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The progress on mapping ubiquitin signaling using photocrosslinking mono and di-ubiquitin probes and other ubiquitin moieties

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The progress on mapping ubiquitin signaling using photocrosslinking mono and di-ubiquitin probes and other ubiquitin moieties

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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Dedication

To my supportive village of family and friends.

Acknowledgement

First, I would like to thank God for helping me every step of the way. This journey has not been easy, but worth it. Through this process, I have matured, learned to persevere, and never give up. I am thankful to be able to see the light at the end of the tunnel. Praise God!

I want to thank my academic advisor Dr. Ashton Cropp. Ashton, you have been very helpful through this entire process and I am so thankful for your support throughout the years. Most importantly, I am thankful for your commitment to hear my concerns and pushing me to go the extra mile. I have genuinely appreciated your nuggets of wisdom and the belief you have in me. Thank you for being very very patient. In addition, I would like to thank my committee members for your support. Thank you for the feedback during poster sessions or the small conversations about my progress while walking through the halls. I am grateful for the words of encouragement from each of you. Furthermore, I would like to thank my collaborators for helping me to achieve my goals and working diligently with me.

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Thank you to all of my family and friends that have supported me through this entire process. The past five years have been challenging, but none of you told me to quit instead you said, "Keep going!" I am thankful for that. Thank you for the listening ears, words of encouragement, and sacrificing your nights to stay up with me. I believe many of you are excited more than I am about obtaining my degree, but that just goes to show how amazing you all are! This degree would not be possible if I did not have you all in my corner every step of the way.

Future Doctors (Deonte, Ariel, and Nikia)! One down and three to go! Thank you all for being supportive and achieving this dream with me. I am thankful my time is over for this season and ready to conquer the next. Just know I am going to be here encouraging and supporting you every step of the way as you all become amazing Doctors.

Finally, I would like to thank my Mom. Many saw me persevere through this process, but you saw the lowest and difficult points through it all. Thank you, for holding me up when I was weak. I appreciate you and love you.

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UBIP	ubiquitous immuopoietic polypeptide
Ub	ubiquitin
PTM	post-translational modification
kDa	kilodaltons
E1	activating enzyme
E2	conjugating enzyme
E3	ligase enzyme
ATP	adenosine 5'-triphosphate
DNA	deoxyribonucleic acid
UAA	unnatural amino acid
AA	amino acids
tRNA	transfer ribonucleic acid
aaRS	aminoacyl-tRNA synthetase
BocLys	N^{ε}-Boc-$_L$-lysine
wtUb	wild type ubiquitin
TFA	trifluoroacetic acid
Alloc	allyloxycarbonyl
MESNA	sodium 2-mercaptoethane sulfonate

Alloc-OSu	allyloxycarbonyl *N*-oxysuccinimide
PCL	photo-crosslinkers
pBpa	*p*-Benzoyl-$_L$-phenylalanine
UV	ultra violet
UBD	ubiquitin binding domains
diUb	ubiquitin dimer
UBA	ubiquitin-associated domains
UIM	ubiquitin-interacting motifs
UBAy	ubiquitin associated domain
UQ1	ubiquilin-1
UBA2	ubiquitin-associated two domain
CBD	chitin binding domain
hHR23a	human homolog of budding yeast RAD 23
Rap80	human receptor-associate protein
tUIM	tandem ubiquitin interacting motifs
ThzK	N^{ε}-$_L$-Thiaprolyl-$_L$-lysine
CysK	N^{ε}-$_L$-Cysteinyl-$_L$-lysine
MjTyRS	*Methannococcus jannaschii* tyrosyl-tRNA synthetase
$tRNA^{tyr}$	tyrosyl tRNA
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis

PCR	polymerase chain reaction
bp	base pairs
EDTA	ethylenediaminetetraacetic acid
LB	Luria-Bertani broth
2×YT	2× Yeast extract and Trytone rich medium
SOC	super optimal catobilite-repression broth
EDTA	ethylenediaminetetraacetic acid
PMSF	phenylmethylsulfonyl fluoride
IPTG	isopropyl-β-D-1-galactopyronaside
OD	optical density
DTT	dithiothreitol
MS	mass spectrometry
polyUb	polyubiquitin
PylT	pyrrolysyl tRNA
PylRS	pyrrolysyl tRNA synthetase
sfGFP	superfolder GFP
DIEA	*N*,*N*-diisopropylethylamine
ESI-MS	electrospray ionization mass spectrometry
ThzK	N^{ε}-L-Thiaprolyl-L-lysine
CysK	N^{ε}-L-Cysteinyl-L-lysine
ThzKRS	thiazolidinyl-lysyl-tRNA synthetase

