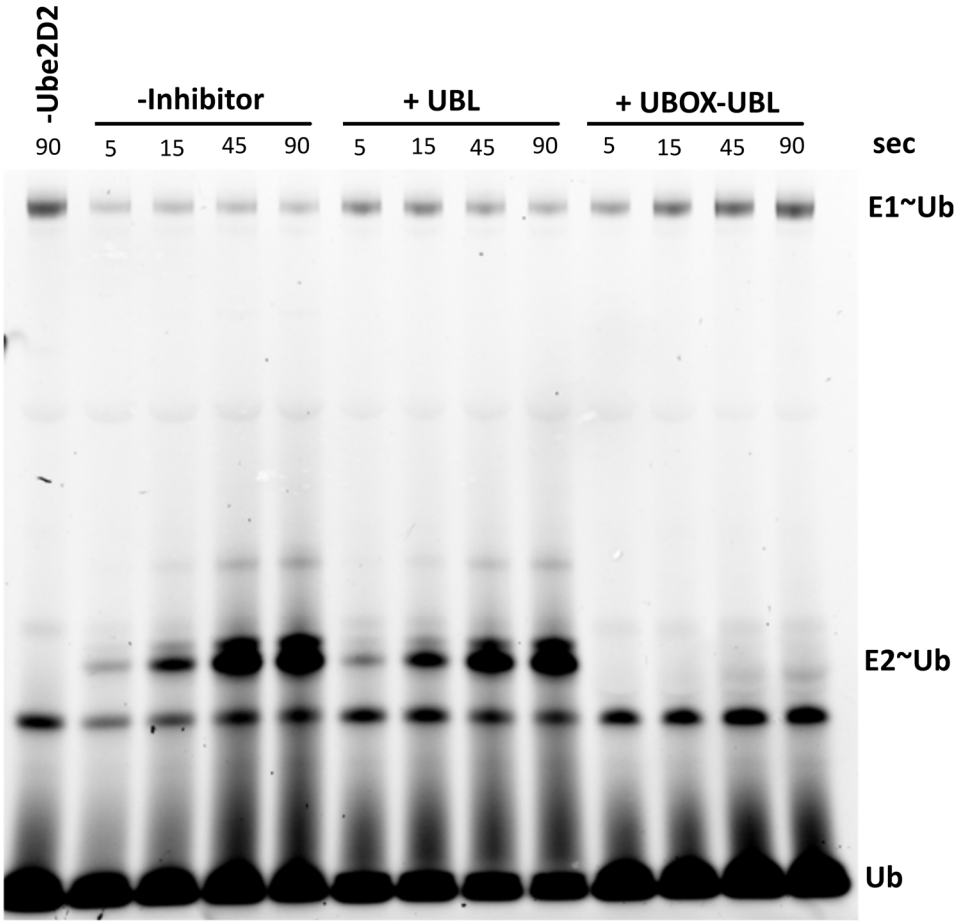



*Figure 24.* E2 loading assays using UBL domain and UU. 55 μM of either UU or UBL domain was added, as well as 2 μM Ube2D2 and 4 μM ubiquitin. Reagents were mixed at RT for 5 minutes. Time points were taken at 5, 15, 45 and 90 seconds. One reaction was performed in the absence of Ube2D2 as a negative control. Ub was fluorescently labelled and imaged with the purpose of monitoring all Ub-protein conjugates within each reaction. Assays were performed in collaboration with Derek Bolhuis and Dr. Nicholas Brown (University of North Carolina, Chapel Hill).

backside binding domain only. We hypothesized that a single UBL domain should not be able to prevent E2 Ub loading.

To test this hypothesis, E2 loading assays were performed in a similar set-up as before, but now we tested ubiquitination reactions in the presence of either UBL domain or Ubox-UBL domain. In the presence of UBL domain only, E2 Ub loading activity was nearly identical to E2 Ub loading activity in the absence of any inhibitor, indicating that UBL domain only was not able to block E2 Ub loading significantly (*Fig.24*). In the presence of Ubox-UBL, E2 Ub loading activity was severely impaired, and nearly all Ub was sequestered on the E1 enzyme (*Fig.24*)

Summarizing the results from both E2 loading assays performed, these results confirm that not only are the linked-domain, specifically UU, inhibitors able to block E1-E2 interactions, the single domain UBL is not able to block E1 interactions. This means that to achieve dual mode of inhibition (blocking both E1 and E3 interactions simultaneously), the linked-domain approach is imperative.

### 3.5 Negative Control Identification for Mammalian Cell Culture

We determined the thermodynamic properties of both the RU~Ube2D and UU~Ube2D interaction, and we characterized their activity *in vitro*. From the evidence we gathered, UU proved to be the more effective inhibitor on all fronts (IAP2 inhibition, E2 loading, etc.) compared to RU. Therefore, we chose to proceed with UU for the remainder of our preliminary studies. To further our understanding of the efficacy of UU, we proposed to perform cell-based assays by transfecting the UU inhibitor gene into HeLa cells. To successfully perform these studies, however, we needed to identify a suitable negative control (besides utilizing untransfected cells) that would account for the cellular effects of the mere act of transfection

itself. Therefore, we designed two different versions of the WT UU inhibitor both of which contained different double mutations.

The first double mutation we introduced was F46V/P1235T (UU16). The second double mutation we introduced was F46V/M1237I (UU17). F46V is a well-characterized mutation located in the UBL domain near the UBL-Ube2D binding interface and previous studies have shown it severely impairs UHRF1's ability to recruit Ube2D (DaRosa et al., 2018a). P1235T and M1237I are both mutations in the U-box domain and were found in COSMIC (Catalogue of Somatic Mutations in Cancer). To qualify as a negative control, the mutants needed to exhibit little to no Ube2D inhibition when compared to UU WT, independent from concentration.

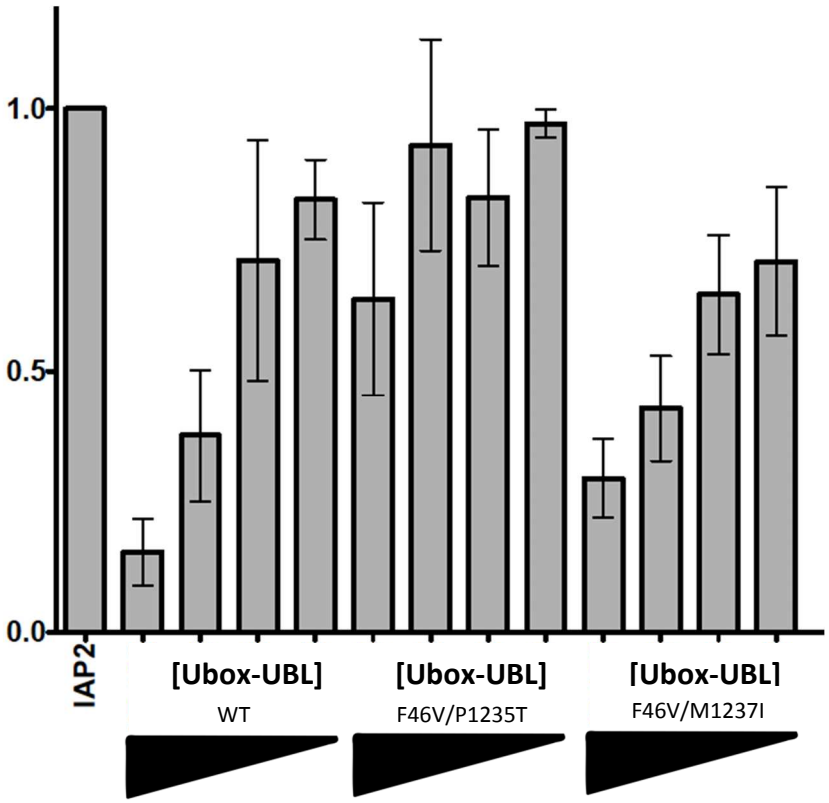



*Figure 25.* IAP2 auto-ubiquitylation levels after the addition of UU WT, UU F46V/ P1235T mutant and UU F46V/M1237I mutant respectively. Each inhibitor was added in concentrations from high to low: 125, 62.5, 15.6, 3.9 µM.

To test our UU mutants, we performed *in vitro* IAP2 ubiquitination assays (same general format as section *3.1*). We performed several assays and measured IAP2 auto-ubiquitination at different concentrations of UU WT, UU16, UU17 and in the absence of any inhibitor. As expected, UUWT reduced IAP2-autoubiquitylation in a concentration dependent manner, showing the lowest IAP2-ubiquitination signal at the highest UU WT concentration. UU F46V/M1237I also reduced IAP2 auto-ubiquitination in a concentration dependent manner, disqualifying it as a negative control for our cell-based studies. UU F46V/P1235T, across all concentrations, consistently restored IAP2-autoubiquitination activity and was therefore selected as a plausible negative control for HeLa cell transfection.

### 3.6 Preliminary HeLa Cell Transfections and Far Western Assays

To investigate whether our UU WT inhibitor would function in cell, we tested HeLa cells transfected with UU WT, UU F46V/P1235T and untransfected HeLa cells. To monitor the effects of potential Ube2D inhibition, we sought to quantify the intracellular levels of ubiquitin, as well as intracellular K48-linked polyubiquitin.

Initially, we performed a western blot using Ub antibody ($Ub^{Ab}$) to monitor general trends and changes in cellular ubiquitin. As expected, cells treated with UU WT inhibitor did not show any significant reduction in (poly)Ub levels when compared to both UU CTRL and untransfected HeLa cells. Ub is controlled by over 600 E3 ligases, ~100 DUB's, over 30 other E2 conjugating enzymes and more (W. Zhang & Sidhu, 2014a). To expect the inhibition of one single E2 enzyme to significantly affect the regulation of the entire ubiquitin population within a cell, would be optimistic at best.

To confirm the successful transfection of our inhibitor we also used FLAG-tag antibody (anti-$FLAG^{Ab}$) (*Fig. 26*). UU WT and UUCTRL are both labelled with the FLAG tag (short

sequence: DYKDDDDK), a tag often used for protein purification or specific pulldown assays with very little background (Gerace & Moazed, 2015). We confirmed the presence of both UUWT and UU CTRL in our HeLa cells and furthermore confirmed that neither was present in the untransfected cells (*Fig.26*).

To get a better idea of Ube2D inhibition within HeLa cells, we looked to monitor K48-linked polyubiquitin levels in the cell. Previous studies have shown that the assembly of K48-linked polyubiquitin chains is partially facilitated by the Ube2D enzyme

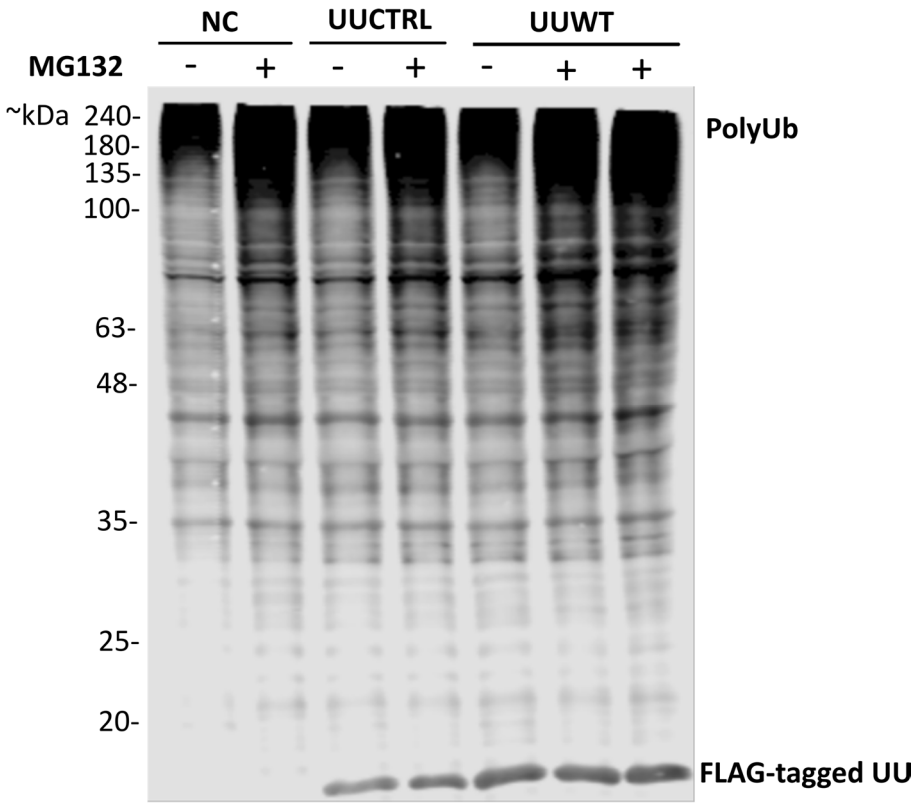



*Figure 26.* HeLa cells transfected with UU WT and UU were lysed and treated with to monitor general intracellular ubiquitin levels. Untransfected HeLa cells, UU F46V/P1235T and UU WT transfected cells were tested for ubiquitylated proteins using. $FLAG^{Ab}$ was also employed to verify successful inhibitor gene transfection. Furthermore, some groups were treated with MG132 to reduce the degradation of ubiquitylated proteins. Polyubiquitinated proteins appear at higher molecular weight.

(Meyer & Rape, 2014; Williamson et al., 2009; Yau, Doerner, Castellanos, Haakonsen, et al., 2017; Li Zhang et al., 2013).

We therefore hypothesized that if UU WT can successfully bind and inhibit the Ube2D enzyme in HeLa cells, we might see a reduction in K48-linked polyubiquitinated proteins in UU WT transfected HeLa cells compared to UU F46V/P1235T transfected HeLa cells and untransfected cells. To visualize and quantify changes in K48-linked polyubiquitin across the different conditions, we performed Far Western Assays and used K48-TUBE, a synthetically engineered molecule that specifically binds K48-linked polyubiquitin chains and simultaneously protects from DUB's and proteasomal degradation.

Most HeLa cells were grown and treated with MG132. MG132, a peptide aldehyde, is a cell-permeable 26s proteasome inhibitor that reversibly binds to the 26s proteasome and arrests its proteolytic activity (Han & Park, 2010; Hougardy et al., 2006; Kisselev & Goldberg, 2001; Palombella et al., 1994). The 26s proteasome is part of the ubiquitin-proteasome system (UPS) (*Fig. 3*), a pathway that selectively degrades K48 and K11-linked polyubiquitinated proteins.

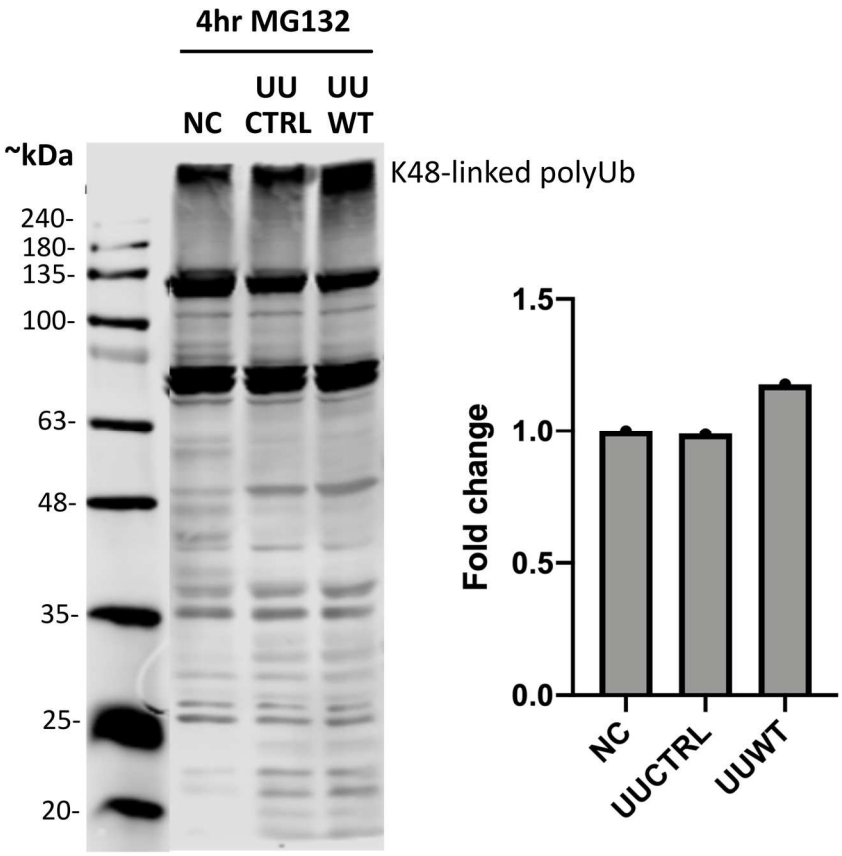



*Figure 27.* Untransfected HeLa cells, UU F46V/P1235T and UU WT transfected cells were treated with MG132 and subsequently tested for K48-linked polyubiquitylated proteins using K48-TUBE (LifeSensors, Malvern, PA, USA)(K48-linked polyubiquitinated proteins are high molecular weight species and are thus concentrated at the top of the western blot. Right: K48-linked polyubiquitinated species are concentrated at the top section of the western blot. To characterize the difference in K48-linked polyUb across groups, the top section of the western blot was quantified.

(Hershko et al., 1980, 1984). By treating the transfected HeLa cells with MG132, we effectively inhibit the 26s proteasome, and consequently halt the degradation of K48 and K11-linked ubiquitinated proteins. This is beneficial especially when performing the K48-TUBE and Ub antibody Far Western, as it will increase the number of K48-linked polyubiquitinated proteins at any given moment, and subsequently enhance the signal acquired from the K48-TUBE western blot.

As expected, K48-linked polyubiquitin levels did not significantly change in cells transfected with UU CTRL, compared to untransfected cells. UU CTRL is not able to bind Ube2D and thus did not cause a reduction in K48-linked polyubiquitin. The fact that UU CTRL transfected HeLa cells did not result in reduced levels of K48-linked polyUb, also confirms that the act of transfection did not affect ubiquitination levels within the cells.

In UU WT transfected cells we hypothesized K48-linked polyUb levels should decrease. Unexpectedly, we observed an increase in K48-linked polyUb levels in HeLa cells transfected with UU WT. It is unclear what might have caused this effect. *In vitro* ubiquitination assays do not mimic the complex mammalian cell environment. As such, these simplified models of the ubiquitination cascade do not allow us to study potential off-target effects of UU WT or Ube2D inhibition in general. Perhaps UU WT triggers other pathways that could affect K48 linked polyubiquitination in HeLa cells. More studies will be necessary to determine the nature and cause of this observation.

### 3.7 UUD1.1 Design and Supporting Inhibition Assays

Besides unchanged Ub levels and an unexpected increase in K48-linked polyubiquitin levels following UU WT HeLa cell transfection, no change in cell viability was observed. To utilize Ube2D as a therapeutic target to treat diseases such as cancer, the administration of a Ube2D inhibitor, should result in noticeable phenotypic changes, e.g., reduced cell viability. Since UUWT did not result in phenotypic changes of note, we hypothesized that the $K_d$ of UUWT (300 nM) might not be low enough to outcompete other potential Ube2D binding partners within the complex mammalian cell environment. With this hypothesis in mind, we wanted to re-design the original UUWT inhibitor to produce a novel inhibitor with improved binding affinity and higher selectivity towards Ube2D.

Two factors were important in the novel inhibitor design: binding affinity and target specificity. Based on the Shannon entropy sequence conservation analysis (*Fig. 14*) we performed on the E2 enzyme family, as well as previous studies, we know that the E3 binding domain residues are highly conserved across the E2 enzyme family (Brzovic et al., 2003b; Eletr et al., 2005; L. Huang et al., 1999b; Sheng et al., 2012; Stewart et al., 2016; Zheng et al., 2000). In accordance with the high residue conservation in this region, most E2 enzymes also show similar electrostatics in the E3 binding domain (Sheng et al., 2012; Stewart et al., 2016). Therefore, optimizing the linked-domain inhibitor at the RING-domain end was not a viable option because the high conservation at the E3 binding domain across E2 enzymes would pose specificity issues.

On the other hand, analysis of the backside binding domain residue conservation in the E2 enzyme family (*Fig. 14*), not only shows low residue conservation amongst merely E2 enzymes, but also shows low residue conservation amongst all 4 Ube2D isoforms. Therefore, the backside binding domain is a much more attractive target especially for encoding specificity.

In section 1.4.2, a 2020 study was outlined which utilized the phage display technique to create "ubiquitin variants" or UbV's. The researchers designed multiple UbV's with the ability to target the backside binding domains of specific E2-conjugating enzymes. Notably, they were able to generate one specific UbV, UbvD1.1, that was able to specifically target the backside of a single isoform of Ube2D; Ube2D1. The researchers performed ELISA assays and determined 1) UbvD1.1 does not inhibit or bind Ube2D2, 3 or 4; 2) The $IC_{50}$ value of UbvD1.1 binding with Ube2D1 was determined to be 65 ± 15 nM. The $IC_{50}$ value is equal to the drug concentration required to achieve 50% target inhibition. $IC_{50}$ values are not direct measures of affinity, rather, they are a measure of the functional strength of an inhibitor (Garg et al., 2020)

(A)


(B)

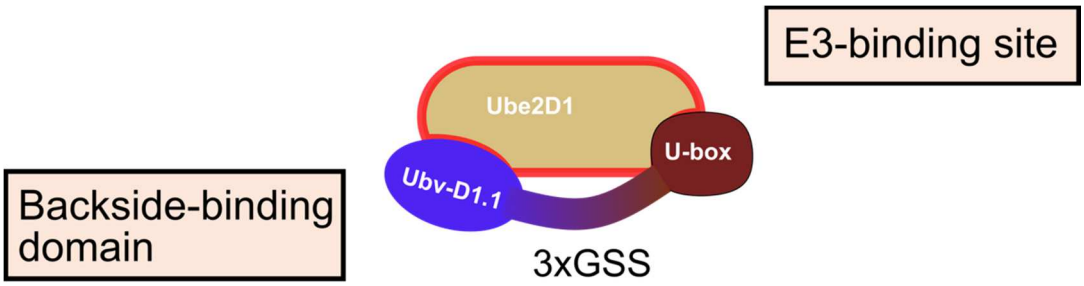



*Figure 28. Optimized inhibitor design.* **(A)** U-box HA (affinity optimized version of U-box domain) (Starita et al., 2013) was covalently attached through a glycine-serine (3xGSS) linker to the phage-display optimized Ubiquitin Variant (Ubv) UbvD1.1 (Garg et al., 2020). UbvD1.1 selectively binds to the backside binding domain on Ube2D1. The inhibitor is referred to as UUD1.1. **(B)** Projected mode of binding of UUD1.1 to Ube2D1.

Since UbvD1.1 was determined to have relatively tight binding with the backside binding domain of Ube2D1 and was also found to be highly selective towards Ube2D1, we wondered whether designing a linked-domain inhibitor containing UbvD1.1 would result in enhanced inhibitor potency. Consequently, we utilized UbvD1.1 and came up with a design in which U-box domain was covalently linked through a GSS-repeat (3x) to the phage-display optimized UbvD1.1 (Garg et al., 2020) (*Fig. 28A*). We projected U-box-UbvD1.1 (UUD1.1) to bind to both the E3-binding domain, as well as the backside binding domain of Ube2D1 simultaneously (*Fig. 28B*) and hypothesized that the UbvD1.1 would increase the binding affinity of UUD1.1

To test the efficacy of UUD1.1, we performed several types of ubiquitylation assays. First, we did a two-way comparison between second generation inhibitor UUWT and UUD1.1. We performed IAP2 inhibition ubiquitination assays in which we either monitored reactions in

the absence of inhibitor, in the presence of UUWT or in the presence of UUD1.1. inhibitors, if applicable, were added in concentrations ranging from 125 μM to 0.196 μM and mixed with 676 nM Ube2D1. Like previous results (*Fig. 18, 25*), UUWT showed concentration dependent IAP2 inhibition, where at 125 and 62.5μM near complete IAP2 inhibition was observed. Around 15.6 μM UUWT, IAP2 auto-ubiquitination was about 50% restored and at 244 nM UUWT, IAP2 activity was entirely restored. In the presence of UUD1.1, IAP2 ubiquitination activity was nearly completely inhibited for all concentrations. At 196 nM, some IAP2 activity was observed, but significantly less compared to UUWT (*Fig. 29A*). These results indicate that UUD1.1 indeed has increased potency compared to UUWT.