CysKRS	cysteinyl-lysyl-tRNA synthetase
Mb	*Methanosarcina bakeri*
Mm	*Methanosarcina mazei*
RF1KNO	release factor 1 knockout
MWCO	molecular weight cut off
GST	glutathione S-transferase
Amp	ampicillin
Chl	chloramphenicol
Kan	kanamycin
Tet	tetracycline
Arg	Arginine
Arab	arabinose

Abstract

THE PROGRESS ON MAPPING UBIQUITIN SIGNALING USING PHOTOCROSSLINKING MONO AND DI-UBIQUITIN PROBES AND OTHER UBIQUITIN MOIETIES

By Courtney N. Braxton, Ph.D.

A dissertation submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2018

Major Director: T. Ashton Cropp. Ph.D., Associate Professor
Department of Chemistry

Ubiquitin (Ub) is a small, 76 amino acid, and post-translational modification (PTM) protein in eukaryotes. Modification of a substrate protein via the covalent attachment of the C-terminal glycine of Ub to the ε-amino group of lysine residues in a substrate is termed ubiquitination. Unlike, other PTM proteins, Ub can form polyUb chains at one or more of its seven lysine residues. (K6, K11, K27, K29, K33, K48, and K68). The consequence of these different polymerization sites is altered biological response with different polyUb linkages conferring different fates to target proteins. Unfortunately, the study of these chains have been limited by the inability to generate homogeneous polyUbs chains linked at known lysine residues. Furthermore, a three step enzymatic cascade consisting of activating-enzymes (E1s), conjugating enzymes (E2s), and ligase

enzymes (E3s) tightly controls this modification. In response, our laboratory has developed a system that creates polyUb chains through bacterial expression and "synthetic" building blocks. Now, the main questions are what do these chains interact with in the cell and how do these interactions mediate biological responses?

In an attempt to answer these questions, this dissertation looks at different molecular techniques created to capture the transient interactions of monoUb and diUb probes with Ub substrates, such as, ubiquitin binding domains (UBDs) and conjugating E2 enzymes. One molecular technique focuses on the use of incorporating a genetically encoded, photo-crosslinker, *p*-Benzoyl-$_{L}$-phenylalanine (pBpa) into diUb probes to capture their interaction with UBDs. This sets the foundation for understanding Ub's cellular signaling recognition of UBDs. Another technique is creating diUb probes that contain lysine derivatives, N^{ε}-$_{L}$-Thiaprolyl-$_{L}$-lysine (ThzK) or N^{ε}-$_{L}$-Cysteinyl-$_{L}$-lysine (CysK), and can form a disulfide bonds with E2 enzymes to capture their complex, opening an opportunity to understand mechanistically the role E2 enzymes have with polyUb chain formation. Herein, these techniques are established to help unravel the complexity of Ub signaling.

Chapter 1: Introduction

1.1 Discovery of Ubiquitin

In 1975, Gideon Goldstein and Colleagues isolated and purified a polypeptide, ubiquitous immuopoietic polypeptide (UBIP) or "Free Protein", from bovine thymus.[1] Due to its discovery, in the thymus it was assumed UBIP was a thymus hormone. However, Goldstein's lab would realize UBIP was present in all tissues and eukaryotic organisms. Two years later, Goldknopf *et al.* discovered a DNA-associated protein, the unique functionality of the protein was its ability to form a covalent bond with an internal lysine of histone H2A protein.[2,3] Within that same year Margaret Dayhoff would identify the abovementioned proteins, as ubiquitin (Ub), because it was ubiquitous in organisms.[3,4] In the 1980s, the interest in understanding ubiquitin grew exponentially and Hershko *et al*. discovered the enzymatic cascade of Ub.[3,5] Ubiquitin researchers, Aaron Ciechanover, Avram Hershko, and Irwin Rose, were notably recognized and awarded the Noble Prize in Chemistry "for the discovery of ubiquitin mediated protein degradation." The aforementioned research is just a tiny glimpse of research that has paved the way for continual exploration of Ub and its unique role it plays in organisms. Herein, the role, functionality, and developing research of Ub will be discussed.