To get a better idea of the binding affinity of UUD1.1 we wanted to reduce the Ube2D1 (target protein) concentration in the ubiquitination assay. If we could reduce the Ube2D concentration significantly, we could achieve Ube2D~Inhibitor saturation within our

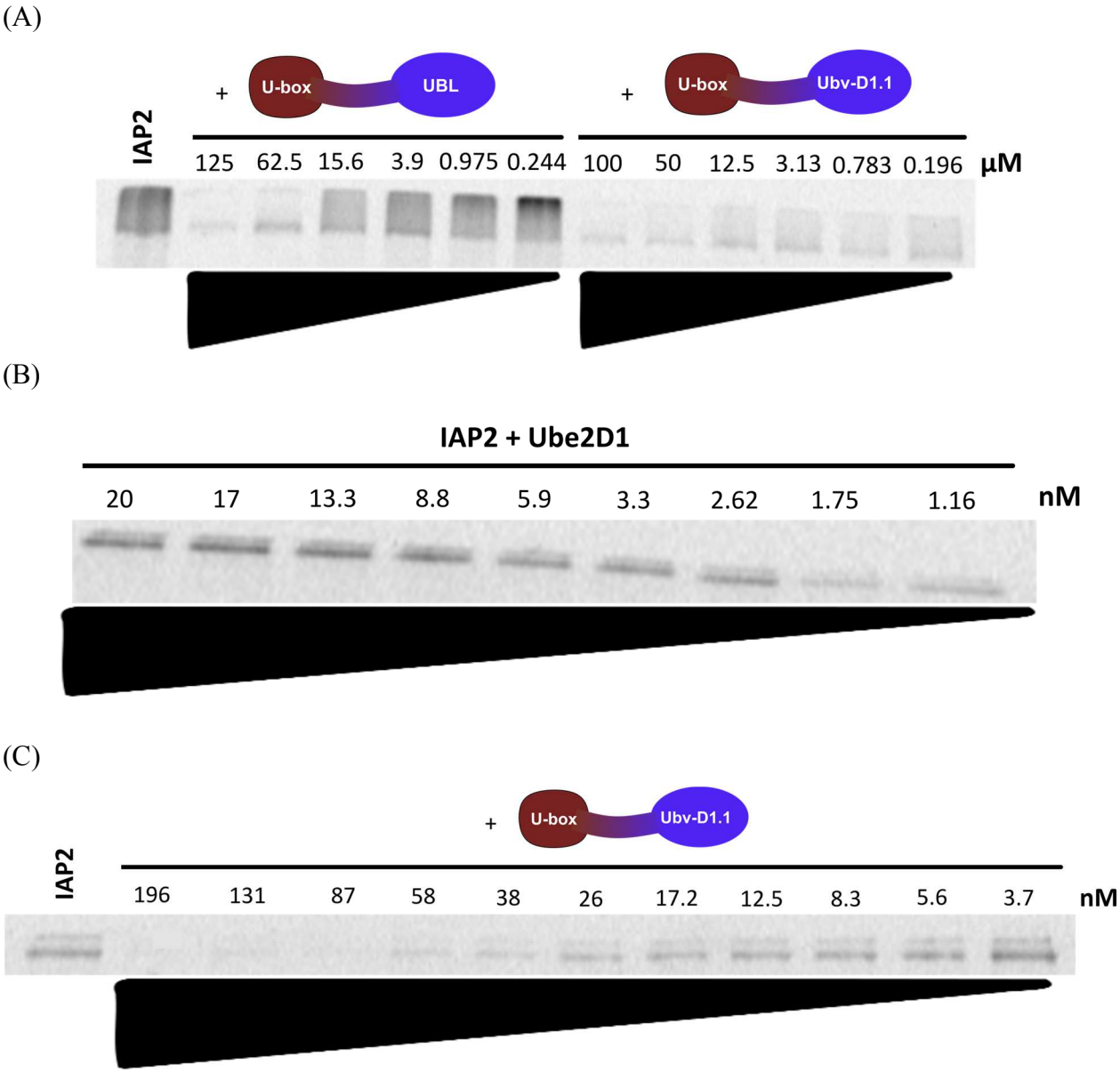



*Figure 29*. IAP2 inhibition ubiquitination assays and Ube2D1 titration. **(A)** Assays were set up in the presence of either UU WT or UUD1.1 inhibitor. Inhibitor concentration ranges from 125 µM to 0.196 µM. Ube2D1 was added at 676 nM. Reactions proceeded at RT for 20 min (**B)** Ube2D1 titration. Reactions were performed in the absence of inhibitors. Ube2D1 was added at 20, 17, 13.3, 8.8, 5.9, 3.9, 2.62, 1.75 and 1.16 nM. Reactions proceeded at RT for 20 min. **(C)** UUD1.1 IAP2 inhibition assays. UUD1.1 was added at 196, 131, 87, 58, 38, 26, 17.2, 12.5, 8.3, 5.6 and 3.7 nM. 3 nM Ube2D was utilized, and reactions were done at 37°C for 20 min.

ubiquitination assays. Like isothermal titration calorimetry, where target saturation is vital for the determination of the $K_d$ value, we would be able to draw some more substantial conclusions

about UUD1.1 binding affinity. To determine at what Ube2D1 concentrations we would still observe sufficient IAP2 ubiquitination signal, we performed a Ube2D1 titration assay (*Fig. 29B).* We added Ube2D1 in concentrations ranging from 20 nM to 1.16 nM and monitored IAP2 auto-ubiquitination signal. We observed signal of acceptable intensity up until about 3 nM Ube2D1.

Next, we performed a third ubiquitination assay in which we combined UUD1.1 inhibitor at concentrations ranging from 196 nM to 3.7 nM, with 3 nM of Ube2D1 at 37°C for 20 min and monitored IAP2 ubiquitination levels. We found that UUD1.1 was able to significantly perturb IAP2 auto-ubiquitination at concentrations as low as about 20 nM. Overall, these results indicate a significantly enhanced potency of UUD1.1 compared to UUWT.

### 3.8 ITC and $K_d$ Determination UUD1.1

To determine the exact binding affinity of UUD1.1 with Ube2D1, we performed isothermal titration calorimetry. We titrated 100 μM UUD1.1 (syringe) into 10 μM Ube2D1 (target cell) and recorded heat released per injection for 35 injections. After determining the area under the curve for each heat release, we fit the data to an independent model and extracted all thermodynamic properties, shown in Table 2.

The $K_d$ was calculated to be around 5 nM, a nearly 60-fold improvement with respect to UU WT, which has a $K_d$ of ~300 nM. 5 nM is in accordance with the results from our IAP2 inhibition assays, as a higher binding affinity does explain the IAP2 ubiquitination perturbations observed, even at relatively low concentrations of UUD1.1 compared to UUWT. Interestingly, the N-value determined through ITC was calculated to be 1.492. Generally, the N-value is used to denote stoichiometry (mole ratio). An n-value around 1 equals a 1:1 binding ratio UUD1.1:Ube2D1. The fact that our n-value is closer to 1.5, suggests something else might be

occurring. Perhaps the mechanism of inhibition of UUD1.1 is slightly more complicated initially thought.

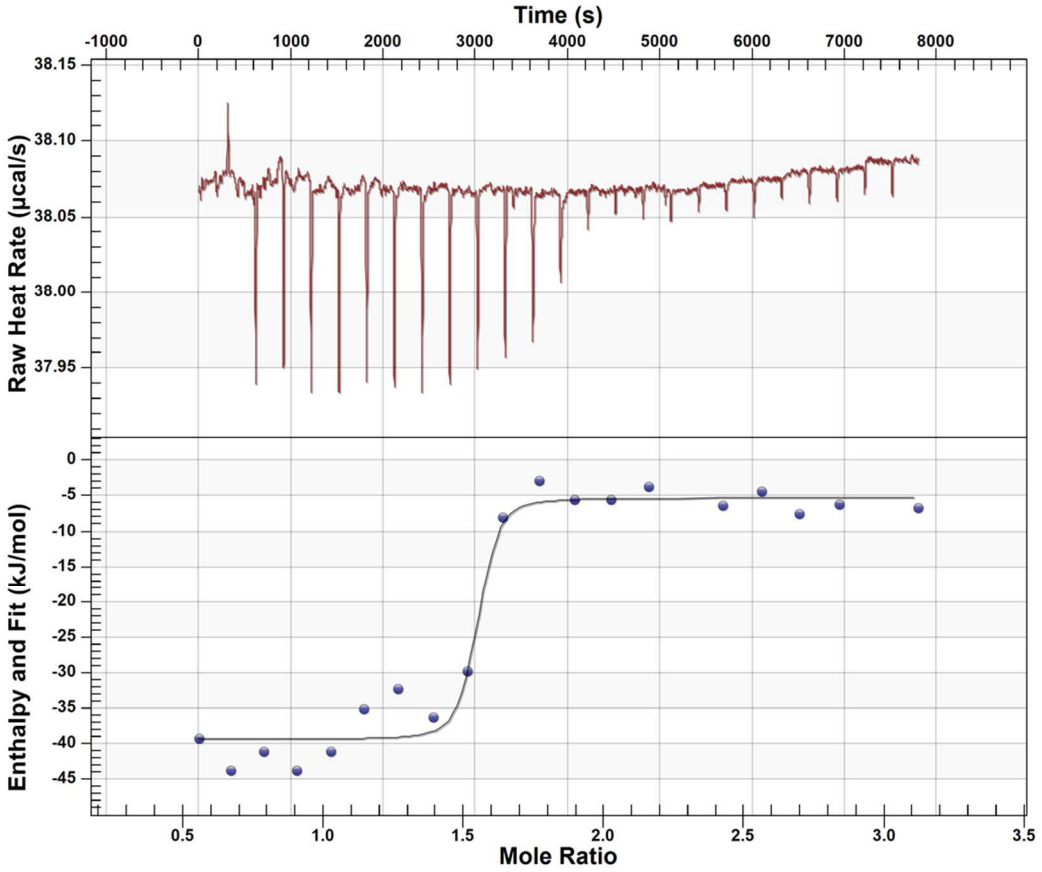



*Figure 30.* UUD1.1 to Ube2D1 titration. Top: raw heat burst curve UUD1.1~Ube2D1 in µcal/s. Bottom: Enthalpy vs. mole ratio (UUD1.1:Ube2D1). Data was fit to an independent model.

Table 2
*Thermodynamic Properties of UUD1.1~Ube2D1 Interaction*

| | **Blank (µJ)** | **$K_d$ ($M^{-1}$)** | **n** | **ΔH (kJ/mol)** | **ΔS (J/mol•K)** |
|---|---|---|---|---|---|
| UUD1.1~Ube2D1 | -1.05 ± 0.32 | $5.0x10^{-9} \pm 8.6x10^{-9}$ | 1.49 | -34.2 ± 2.648 | 44.14 |

### 3.9 UHRF1 Inhibition Assays

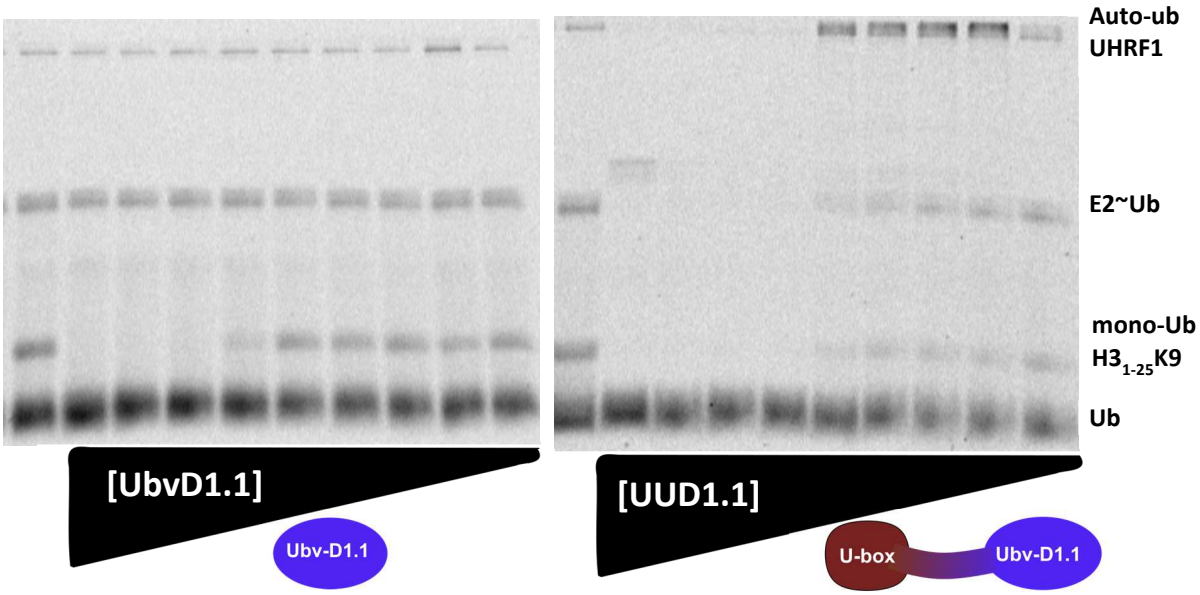
In the 2020 study by Garg and his colleagues, the emphasis was more so on the actual development of the Ubv molecules, rather than their functionality *in vitro* let alone in cells. Limited biochemical assays were performed to test the Ubv efficacy (Garg et al., 2020). Since we wanted to test UUD1.1 to establish whether it could inhibit other types of ubiquitination reactions, we took the opportunity to test UbvD1.1 also, and simultaneously compare the efficacy of UbvD1.1 versus UUD1.1. In earlier experiments (refer to *Fig. 24*), it was observed that UBL domain alone was not able to block E2 Ub loading, whereas UUWT was. It seemed logical that when testing UbvD1.1 (a Ub-like structure), it would not be able to inhibit E2 activity and UUD1.1 would. To assess the potency of UUD1.1, UbvD1.1 and UUWT in a three-way comparison, we performed UHRF1 inhibition assays. UHRF1 is an E3 ligase and is heavily involved in epigenetic regulation. It is vital in maintaining DNA methylation patterns across the genome(Kim et al., 2018). Its SRA domain can bind hemi-methylated DNA (HeDNA) during DNA replication. At the same time, TTD and PHD domain can bind trimethylated histone 3 (H3) in the nucleosome. UHRF1's RING domain then ubiquitylates H3, which recruits DNA methyltransferase (DNMT1) to HeDNA, resulting in DNA methylation (DaRosa et al., 2018b; Kim et al., 2018)

During UHRF1 inhibition assays, there are three main species that can get ubiquitylated: UHRF1 itself ($UHRF1^{auto\text{-}Ub}$), H3 peptide (n-Ub $H3_{1\text{-}25}K9$) and Ube2D1 (E2~Ub). We combined UHRF1, HeDNA, H3 peptide and performed ubiquitination reactions in the absence of inhibitor, in the presence of UbvD1.1, UUWT, or UUD1.1.

We found that UbvD1.1, across all concentrations, was unable to block E2 ubiquitination and UHRF1 auto-ubiquitination. At higher concentrations, we observed the loss of peptide

ubiquitylation. Peptide ubiquitylation was restored at lower UbvD1.1 concentrations (*Fig.31,* top left). UUD1.1 on the other hand, at higher concentrations induced complete loss of both UHRF1 auto ubiquitination, E2 ubiquitination and peptide ubiquitination. UHRF1 auto-ubiquitination increased at lower UUD1.1 concentrations. Both E2 ubiquitination and peptide ubiquitination were slightly restored at lower UUD1.1 concentrations (*Fig. 31*, top right). UUWT showed similar patterns to UUD1.1: at high concentrations UHRF1, E2 and peptide ubiquitination were entirely blocked. At lower UUWT concentrations all three ubiquitinated species were restored (*Fig.31*, bottom left).

Figure 32 shows a visual representation of the mode of inhibition for each inhibitor, based on the UHRF1 inhibition assay results. Both UUD1.1 and UUWT bind both E3 binding domain and backside binding domain on Ube2D1, effectively preventing Ub transfer from E1 onto E2 itself, UHRF1 and H3 peptide. UbvD1.1 on the other hand is not able to block E2 ubiquitylation or UHRF1 auto-ubiquitylation. It is, however, able to interfere with H3 peptide



(Figure 31 Continued)

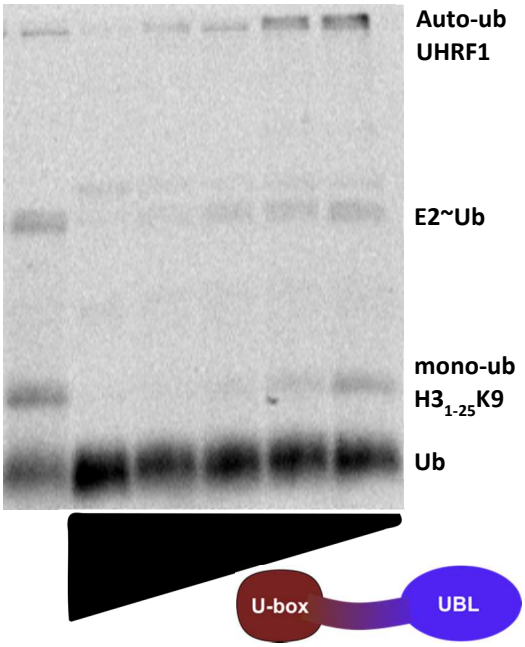



*Figure 31*. UHRF1 inhibition assays were performed using 3 different Ube2D inhibitors. Inhibitors were mixed with 600 nM UHRF1, 19 µM peptide, 1 µM HeDNA. Reactions ran for 20 min at RT. UHRF1 inhibition assays are shown as follows: top left: UbvD1.1; top right: UUD1.1; bottom left: UUWT. Generally, inhibitors were added at concentrations ranging from 62 µM to 0.038 µM. Only UU WT was added at 62.5, 15.6, 3.9, 0.975, 0.244 µM.

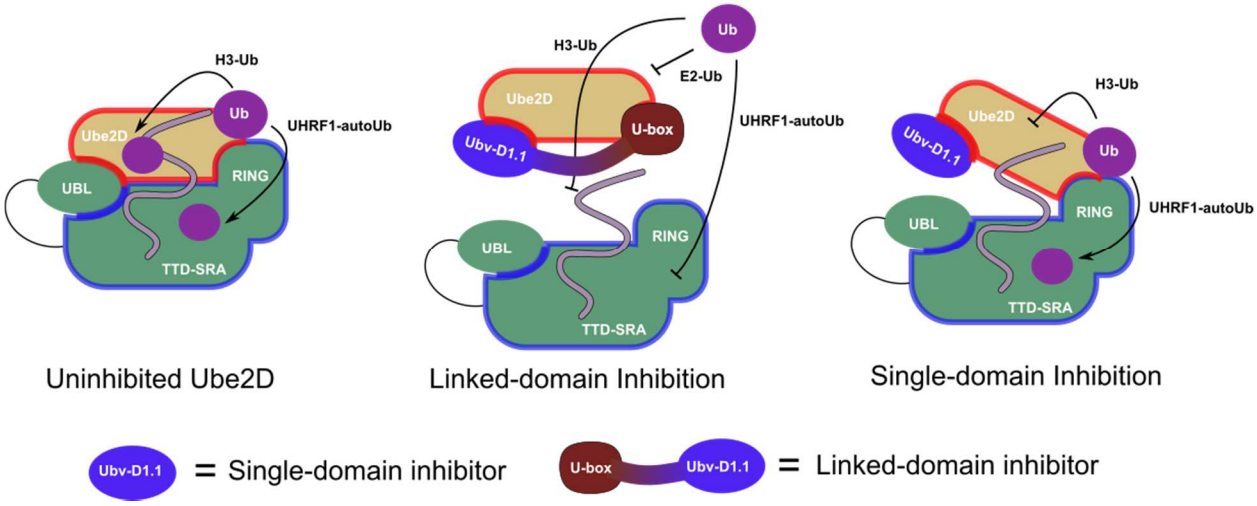



*Figure 32.* Mode of inhibition of single domain and linked-domain inhibitors based on UHRF1 inhibition assays**.** Left: UHRF1 activity when Ube2D is uninhibited; middle: Ube2D is inhibited by linked-domain UUD1.1; right: Ube2D is inhibited by single-domain UbvD1.1.

ubiquitylation. This is in accordance with recent studies, that show that the UHRF1's UBL domain plays a critical role in targeting Ub to histone H3, by binding the Ube2D1 backside binding surface (DaRosa et al., 2018b).

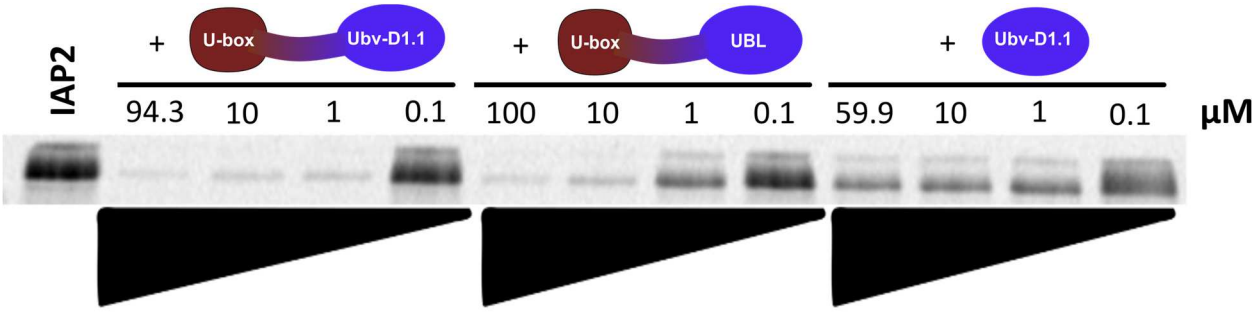
### 3.10 UbvD1.1 vs. UUD1.1 vs. UUWT Assay Comparison

The UHRF1 inhibition assays showed us the relative relationships between 3 inhibitors UUD1.1, UUWT and UbvD1.1. In order of inhibition potential from high to low: UUD1.1 > UUWT > UbvD1.1. To confirm that this order holds true, we performed one last IAP2 ubiquitination assay in which we mixed all enzymes involved in the ubiquitination cascade (E1, 100 nM of Ube2D1, IAP2) as well as fluorescein-Ub, with either UUD1.1, UUWT or UbvD1.1. We added inhibitors in concentrations ranging from 100 μM to 100 nM and left the reactions for 20 min. at RT.

At ~100 and 10 μM of UUD1.1 or UUWT, near complete IAP2 ubiquitination inhibition was observed. IAP2 activity was still significantly hindered at 1 μM of UUD1.1. Upon the addition of 0.1 μM of all three inhibitors, about 75% of IAP2 activity was restored. Unexpectedly, at ~60, 10 and 1 μM of single domain UbvD1.1, IAP2 activity was reduced to ~20-30% activity compared to the positive control. Since UbvD1.1 does not block the E3 ligase binding domain on Ube2D1, theoretically Ub transfer from the Ube2D1 active cysteine to IAP2 and eventually substrate should not be significantly hindered. If that were the case, we should see little to no IAP2 activity inhibition upon the addition of UbvD1.1. The fact that we still observe IAP2 activity hindrance, implies that the backside binding domain somehow plays an important role in Ube2D1 and IAP2 function, as well as E2 to E3 Ub transfer. Previous studies have shown that noncovalent Ub interactions at the backside binding domain can activate E2 enzyme function and these interactions have been shown to be vital for polyubiquitin chain

assembly and processivity by multiple E3 ligases (Brzovic et al., 2006a; Miura et al., 1999; Schumacher et al., 2013a; Wright et al., 2016).

(A)




(B)

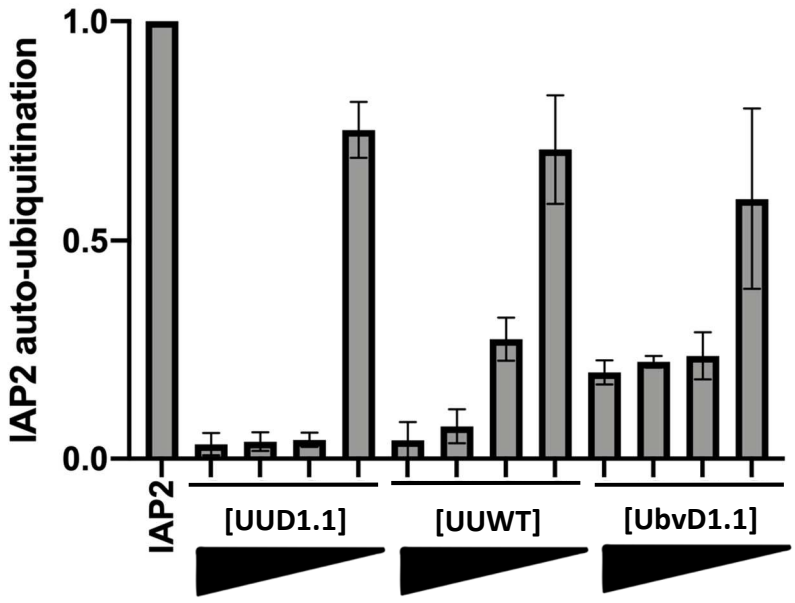



*Figure 33.* Three-way comparison between UUD1.1, UUWT and UbvD1.1 during IAP2 Inhibition assays. (A) UUD1.1, UUWT and UbvD1.1 were added in concentrations approximately 100, 10, 1 and 0.1 µM. Reactions were run for 20 min at RT. IAP2 activity was monitored by visualizing all Ub-protein conjugates. (B) IAP2 activity for every inhibition reaction as a fraction of the signal quantified for our positive control (uninhibited reaction). Signal quantified for the Ub reaction in the absence of inhibitor was set to 1.

In figure 34, we show the expected mode of inhibition of UbvD1.1 based on the IAP2 inhibition assays (*Fig.33*). While IAP2 does show more activity in the presence of UbvD1.1 compared to UUWT and UUD1.1 at similar concentrations, it seems as if backside binding domain inhibition still significantly impairs IAP2 activity. Perhaps UbvD1.1 interferes with polyUb chain formation and processivity, or something occurs that we did not account for.