1.2 Ubiquitin

Ubiquitin is a small, 8.5 kDa, 76 amino acid protein found in eukaryotes that controls, cellular processes, such as, protein degradation and trafficking.[6,7] Biologically, Ub functions as a post-translation modification protein (PTM), by the process of ubiquitination, the covalent conjugation of Ub's c-terminal glycine residue (distal monomer) to the ε-amino group of a lysine in Ub (proximal monomer) or a substrate.[6,8] The covalent conjugation of Ub happens through a three step enzymatic cascade, consisting of an activating enzyme (E1), conjugating enzyme (E2), and ligase enzyme (E3) (**Figure 1.1**).[6,8] In a concerted fashion, the E1 enzyme activates the adenylation of Ub by ATP and forms an Ub thioester (Ub-S-E1). Next, Ub transfers to the thiol group of an E2 enzyme (Ub-S-E2) for transportation to ubiquitination sites. Lastly, the E3 ligase forms a complex with Ub-S-E2, mediating the transfer of Ub to the substrate lysine.

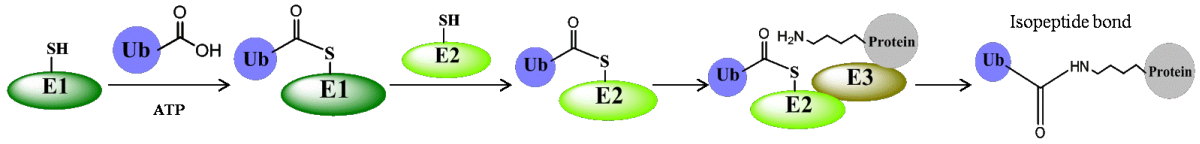


Figure 1.1. Ubiquitin Cascade

Structurally, Ub contains seven lysine resides (K6, K11, K27, K29, K33, K48, and K63) and methionine (Met1), allowing for PTM variability (**Figure 1.2**). The PTM variability consists of a single lysine residue (monoUb), multiple lysine residues (multi-monoUb), or one lysine with polymerization of Ub (polyUb) (**Figure 1.3**).[9,10] In addition, polyUb chains can be formed in mixed, branched, and unanchored Ub chains (**Figure 1.3**).[9,10]

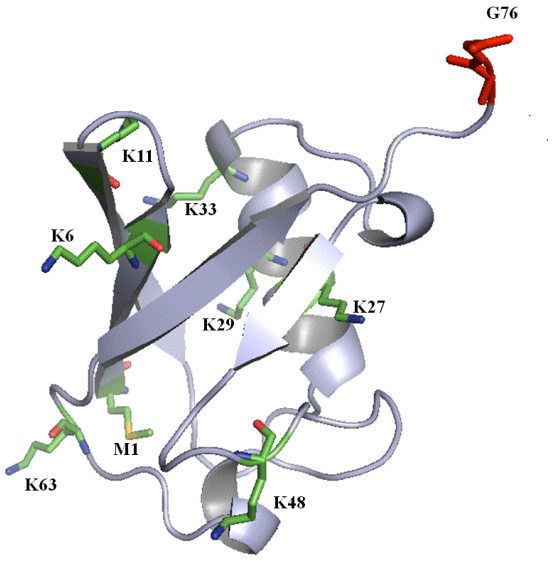


Figure 1.2. Structure of ubiquitin and its labled Met, Lys, and Gly residues.

For the past several decades, the complexity of Ub-signaling for cellular processes has been unveiled.[11] All seven lysine residues and the Met1 group of Ub are used *in vivo* to create polyUb chains. Each linkage having their own functional activity within cells.[10,12–17] Obscurity is observed in understanding each linkages' functional activity (K6, K11, K27 K29, K33)[18–23], except for typical, K48 and K63 linked chains. Moreover, K48-linked polyUb chains regulate proteasomal degradation and K63-linked polyUb chains are involved in DNA repair and signal transduction.[16,24–30]