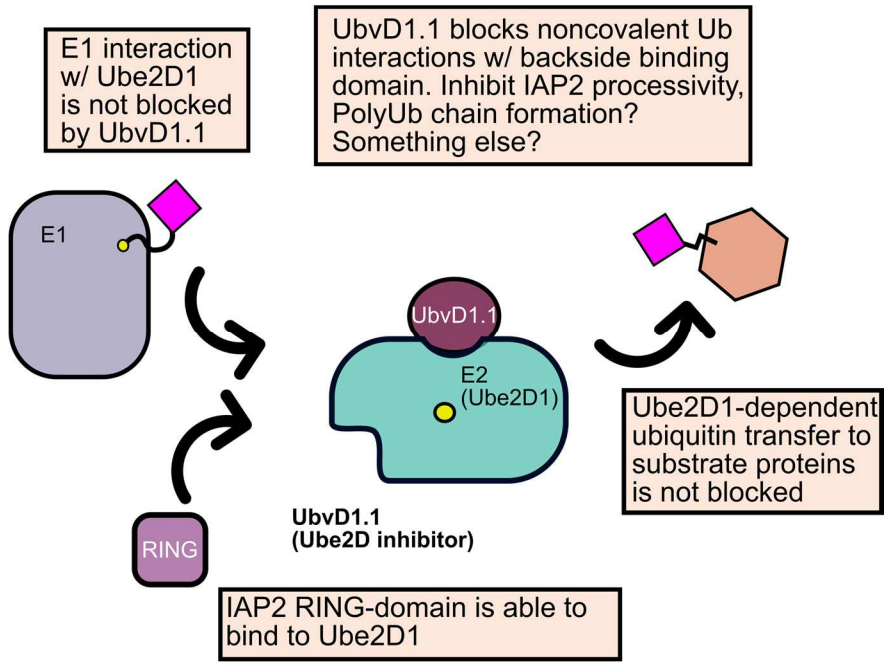



*Figure 34*. Mode of inhibition based on IAP2 inhibition assays utilizing UbvD1.1. It is unclear how UbvD1.1 still achieves moderate IAP2 inhibition.

### 3.11 Protein Crystallization and UUD1.1~Ube2D1 Structure

Up until this point, we have acquired evidence that shows that UUD1.1, and the other linked-domain inhibitors possess the ability to simultaneously block both E1 and E3 interactions. Merely based on those facts, we have assumed that the mechanism of inhibition/mode of binding occurs in a 1:1 binding ratio, in which U-box domain binds the E3 binding site and UbvD1.1 binds the backside binding domain. However, since we cannot state this for certain, we wanted

to crystallize the noncovalent UUD1.1~Ube2D1 complex and utilize x-ray crystallization to solve the complex structure. By doing so, we would be able to determine the exact mechanism of binding of UUD1.1, inhibitor orientation as well as well as evaluate whether the 3xGSS covalent linker is of optimal length.

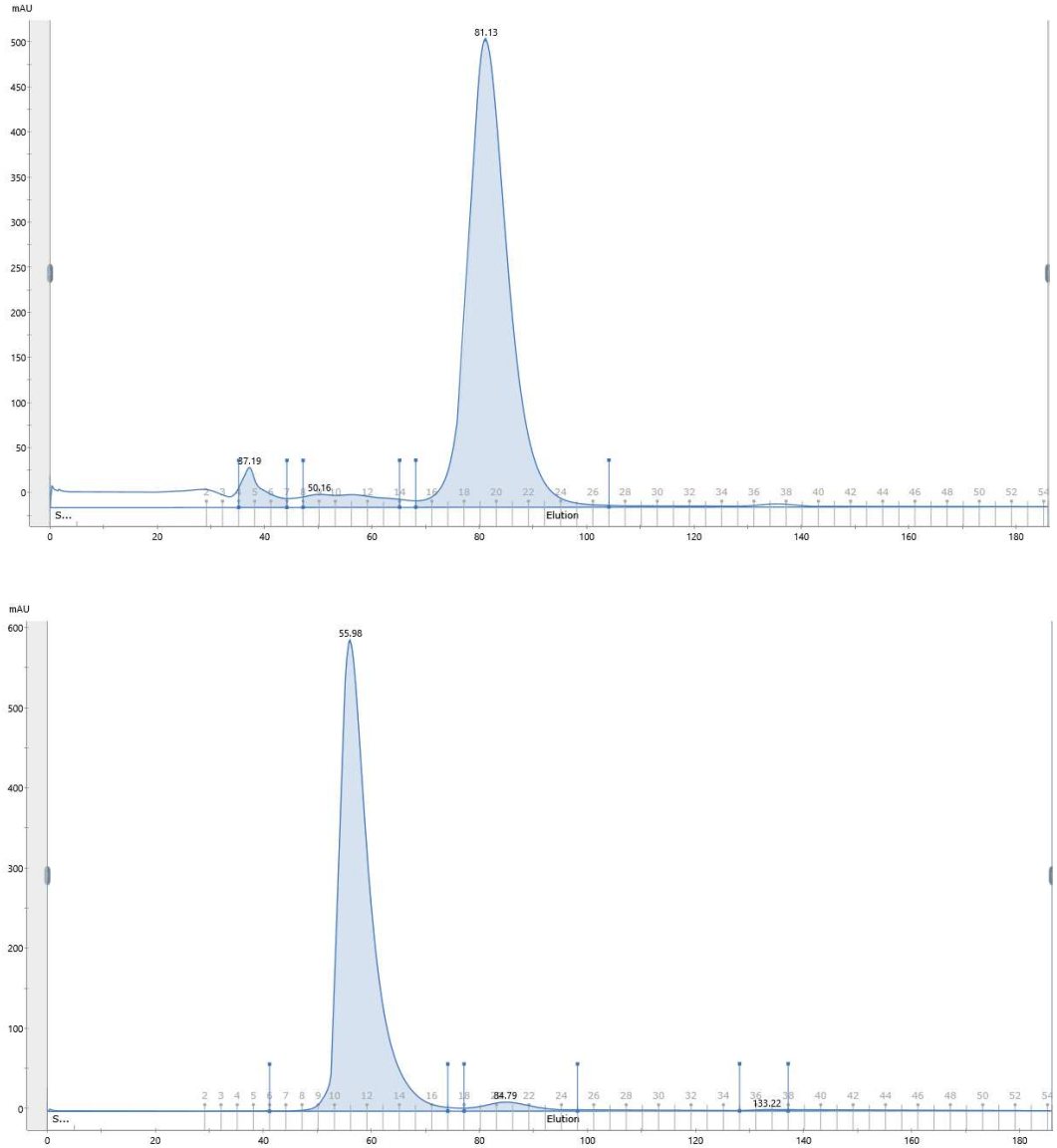



*Figure 35*. s200 Sephacryl column chromatography traces. Top: SEC trace for the purification of Ube2D1. Ube2D1 eluted in fractions 16-23. Bottom. SEC trace for the purification of UUD1.1~Ube2D1. A large peak eluted in fractions 10-15. Peak shift for UUD1.1~Ube2D1 complex indicates co-elution of E2 and inhibitor.

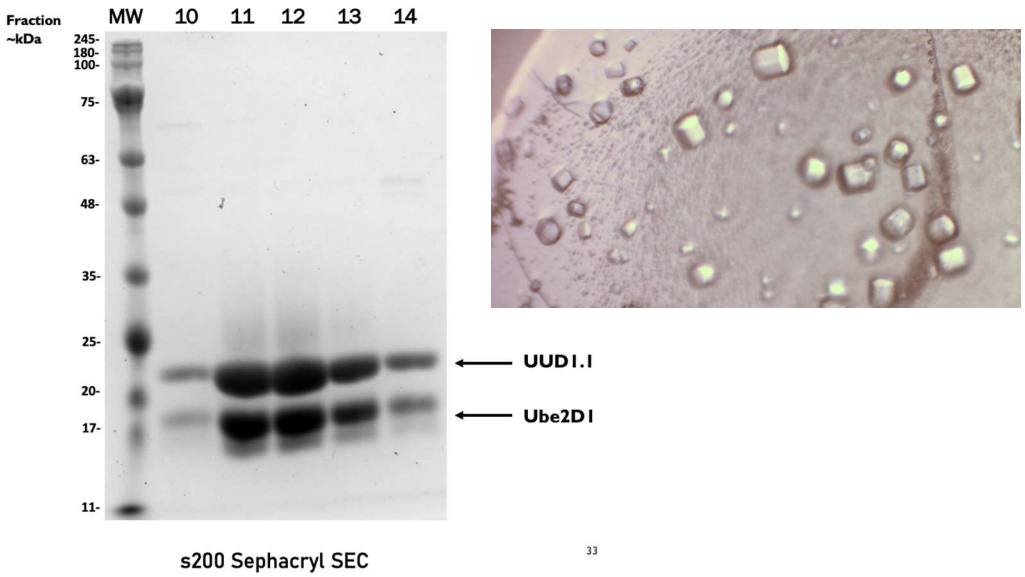



*Figure 36.* Preliminary crystallization trials on UUD1.1~Ube2D1. Left: s200 Sephacryl SEC purification of UUD1.1~Ube2D1 complex. Right: formation of hexagonal crystals in 1500 mM ammonium sulfate, 100 mM HEPES pH 7.5. From these crystals we were able to extract structural data with a resolution of 6 Å.

We individually purified UUD1.1 and Ube2D1 and combined them at a 1:1 ratio. We then ran an s200 Sephacryl SEC column and co-purified UUD1.1~Ube2D1 complex. Figure 36 shows the successful purification of UUD1.1~Ube2D1. We used the Berkeley Screen to screen crystallization conditions. We were able to grow crystals in condition E4: 100 mM HEPES free acid/ NaOH, pH 7.5, 1500 mM ammonium sulfate. Crystal samples were analyzed at the Berkeley Center for Structural Biology beamlines. Unfortunately, we were not able to obtain usable datasets from the crystal samples. Consequently, we have not been able to solve the structure of UUD1.1~Ube2D1. We will need to optimize the crystallization conditions further in future work.

# CHAPTER 4: DISCUSSION

For decades now, researchers have been attempting to develop drugs that can inhibit the ubiquitination cascade by targeting the participating enzymes. These efforts have yielded multiple successful attempts as evidenced by the introduction of Bortezomib based 26s proteasome inhibitors that are utilized to treat multiple myeloma (Accardi et al., 2015; Field-Smith et al., 2006). Within the ubiquitination cascade, the E2 enzyme is extremely important as it is in charge of multiple facets of polyUb chain formation including processivity, selection and switching from chain initiation to elongation (Ye & Rape, 2009). Because of this vital role, it poses an amenable target to selectively inhibit biological processes by blocking polyUb chain formation.

Even with this potential, few molecules have been developed that can specifically target individual members of the E2 enzyme family. The molecules that do exist, either use a strategy that cannot be applied to other E2 enzymes (Ceccarelli et al., 2011; Pulvino et al., 2012), or do not shut down E2 enzyme functionality completely (Garg et al., 2020). Historically, targeting the E2 enzyme comes with specificity issues (Petroski, 2008). All E2 enzymes contain a highly conserved, shallow catalytic cysteine, as well as a conserved UBC core domain (Brzovic et al., 2006a; L. Huang et al., 1999b), making it difficult to develop structures that can distinguish between E2 enzymes. Ube2D, one E2 enzyme family member is known as the non-specific E2 since it is able to interact with the most E3 ligases and can facilitate the most polyUb chain linkages (Christensen & Klevit, 2009; Kar et al., 2012). It is involved in diseases such as cancer, atherosclerosis and hemochromatosis (Alpi et al., 2016). Whilst Ube2D is ubiquitously active, potential cellular phenotypes associated with Ube2D inhibition are mostly unexplored.

Furthermore, little is known about the potential of Ube2D as a therapeutic target for cancer treatments.

Our studies show that targeting the Ube2D backside binding domain in concert with the E3 binding domain utilizing ubiquitination cascade native protein domains such as UHRF1-UBL, UHRF1-RING, U-box and Ubiquitin Variants such as UbvD1.1, achieves near complete Ube2D functional inhibition in the case of UU WT and UUD1.1. RU's lesser effectiveness can be explained by the higher affinity of U-box domain for Ube2D, compared to RING-domain.

We hypothesized that the linked-domain inhibitors would inhibit both E1, E3 and backside interactions and our results confirmed. Since the E1 and E3 binding domain on Ube2D overlap, the RING/U-box domain moiety of our inhibitor should block both interactions. E2 loading assays showed that in the presence of both RU and UU, E2~Ub levels decreased dramatically compared to the reaction in the absence of inhibitor. Further E2 loading assays where we compared the efficacy of single UBL domain versus Ubox-UBL, revealed that UBL domain alone did not prevent E2 loading whereas UU did, confirming that in the absence of a moiety that binds the E3 binding domain on Ube2D, E1 can interact with Ube2D and transfer Ub. Combined, we show that the linked-domain approach is vital to achieving a dual-mode of inhibition

Even though we had hypothesized that RU and UU would be specific towards Ube2D given their direct derivation from UHRF1 (a Ube2D-specific E3 ligase)(DaRosa et al., 2018a), to confirm this notion, we performed multiple ubiquitination inhibition assays utilizing two different E3 ligases. We found that UU and RU were able to prevent APC/C substrate ubiquitylation in the presence of Ube2D, but not in the presence of Ube2S or Ube2C. Similarly, UU and RU were able to disrupt beta-catenin ubiquitylation by Skp2/Cul1/F-box E3 ligase only

in the presence of Ube2D, not Ube2R. These studies show that both UU and RU prefer to interact with Ube2D over other E2 enzymes. However, UU and RU do not specifically inhibit any single Ube2D isoform as inhibition was achieved in the presence of both Ube2D1 and Ube2D2. This observation is consistent with the fact that UHRF1 can also interact with Ube2D1, 2 and 3 (DaRosa et al., 2018a; Foster et al., 2018). While specificity assays have not yet been performed utilizing UUD1.1, we expect that UUD1.1 will show isoform specificity towards Ube2D1. In a previous study, the specificity bearing moiety of UUD1.1, UbvD1.1, was found to be selective for Ube2D1 in competitive ELISA assays (Garg et al., 2020). This selectivity was attributed to a His at position 20 in Ube2D1 compared to a Gln at position 20 for Ube2D2,3 and 4. His20 of Ube2D1 points away from Trp9 in UbvD1.1, whereas the Gln20 was found point towards Trp9, inducing steric hindrance associated with unfavorable UbvD1.1 binding to these Ube2D isoforms (Garg et al., 2020). By default, we therefore expect that UUD1.1 will also be specific for Ube2D1. To determine whether UU, RU and UUD1.1 have absolute specificity towards Ube2D, more studies will have to be performed e.g. an E2~inhibitor screen utilizing yeast two-hybrid assays.

ITC $K_d$ measurements resulted in Kd values from highest affinity to lowest affinity: UUD1.1 (5 nM) > UU WT (300 nM) > RU WT (3 μM). These values corroborate our experimental observations as UUD1.1 was able to inhibit both IAP2 and UHRF1 auto-ubiquitination activity more completely compared to both UU WT and RU WT. UUD1.1 also showed inhibition activity at lower concentrations than both UUWT and RU WT.

When testing single domain back-side binding UbvD1.1 against UUWT and UUD1.1 in UHRF1 inhibition assays, we found that only the linked-domain inhibitors showed robust inhibition of E2 auto ubiquitination. This can be rationalized by the fact that the E1 and E3

binding domains on Ube2D overlap, such that when the E3 binding domain is blocked the E1 binding domain is simultaneously blocked also. UbvD1.1 was unable to block E2~Ub formation, since the E1 is free to bind to Ube2D in this case and can successfully transfer Ub onto the catalytic cysteine of Ube2D. It is, however, able to interfere with H3 peptide ubiquitylation. This is in accordance with recent studies, that show that the UHRF1's UBL domain plays a critical role in targeting Ub to histone H3, by binding the Ube2D1 backside binding surface (DaRosa et al., 2018b; Foster et al., 2018).

As expected, in three-way comparative IAP2 inhibition assays utilizing UUD1.1, UU WT and UbvD1.1, UUD1.1 inhibited IAP2 activity the most, followed by UU WT and UbvD1.1. UbvD1.1, while being the least potent, was still able to inhibit IAP2 auto-ubiquitination fairly well, suggesting a more important role of Ube2D BBD interactions in IAP2 facilitated substrate ubiquitination. Indeed, recent studies investigating noncovalent Ube2D2~Ub BBD interactions during IAP1 facilitated ubiquitination indicate that the RING-domain of IAP1 recruits and activates the Ube2D~Ub complex. Noncovalent Ub interaction at the Ube2D BBD stimulates a closed E2~Ub conformation and in turn stabilizes the IAP1~Ube2D~Ub complex, facilitating Ub transfer onto substrate (Patel et al., 2019; Pruneda et al., 2012). This explains why UbvD1.1 can still moderately inhibit IAP2 auto ubiquitination.

In our preliminary *in vivo* HeLa cell transfections and subsequent far western assays using K48-TUBE, we curiously found that UUWT transfected HeLa cells showed a slight increase in K48-linked polyUb when compared to UUCTRL and untransfected HeLa cells. We had expected a decrease in K48-linked polyUb. While seemingly unusual, we wondered whether this observation could hint towards a ubiquitinome that can reprogram, in other words, whether the UPS contains multiplicity. Multiplicity is a phenomenon that is well-characterized within the

kinome (J. S. Duncan et al., 2012; Johnson et al., 2014; Stuhlmiller et al., 2015; Xiaohong Zhao et al., 2018). For instance, in triple-negative breast cancer (TNBC), tumor cells are able to circumvent treatments that target specific kinases by reprogramming. MEK inhibition resulted in the loss of ERK activity but was instantly followed by c-Myc degradation which stimulated the activation of a host of receptor tyrosine kinases. This series of events resulted in kinase inhibitor drug resistance in TNBC tumors (J. S. Duncan et al., 2012; Stuhlmiller et al., 2015)

Recently a similar discovery was done in the ubiquitination cascade when studying CRL E3 ligases. In humans, 6 E2 enzymes are able to facilitate CRL ubiquitination activity. Hill et al. found that the combinatorial siRNA knockout of both Ube2R1 and 2 did not affect SCF substrate levels (Hill et al., 2019). When performing a genome wide E2 CRISPR knockout screen, they found that only cells lacking Ube2R1/2 and Ube2G were lethally disabled. This suggested a buffering function of Ube2G in scenarios where Ube2R1/2 are found dysfunctional (Hill et al., 2019). To assess whether ubiquitinome reprogramming is also occurring after Ube2D inhibition we will need to perform more cell culture and proteomic studies.

Based on the E2 enzyme family sequence conservation analysis we performed, we believe that a linked-domain inhibitor approach can be standardized and applied to selectively inhibit other E2's as well, which could provide valuable mechanistic and functional insights into the E2 enzyme family. Whether our linked-domain inhibitors will also be useful in a clinical setting to treat E2-related diseases, will have to be established by further *in vivo* studies. However, the approach does seem to have potential, especially considering that the binding affinity of UUD1.1 (5 nM), resides within an acceptable range for potential drug applications. To put into context, the $IC_{50}$ value of CC0651, thus far the most well-known and promising specific E2 inhibitor, is 2 μM (Kotz, 2011).

# CHAPTER 5: CONCLUSION

- E2 enzyme family sequence conservation analysis showed the E2 backside binding. domain to be most divergent amongst across E2 enzymes, illustrating its potential for future E2 inhibitor designs.

- We successfully designed, expressed and tested 3 novel Ube2D inhibitors.

- Linked-domain inhibitors can prevent E1, E3 and backside binding domain interactions simultaneously.

- Preliminary investigations show some evidence for UU and RU specificity towards Ube2D.

- $K_d$ values were determined at 5 nM, 300 nM and 3 μM for UUD1.1, UU and RU respectively.

- IAP2 and UHRF1 inhibition assays corroborated the $K_d$ values and showed that UUD1.1 interfered with E3 auto-ubiquitination the most.

- UHRF1 inhibition assays show UUD1.1 (linked-domain) has a higher efficacy compared to UbvD1.1 (single-domain) inhibitor.

- UbvD1.1 is unable to prevent E2 auto-ubiquitination, whereas UUD1.1 shows robust E2 auto-ubiquitination inhibition.

# CHAPTER 6: MATERIALS & METHODS

## 6.1 Vector and Primer Designs

Primers were obtained from Eurofins. Primers were designed for transformation into bacterial cell vector pQE-80L MBP-SspB Micro (pQE-80L MBP-SspB Micro was a gift from Brian Kuhlman (Addgene plasmid #60410; http://n2t.net/addgene:60410; RRID: Addgene_60410)) and mammalian cell vector pcDNA3.1 (+) (Invitrogen, cat. #V79020). List of primers utilized are featured in Appendix B.

## 6.2 Inhibitor Constructs

A range of inhibitors were created. Inhibitor amino acid sequences can be found in Appendix C.

## 6.3 Cloning

Gene cloning was performed using a Gibson Assembly Master Mix containing 699 µL $H_2O$, 320 µL 5X ISO buffer (3 mL 1M Tris-HCl pH 7.5, 150 µL 2M $MgCl_2$, 60 µL 100 mM dATP, 60 µL 100 mM dTTP, 60 µL 100 mM dGTP, 60µL 100 mM dCTP, 300 µL 1M DTT, 1.5 g PEG-8000, 300 µL 100 mM NAD, 2.01 mL $H_2O$), 0.64 µL 10 U/µL T5 exonuclease (New England BioLabs, Ipswich, MA, USA), 20 µL 2 U/µL Phusion DNA polymerase (Thermo Fisher Scientific, Rockford, IL, USA), 160 µL 40 U/µL Taq DNA polymerase (Thermo Fisher Scientific, Rockford, IL, USA). 15 µL of Gibson Assembly Master Mix was combined with 5 µL of insert and vector at the appropriate concentrations. The mixture was incubated at 50 °C for 1 hr.

## 6.4 Protein Purification

All plasmids were transformed into RIPL cells at 42°C for 42 seconds and subsequently recovered on ice for 3 minutes. Starter cultures were then grown at 37°C for 1hr, after which they were transferred into LB-Amp selective medium and grown O/N. Both Amp (100 μg/μL) and cell culture were added in 1:1000-fold dilution. Cells were then incubated at a 1:100 dilution in 1L LB-Amp selective media and grown for 4 hrs at 37°C in a shaking incubator. Temperature was lowered to 16°C and cells were induced O/N using IPTG (200μg/μL) at 1:1000 dilution. Cells were spun down by centrifuging at 4200 rpm, 4°C for 30 minutes and resuspended into ~20 mL DI $H_2O$. They were then spun again to yield cell pellets and stored at -20°C.

All cell pellets were resuspended in 25 mL 15 mM Tris-HCl, 50 mM NaCl, pH 8 and subsequently sonicated to complete lysis. Cells were than clarified and spun down by centrifuging for 1 hr at 10000 rpm and 4°C. Clarified lysate was run over an Ni-NTA column, washed using 50-100mL of 50 mM Tris-HCl, pH 8, 1M NaCl, 5 mM imidazole. Target proteins were eluted using 10 mL of 50 mM Tris-HCl, pH 8, 100 mM NaCl, 250 mM imidazole.

### 6.4.1 Ubox-Ubl WT, 16, 17

Protein was dialyzed in dialysis tubing at MWCO 10,000 in 25mM HEPES, pH 7, 50 mM NaCl, 1mM DTT, 1 mM EDTA at 4°C. MBP tag was cleaved using TEV protease (added at 1:100 dilution). Dialysis took place O/N. ÄKTA Start FPLC was used to run protein over anion exchange (Buffer A: 25 mM HEPES pH 7, 50 mM NaCl; Buffer B: 25 mM HEPES pH 7, 150 mM NaCl, 1 mM DTT. Parameters: a) Linear, 25%B, CV=10.00; b) Step, 25%B, CV = 10.00; c) Linear, 100%B, CV=10.00). Fractions corresponding to the Ubox-Ubl peak (as determined by SDS-PAGE gel) were collected and ran over another Ni-NTA column. Flow-

through was collected and the column was washed with ~40 mL of 50mM tris-HCl pH 8.00, 100mM NaCl, 15 mM imidazole to collect any protein that was left behind. Protein was then concentrated using a concentrating conical with MWCO 10,000, and stored at -80°C.

**6.4.2 Ube2D1**

Protein was dialyzed in dialysis tubing at MWCO 10,000 in 50mM HEPES, pH 7, 100 mM NaCl, 1mM DTT at 4° O/N. The sample was then injected into the ÄKTA Start and ran over an s200 Sephacryl Size Exclusion Column (Buffer: 50 mM HEPES pH 7, 100 mM NaCl, 1 mM DTT). Fractions corresponding to Ube2D1 were collected, concentrated and flash frozen to be stored at -80°C.

**6.4.3 Ring-Ubl WT, 14, 15**

Protein was dialyzed in dialysis tubing at MWCO 10,000 in 50mM Tris-HCl, pH 8, 50 mM NaCl, 1mM DTT, 1 mM EDTA at 4°C. MBP tag was cleaved using TEV protease (added at 1:1000 dilution). Dialysis took place O/N. ÄKTA Start FPLC was used to run protein over anion exchange (Buffer A: 50 mM Tris-HCl, pH 8, 50 mM NaCl; Buffer B: 50 mM Tris-HCl pH 8, 500 mM NaCl, 1 mM DTT. Parameters: Linear, 100%B, CV=20.00). Fractions corresponding to the Ring-Ubl peak (as determined by SDS-PAGE gel) were collected and concentrated using a concentrating conical with MWCO 10,000 and stored at -80°C.

**6.4.4 Ubox-Ubv-D1.1**

Protein was dialyzed in dialysis tubing at MWCO 10,000 in 25mM HEPES, pH 7, 50mM NaCl, 1mM DTT, 1mM EDTA at 4°C. MBP tag was cleaved using TEV protease (added at 1:1000 dilution). Dialysis took place O/N. The sample was then run over another Ni-NTA column. Flow-through was collected and the column was washed with 40 mL of 50mM tris-HCl pH 8.00, 100mM NaCl, 20mM imidazole to collect any protein that was left behind. Anion

exchange was then performed using the same buffer conditions as for Ubox-Ubl (section *5.4.1*). Method parameters: a) Linear, 25%B, CV=10.00; b) Step, 25%B, CV = 8.00; c) Linear, 100%B, CV=10.00, d) Step, 100%B, CV=6.00). Finally, the sample was run over a Ni-NTA column. Flow-through was collected and the column was washed with ~40 mL of 50mM tris-HCl pH 8.00, 100mM NaCl, 20mM imidazole to collect any protein that was left behind. The sample was then concentrated using a concentrating conical at MWCO 10000, flash frozen and stored at -80°C.