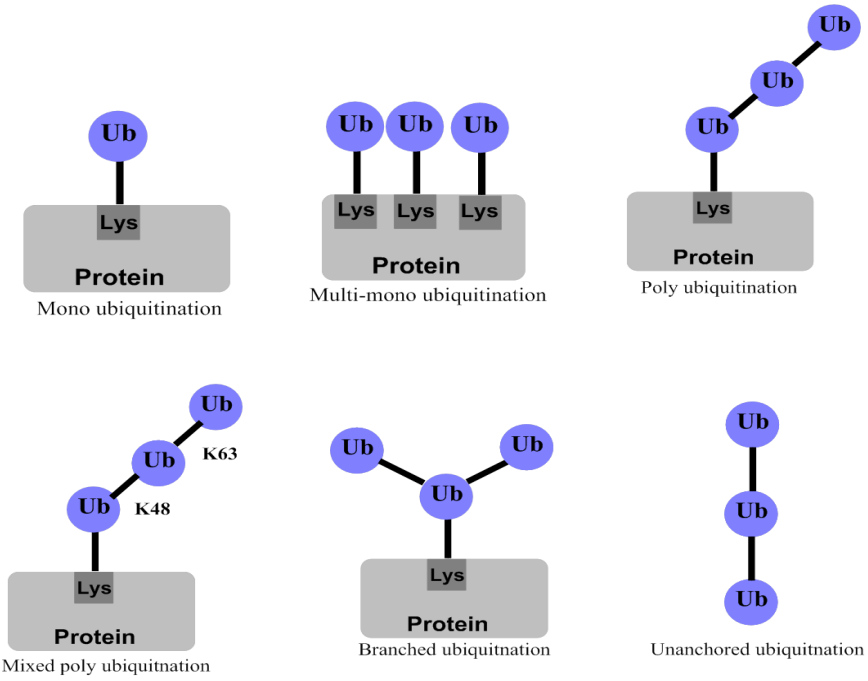


Figure 1.3. Ubiquitination variability.

Ubiquitin modifications play a complex role in the signal response of target proteins. More specifically, polymeric chain formation enhances the complexity of intracellular Ub signaling networks due to the assembly of chains with different linkages and lengths that are unique for different biological processes.[10,12–17] A main question in ubiquitin biology is understanding, how the broad functional range of Ub signaling is achieved?[31] Several studies focus on understanding how Ub signaling is formed, recognized, and translated into various biological processes; however, the mechanism behind polyUb chains acting as a molecular signal for various cellular processes is not well understood.[31] The specificity of the recognized signal by a particular polyUb chain is determined by unique conformations that a particular chain can adapt, which are dictated by the linkage type.[16]

To study the relationship between the linkage, structure, and function of polyUb signals, polyUb chains need to be generated with native connectivity, controlled length, defined linkage and composition, and sufficient (mg scale) quantities.[31] Enzymatically, this approach can be challenging due to the lack of available lysine specific E2 enzymes for all linkages and *in vitro* ubiquitination of target proteins substrate-specific Ub-ligases (E3) are needed and not often known. Furthermore, enzymatic construction of polyUb chains of specific linkages for function must meet specific requirements: (1) the enzyme must exhibit linkage specificity and (2) the enzyme must be active with free Ub. There are three known E2 conjugating enzymes that fulfill these requirements: K48-specific E2-25K[32], K63-specific Ubc13/Mms2[33,34], and K11 specific UBE2s.[35] Knowing and having these three E2 conjugating enzymes makes it readily available to enzymatically create K48-linked, K63-linked, and K11-linked polyUb chains.

Due to the lack of accessibility and understanding of E2/E3 enzymes interest has grown in making polyUb chains nonezymatically.[18,36–45] Several nonenzymatic approaches, such as, photoremoveable auxiliary[36] and δ-mercaptolysine-mediated peptide ubiquitination[38], total chemical synthesis[18,39,41–43], and native chemical ligation[18,37,42,46] have successfully created monoUb or polyUb chains. The studies focused on nonenzymatic polyUb, chain assembly meet the requirements to successfully study polyUb signaling.[18,39–41,43,47] However, the concern with the aforementioned approaches are their efficiency of making them in a laboratory. These approaches rely heavily on chemical synthesis, which can be outside of the range for many biochemical approaches. To circumvent this issue, the Cropp lab in collaboration with the Fushman lab from University of Maryland (UMD), previously developed the nonenzymatic assembly of polyUb chains by bacterial expression and a silver mediated condensation reaction.[31,48]