**6.4.5 Ube2D1~UUD1.1 Complex for X-Ray Crystallization**

For individual purification methods for both Ube2D1 and UUD1.1 refer to sections *5.2.2* and *5.2.4* respectively. Proteins were combined stoichiometrically and incubated on ice for 45 minutes. They were then concentrated to produce a final volume of 1.25 mL. The sample was then injected into the ÄKTA Start and ran over an s200 Sephacryl Size Exclusion Column (Buffer: 25 mM HEPES pH 7, 100 mM NaCl, 1 mM DTT). Fractions corresponding to Ube2D1~UUD1.1 complex (as determined by SDS-PAGE gel and SE chromatogram peak shifts), were collected and concentrated using a concentrating conical at MWCO 3000.

**6.4.6 Ubv-D1.1**

Protein was dialyzed in dialysis tubing at MWCO 3500 in 25mM HEPES, pH 7.5, 100 mM NaCl, 1mM DTT at 4°C. MBP tag was cleaved using TEV protease (added at 1:1000 dilution). Dialysis took place O/N. ÄKTA Start FPLC was used to run protein over anion exchange (Buffer A: 25 mM HEPES, pH 7.5, 100 mM NaCl; Buffer B: 25 mM HEPES pH 7.5, 300 mM NaCl, 1 mM DTT. Fractions corresponding to UbvD1.1 (and MBP contamination) were collected, concentrated, and subsequently ran over the Superdex s75 SEC column (25 mM

HEPES pH 7.5, 100 mM NaCl, 1 mM DTT). Fractions corresponding to UbvD1.1 were collected, concentrated and flash frozen to be stored at -80°C.

## 6.5 *in vitro* Ubiquitylation Assays

Typically, cell-free ubiquitination assays were carried out in a total reaction volume of 20 µL. All reactions contained 50 mM HEPES pH 7.4, 2.5 µM $MgCl_2$, 2.5 µM DTT, 60 µM NaCl, 10 µM ATP, 5 µM of fluorescein-Ub, 1.25 µM IAP2 and 675 nM of Ube2D1, unless indicated otherwise. Reactions were quenched with SDS-PAGE sample loading buffer. Equal amounts of samples were loaded onto 12-15% SDS-PAGE gels and subsequently imaged using the STORM 860 Molecular Imager (Molecular Devices, San Jose, CA, USA) and gel fluorescent bands were quantified using densitometric analysis through ImageQuant Software (v5.2). All bands were adjusted to reflect background noise and were subsequently normalized to the positive control reaction.

### 6.5.1 IAP2 Ube2D1 Titration Assays

IAP2 was combined with 20, 17, 13.3, 8.8, 5.9, 3.3, 2.62, 1.75 or 1.16 nM of Ube2D1. Reactions ran for 20 min at 37°C.

### 6.5.2 IAP2 Inhibition Assays

Reactions were performed at the indicated concentrations and mixed at RT for 20 minutes. For the UUD1.1 IAP2 inhibition assay (*Fig. 28C*), Ube2D1 was added at 3 nM

### 6.5.3 UHRF1 Inhibition Assays

600 nM of UHRF1 was combined with either UU WT (62.5, 15.6, 3.9, 0.975 or 0.244 µM), UbvD1.1 (45, 15.6, 3.9, 0.975, 0.244, 0.131, 0.087, 0.058 or 0.038 µM) or UUD1.1 (60, 15.6, 3.9, 0.975, 0.244, 0.131, 0.087, 0.058 or 0.038 µM), 19 µM $H3_{1-25}K9$ peptide, 1 µM HeDNA and 675 nM Ube2D1. Reactions proceeded for 20 min at RT.

### 6.5.4 E2 Loading Assays

55 μM inhibitor (if applicable), 2 μM Ube2D2 and 4 μM ubiquitin were mixed at RT for 5 minutes. Time points were taken at 5, 15, 45 and 90 seconds. One reaction was performed in the absence of Ube2D2 as a negative control.

### 6.5.5 APC/C CDH1 Inhibition Assays

20 μM of inhibitor (if applicable), 200 nM (Ube2S, Ube2C or Ube2D2), 100 nM E1, 30 nM APC/C, 500 nM CDH1, 200 nM Ub-Cyclin B, 100 μM Ub WT, 5 mM ATP and BSA were mixed at RT for 12 minutes. One reaction was performed in the absence of APC/C (negative control) and another in the absence of inhibitor (positive control).

### 6.5.6 Cullin1 Beta-Catenin Inhibition Assays

Single-encounter ubiquitylation assays were performed using a KinTek RQF-3 Rapid Quench-Flow instrument. Ube2R2 (20 μM) was charged in the presence of $(His)_6$-tagged ubiquitin (30 μM), E1 (1 μM), and reaction buffer, followed by the addition of unlabeled β-catenin peptide (200 μM). This reaction mixture was then mixed equally with SCF (1 μM) that had been preincubated with $^{32}$P-labeled Ub–β-catenin peptide (0.2 μM). Reaction mixtures were quenched in reducing SDS-PAGE buffer and substrate, and products were resolved on a 4% to 20% gradient SDS-PAGE gel. Reactions were run in duplicate, and each product band was quantified as a percentage of the signal from the total lane for each time point. The rates of ubiquitin transfer and substrate dissociation from SCF were determined using both KinTek Explorer global fitting software and nonlinear curve fitting to analytical closed-form solutions as described by Pierce et al. (Pierce et al., 2009).

## 6.6 Isothermal Titration Calorimetry

Ube2D1 and UUD1.1 were dialyzed into 25mM HEPES pH 7.0, 100mM NaCl, 1mM TCEP at MWCO 10,000. ITC experiments were performed using the Affinity ITC LV (Waters, TAInstruments). 2 µL aliquots of UUD1.1 (100 µM) were injected from a 264 µL rotating syringe (125 rpm) into an isothermal cell containing 185 µL of Ube2D1 (10 µM). Experiments were performed at 25°C. The delay between each injection was 300 seconds. A heat-burst curve was generated (micro calories/second vs. seconds) for each injection and the area under the curve was calculated for each injection using NanoAnalyzer software (version 3.8.0) to determine the heat (kJ/mol) associated with each injection. The last 5 injections were used to determine a blank constant that was used to adjust the raw measurements. The dissociation constant was also determined using NanoAnalyzer Software (version 3.8.0) after fitting the adjusted measurements to an independent model.

## 6.7 X-ray Crystallography Experiments

### 6.7.1 Crystallization of Protein Complex

Ube2D1~UUD1.1 complex protein was purified as outlined in section *5.2.5*. Proteins were concentrated to ~ 15 mg/mL in 25 mM HEPES pH 7, 100 mM NaCl. Samples were screened using the sparse matrix method (Jancarik and Kim, 1991) with a Phoenix Robot (Art Robbins Instruments, Sunnyvale, CA) utilizing the Berkeley Screen of conditions (Lawrence Berkeley National Laboratory). The optimum conditions for crystallization of the complex were found as follows: 100mM HEPES free acid/NaOH, pH 7.5, 1500 mM ammonium sulfate. Crystals were obtained after 3 days by the sitting-drop vapor-diffusion method with the drops consisting of a 1:1 mixture of 0.2 µL protein solution and 0.2 µL reservoir solution.

## 6.8 Cell Culture Experiments

### 6.8.1 HeLa Cell Growth and Transfection

HeLa cells were maintained in DMEM (Thermo Fisher Scientific, Rockford, IL, USA) supplemented with 10% fetal bovine serum, 2 mM GlutaMAX-I, 10 U/mL of penicillin, and 10 mg/mL of streptomycin (Invitrogen, Carlsbad, CA, USA) at 37 °C and 5% $CO_2$.
Cells were transfected with expression plasmids using the EndoFectin reagent (GeneCopoeia Rockville, MD, USA) according to the manufacturer's instructions in 6-well plates. Cells were incubated for 48 hrs. Fresh complete medium was exchanged 24 hrs after transfection and cells were treated with MG132 (Cayman Chemical, Ann Arbor, MI, USA) for 4 hours prior to cell harvest.

### 6.8.2 Ub Immunoblot Western Analysis

Cells were harvested using cold 1X PBS by mechanical scraping. Cell pellets were resuspended using cold lysis buffer (25 mM HEPES, pH 7.4, 0.4 M KCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 1% NP-40, 1 mM PMSF, and 2 μg/mL of leupeptin) of 3 times the volume of the cell pellets. After three cycles of freeze/thaw, lysates were kept on ice for 30 min and were then centrifuged at 16,000× *g* for 10 to 20 min at 4 °C. Sample protein concentrations were determined using a BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA).

15% SDS-PAGE gels were transferred using a Bio-Rad transfer kit at 300 mA, 160 V for 3 hours at 4 °C. After the wet transfer total protein staining was performed using LI-COR REVERT Total Protein Stain (LI-COR Bioscience Lincoln, NE, USA). The transferred nitrocellulose membrane was blocked in PBS containing 5% BSA and 0.1% Tween-20 for 1 h. Primary antibody incubation utilizing anti-ubiquitin P4D1 mouse IgG (1:1000) (Santa Cruz Biotechnology, Dallas, TX, USA) was performed in blocking buffer overnight at 4°C. The

membrane was then washed with PBS containing 0.1% Tween-20 for four times. Secondary antibody incubation utilizing LI-COR donkey anti-mouse IgG IRDye 680 (1:10000) (LI-COR Bioscience Lincoln, NE, USA) was done in blocking buffer for 1 hr at room temperature. After washing four times with PBS containing 0.1% Tween-20 and once with PBS alone, the membrane was dried and analyzed using a LI-COR Odyssey CLx imaging system. Western bands were normalized using total protein stain.

### 6.8.3 K48-TUBE Far Western Analysis

Cells were lysed as described in section *6.8.*2 with 4 times the volume of the pellet size. Three deubiquitylase inhibitors: 1,10-phenanthroline (5 mM), NEM (10 mM), and PR-619 (50 μM) were added to the lysis buffer for K48-TUBE Far-western analysis. About 1.5-2.0 milligrams of whole-cell lysates were used and added to SDS-PAGE sample buffer, followed by Far-western analysis. K48-TUBE biotin (1:1000) (LifeSensors, Malvern, PA, USA) was incubated with the nitrocellulose membrane (LI-COR Bioscience Lincoln, NE, USA) for 1 h at room temperature and then with IRDye-800 conjugated streptavidin (1:10,000) (LI-COR Bioscience Lincoln, NE, USA) for 2 h at room temperature. The wash steps between incubations were the same as in Western analysis described in *section 6.8.2*. Results were obtained and analyzed using a LI-COR Odyssey CLx imaging system.

## 6.9 Multiple Sequence Alignments and Consensus Sequences

To acquire a list of homologous sequences, we conducted a BLAST search using each human E2 enzyme gene query sequence, extracted from UNIPROT (PMID: 14681372). We took the top 10,000 hits and inspected each set family set manually and established a cut-off that included all hits corresponding to the E2 family member. Some E2 enzymes have several isoforms. In this case, all corresponding sequences were grouped together in one dataset. To

limit potential bias in the alignment, redundant sequences were removed from the dataset with use of the MEME Suite (v5.1.1, build 2020-02-01) Purge tool (PMID: 19458158). A score threshold of 900 was used. The resulting sequences in the dataset were then aligned using the multiple sequence alignment tool Mafft (v7.471, build 2020-07-03) (PMID: 23329690). To enhance the quality of the multiple sequence alignment (MSA), and remove poorly aligned regions and leftover spurious sequences, we employed the trimming tool triMAl (v1.4.rev.22, build 2015-05-21) (PMID: 19505945). The alignment was trimmed using a gap threshold of 0.75 resulting in the removal of positions with gaps that occurred in more than 25% of the sequences. A consensus sequence for each E2 enzyme was then created from the trimmed MSA using the EMBOSS package Cons (v6.6.0.0, build 2013-07-15) (PMID: 10827456). Consensus residues at each position were determined through Cons using a plurality value of 0.1. All steps described up to this point were repeated for all members of the E2 enzyme family, resulting in 23 E2 enzyme consensus sequences. Finally, we utilized the PROMALS3D server to create a MSA of these consensus sequences (PMID: 18287115). PROMALS3D was used since it takes both structural and sequence information into account, resulting in a higher quality MSA.

### 6.10 Phylogenetic Tree, Weblogos and Shannon Entropy

A maximum likelihood phylogenetic tree was constructed using Protest3, which used Blosum62+G+F substitution matrix. We then plotted the tree as an unrooted radial phylogram. To illustrate the amino acid charge conservation variation at each position in the protein sequences for each E2 enzyme family member we constructed weblogos using Weblogo3 (PMID: 15173120). We also conducted a Shannon Entropy calculation on this alignment.

To calculate the amino acid (AA) variation at all positions in all 23 E2 consensus sequences, we utilized the *Calculate_AA_Variation* function in the R Package BALCONY

(v0.2.10**,** build 2019-02-28**)** and applied it to each E2 family member MSA (PMID: 30107777**).** Based on the AA variability analysis, we were able to calculate the average charge for each position in all E2 consensus sequences using the formula (K%+R%+H%)-(D%+E%). We then colored each E2 HTH according to this score.

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# APPENDIX A: ANALYSIS PIPELINE RESULTING IN THE E2 ENZYME FAMILY PHYLOGENETIC TREE

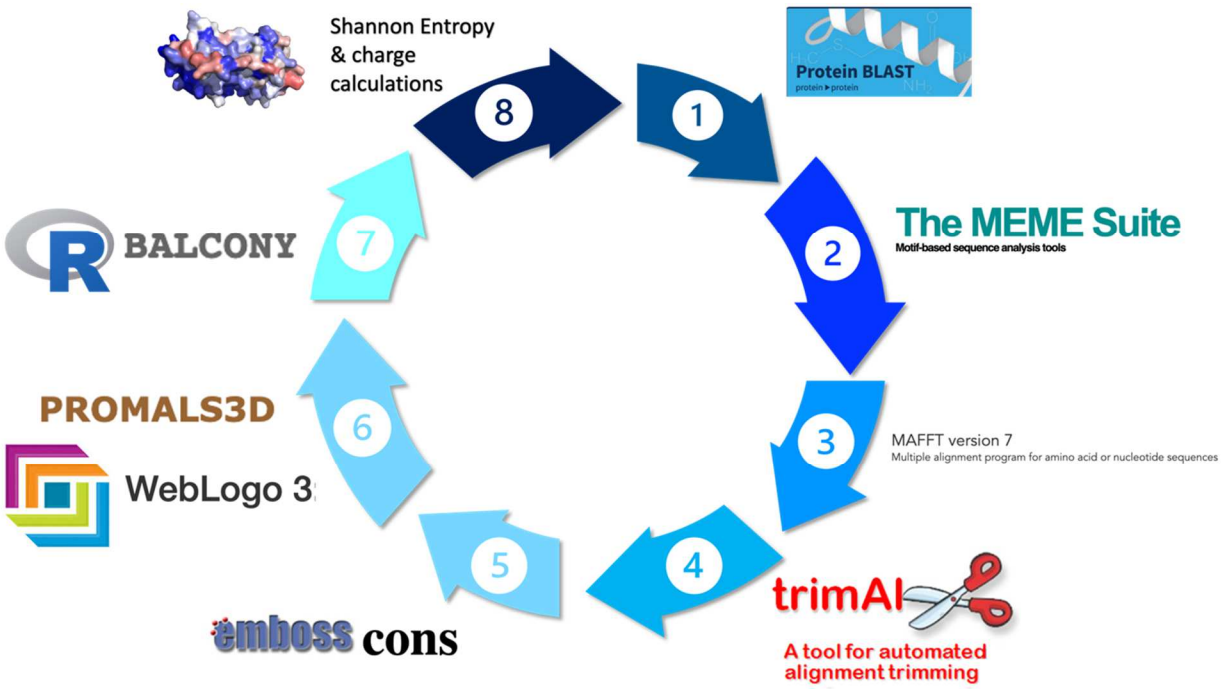


*Figure 36*. Analysis pipeline resulting in the E2 enzyme family phylogenetic tree and Shannon entropy values.

# APPENDIX B: TABLE OF PRIMER DESIGNS

Table 3

*Primer Designs. All primers were ordered through Eurofins sciences. PQE-80L MBP-SspB Micro was a gift from Brian Kuhlman (Addgene plasmid #60410; http://n2t.net/addgene:60410; RRID: Addgene_60410).*

| Name | Vector | F/R | Sequence 5'-3' |
|---|---|---|---|
| Ubv.D1.1 | pQE-80L MBP-SspB Micro[1] | F | CAGGGATCCTCTAGAGTCGAACATGCAGATCTTTGTCAAAAATTC |
| UUD1.1 | pcDNA 3.1[2] | F | AAGCTTGGTACCGAGCTCGGATCCAGGAGATGGACTACAAAGACGATGACGACAAGGCAGAAATCGACTATAGCGACGCG |
| UUD1.1 | pcDNA3.1 | R | AGAATTCTGCAGATATCCAGCACA |

# APPENDIX C: TABLE OF INHIBITOR PROTEIN SEQUENCES

Table 4
*Amino Acid Sequences of All Inhibitor Designs, Including the Control Sequences*

| Name | AA sequence |
|---|---|
| UU WT | MDYKDDDDKAEIDYSDAPDEFRDPIMDTLMTDPVRLPSGTVVDRSIILR HLLNSPTDPFNRQMLTESMLEPVPELKEQIQAWMREKQSSDHGGSSGSS GGSSMWIQVRTMDGRQTHTVDSLSRLTKVEELRRKIQELFHVEPGLQRL FYRGKQMEDGHTLFDYEVRLNDTIQLLVRQS |
| UU 16 | MDYKDDDDKAEIDYSDAPDEFRDTIMDTLMTDPVRLPSGTVVDRSIILR HLLNSPTDPFNRQMLTESMLEPVPELKEQIQAWMREKQSSDHGGSSGGS SGGSSMWIQVRTMDGRQTHTVDSLSRLTKVEELRRKIQELFHVEPGLQR LVYRGKQMEDGHTLFDYEVRLNDTIQLLVRQSEFLKTKGP |
| RU WT | SLTAQQSSLIREDKSNAKLWNEVLASLKDRPASGSPFQLFLSKVEETFQCI CCQELVFRPITTVCQHNVCKDCLDRSFRAQVFSCPACRYDLGRSYAMQ VNQPLQTVLNQLFPGYGNGRGGSSGGSSGGSSGGSSMWIQVRTMDGRQ THTVDSLSRLTKVEELRRKIQELFHVEPGLQRLFYRGKQMEDGHTLFDY EVRLNDTIQLLVRQS |
| UUD1.1 | MDYKDDDDKAEIDYSDAPDEFRDPIMDTLMTDPVRLPSGTVVDRSIILR HLLNSPTDPFNRQMLTESMLEPVPELKEQIQAWMREKQSSDHGGSSGGS SGGSSMQIFVKKFWGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFA GKQLEDGRTLSDYNIQKKFTLYLAYGLRAG |
| UU 17 | MDYKDDDDKAEIDYSDAPDEFRDTIMDTLMTDPVRLPSGTVVDRSIILR HLLNSPTDPFNRQMLTESMLEPVPELKEQIQAWMREKQSSDHGGSSGGS SGGSSMWIQVRTMDGRQTHTVDSLSRLTKVEELRRKIQELFHVEPGLQR LVYRGKQMEDGHTLFDYEVRLNDTIQLLVRQS |
| RU F/V | SLTAQQSSLIREDKSNAKLWNEVLASLKDRPASGSPFQLFLSKVEETFQCI CCQELVFRPITTVCQHNVCKDCLDRSFRAQVFSCPACRYDLGRSYAMQV NQPLQTVLNQLFPGYGNGRGGSSGGSSGGSSGGSSMWIQVRTMDGRQT HTVDSLSRLTKVEELRRKIQELFHVEPGLQRLVYRGKQMEDGHTLFDYE VRLNDTIQLLVRQS |

APPENDIX D: WNT SIGNALING UTILIZING UBE3A IN THE PRESENCE OF RU WT (#1), RU F/V (#2) OR UU WT (#3/#4).

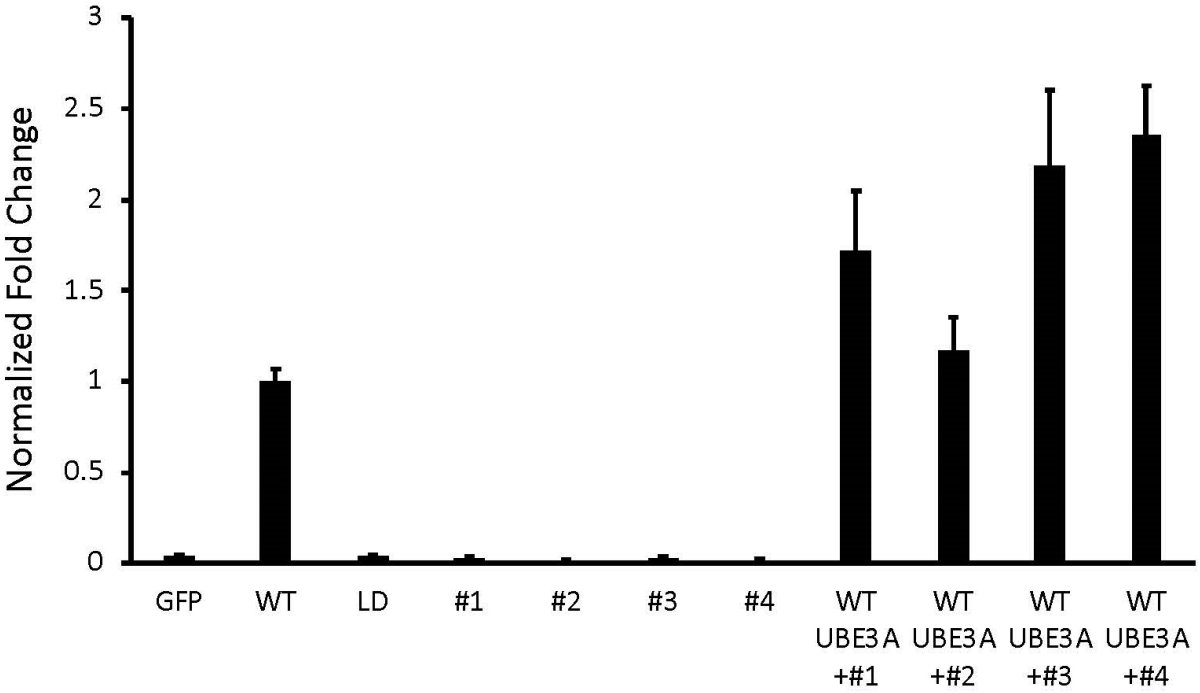


*Figure 37*. Wnt signaling utilizing Ube3A in the presence of RU WT (#1), RU F/V (#2) or UU WT (#3/#4). Fold changes is normalized to WT Ube3A activity.

APPENDIX E: IAP2 UBIQUITYLATION ACTIVITY AFTER THE ADDITION OF EITHER RU F/V (NEGATIVE CONTROL) OR RU WT.

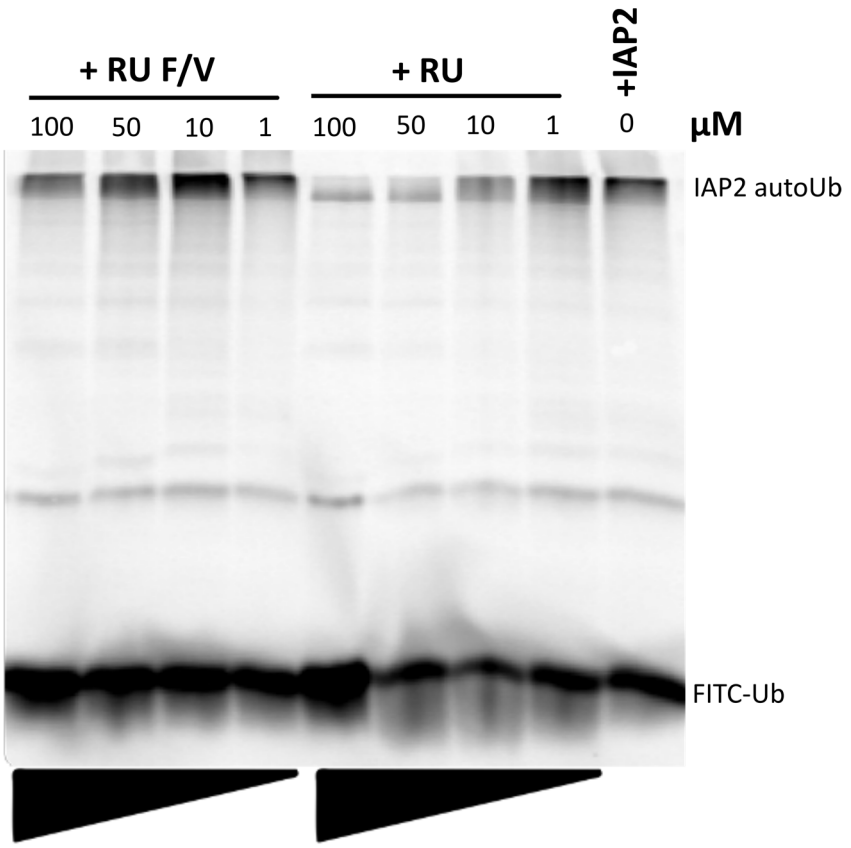


*Figure 38*. Negative control identification for mammalian cell culture. IAP2 auto-ubiquitination was measured in the presence of either RU F/V or RU WT in concentrations ranging from 100 µM to 1 µM. Signal was normalized to uninhibited IAP2 auto-ubiquitination activity.