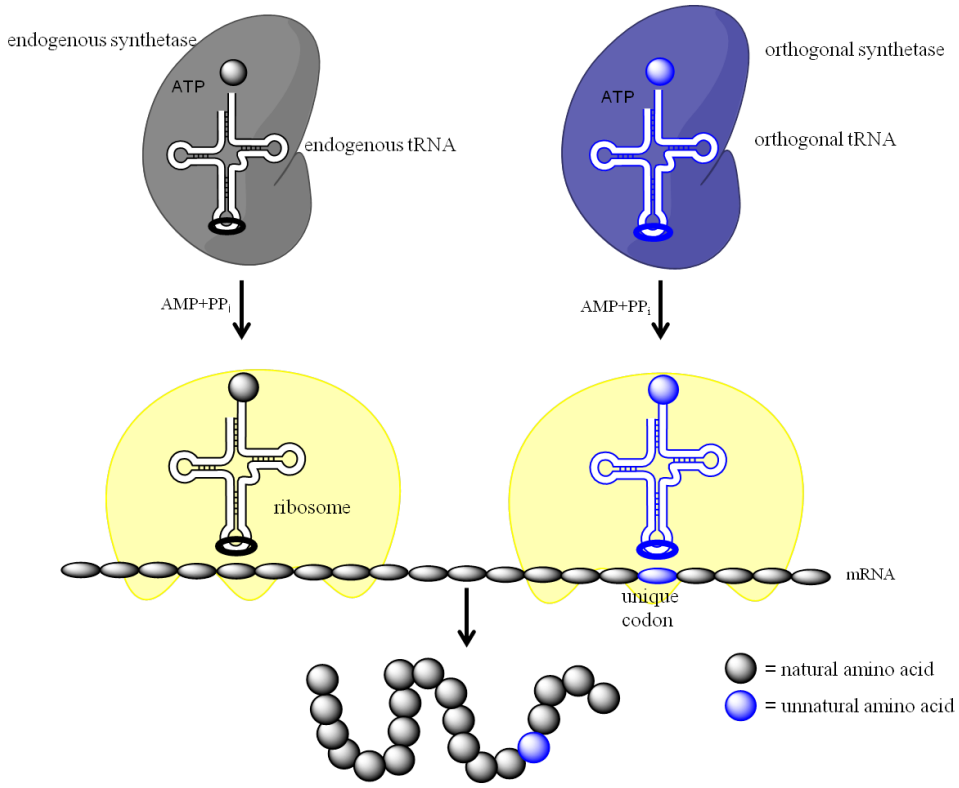


Figure 1.4. Incorporation of an UAA in response to an amber stop codon, UAG.

Following the methodology, developed by the Schultz lab, Ub monomers are bacterially expressed to contain an unnatural amino acid (UAA).[49] Traditionally, 64 codon triplets encode for the 20 naturally occurring amino acids (AA). Each native amino acid has an endogenous tRNA and aminoacyl-tRNA synthetase that incorporates the amino acid into a growing peptide chain (**Figure 1.4**). For the incorporation of unnatural amino acids an orthogonal tRNA/aminoacyl-tRNA synthetase (aaRS) pair is needed (**Figure 1.4**). The orthogonality of the pair prevents the tRNA from being a substrate for native aminoacyl synthetases and the synthetase only recognizes the tRNA engineered for the UAA (**Figure 1.4**). The tRNA recognizes an amber suppressor codon, TAG, encoded in the protein (**Figure 1.4**). Our lab has created plasmids that contain the

pyrrolysine translational tRNA/aaRS machinery to incorporate UAAs, such as, N^{ε}-Boc-$_L$-lysine (BocLys), in response to the TAG codon, encoded in Ub. The Ub mutants expressed can then be assembled into polyUb chains.[31]

Chain formation consists of two Ub monomers being ligated. One monomer (proximal) containing an UAA, BocLys, and the other monomer is wild type Ub (WTUb) (distal) (**Figure 1.5**). The wild type Ub monomer is reacted with MESNA, ATP, and E1 to form a C-terminus thioester group.[31] Both monomers are reacted with Alloc-OSu, to add an orthogonal allyloxycarbonyl (Alloc) protecting group to the amine groups of the remaining lysines and N-termini (**Figure 1.5**). After, Alloc protection the proximal monomer is treated with diluted TFA to deprotect the BocLys (**Figure 1.5**). The two monomers are ligated together through a silver mediated condensation reaction[48] and alloc-deprotected to give an nonezymatically linked Ub chain (**Figure 1.5**). Chains can contain monomers that have different isotopic labeled AAs, amino acid mutations[31], and photo-crosslinkers (PCL)[50,51].

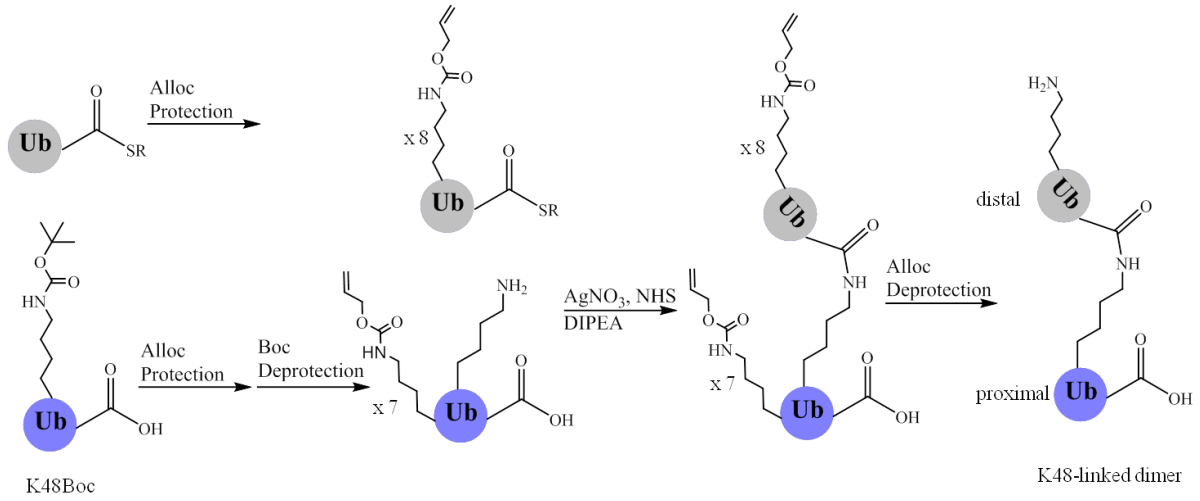


Figure 1.5. Example of the non-enzymatic method for the production of K48-linked diUb. "building blocks" are bacterially expressed.

1.3 Understanding Ubiquitin Signaling

In this dissertation, we are interested in developing technologies that covalently capture the interaction between monoUb and diUb probes with ubiquitin substrates to understand Ub signaling. Specifically, diUb probes are created enzymatically and non-enzymatically to contain UAAs, such as, photo-crosslinkers and lysine derivatives, that react to capture the transient interactions of Ub probes with their Ub substrates.

In biological systems, photo-crosslinkers are photochemical reactive groups that get excited from UV exposure and form a covalent bond between two macromolecules. Photo-crosslinkers incorporated into a protein can help with identifying protein binders of the protein. There are different classes of PCL and they include aryl azides[52,53], diazarines[52,53], and benzophenones[51–54] (**Figure 1.6**). Biologically, each class has their benefits and qualms for use. Aryl azides are easily synthesized, commercially available; stable in dark, highly reactive, but their max UV irradiation is less than 300 nm, which is problematic because it causes damage to biological systems.[53] Diazarenes are stable at room temperature, highly reactive (350-380 nm); however, the synthetic process is complex.[53] Benzophenones are commercially available, chemically stable, can be manipulated under ambient light, UV radiation is non-damaging (350-360 nm), reacts with C-H bonds and is reversible, but require longer irradiation and are bulky.[53] Our lab is interested in using a benzophenone photo-crosslinker, *p*-Benzoyl-$_L$-phenylalanine (pBpa) (**Figure 1.6**).

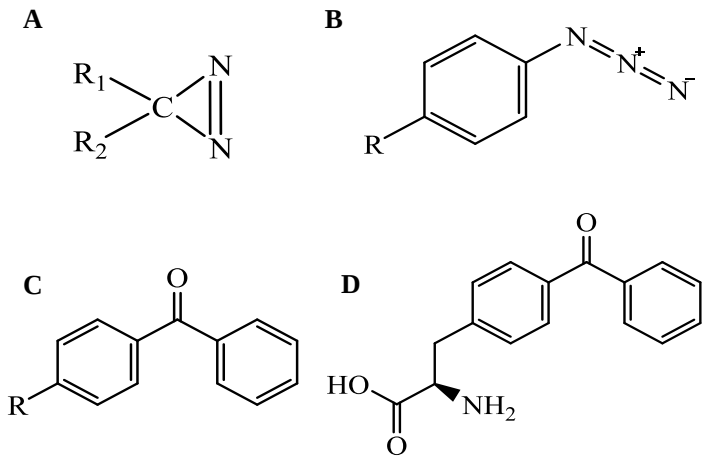


Figure 1.6. Chemical structures of the photo-crosslinkers. **A)** diazirine **B)** aryl azide **C)** benzophenone. **D)** Structure of unnatural amino acid, *p*-Benzoyl-L-phenylalanine (pBpa).

The novelty, of pBpa came about by trying to simplify the synthesis of photoreactive peptides, as a means, to make a new photo-crosslinking amino acid that is easily accessible to incorporate into peptides by solid phase synthesis.[55,56] Successful achievement of incorporating pBpa into peptides paved the way for performing experiments to understand peptide-protein interactions.[56] Through the experiments, the viability of pBpa's crosslinking ability between the peptide and protein was 50 % and 100 %, thus establishing pBpa as an efficient photo-crosslinker.[56] Other unique characteristics of pBpa is its inability to photodissociate and its reversibility.[50] Expounding on its reversibility, pBpa becomes a triplet biradical when exposed to UV (350-360 nm), and preferentially reacts with weak C-H bonds[50,57,58] The covalent bond forms through H-abstraction.[56] Furthermore, due to pBpa being reversible it can undergo many excitation and relaxation cycles until a covalent bond forms.[56] Understanding, pBpa's role in peptide-protein interactions sparked the interest and development of incorporating pBpa into larger proteins by the

suppressor tyrosyl tRNA ($tRNA^{tyr}$) and *Methannococcus jannaschii* tyrosyl-tRNA synthetase (MjTyrRS) pair, in response to an amber stop codon, TAG.[50]

In **Chapter 1** we focus on creating a molecular technique, by way of, incorporating pBpa into Ub via our bacterial expression expertise[31], to capture transient interactions by crosslinking of Ub and ubiquitin binding domains (UBDs). Our hope is this technique will contribute to answering the question: How does the broad functional range of Ub signaling occur, moreover with, Ub monomers and polyUb chains?

One way to understand the role of different polyUb chain linkages is identifying what binds to them. Ubiquitin binding domains are small modular proteins that interact noncovalently with Ub, typically around the Ile44 patch.[59,60] Previous experiments have identified UBDs for monoUb and polyUb chains through bioinformatics[61], yeast two-hybrid screens[62,63], and affinity pull down technology[64,65]. UBDs interact with Ub on a broad spectrum of binding affinities, 3μM-2mM.[66] In efforts, to identify novel UBDs we developed a molecular technique to capture UBDs via photo-crosslinking with pBpa. To establish the framework for our technique we selected positions in Ub to incorporate pBpa, to purify monUb and polyUb chains, and selected known UBDs to test the efficacy of our technique, for future use to identify novel UBDs.

There are 16 different classes of UBD[67–69] and the ones of interest here are ubiquitin-associated domains (UBA)[61] and ubiquitin-interacting motifs (UIM)[69]. The UBDs selected were, ubiquitin associated domain (UBAy) of ubiquilin-1 (UQ1)[61], ubiquitin-associated two domain (UBA2) of human homolog of budding yeast RAD 23 (hHR23a)[61], and human receptor-associated protein (Rap 80) of tandem ubiquitin interacting motifs (tUIM)[70]. Ubiquilin-1 is a presenilin-interacting protein and involved in proteasomal degradation of cellular proteins.[71] Structurally, UQ1 has an N-terminal UBL and C-terminal UBA domain (UBAy).[66] Ubiquilin's UBAy domain is a 40-50

amino acid motif and was identified bioinformatically.[61] UBAy has little to no binding specificity for K48-Ub_4, K63-Ub_4, and mixed Ub_4 of K29/K6, meaning UBAy interacts equally well with them.[72] UBAy's binding affinity for monoUb is K_d= 27 μM.[72] Human homolog of budding yeast RAD 23 is involved in nucleotide excision repair and proteasome degradation of cellular proteins.[73–77] Structurally, hHR23a has an N-terminal UBL domain, one internal domain (hHR23a UBA1), and one C-terminal domain (hHR23a UBA2).[66] Similar to UBAy, UBA2 is a 40-50 amino acid motif and was identified bioinformatically.[61] UBA2 has a weak binding affinity, K_d= 400-500 μM[29,78], for monoUb, but has a greater binding affinity of K_d= 8 μM[79] for K48-linked diUb chains. Human receptor-associated protein 80 is involved in DNA damage response and has a tandem UIM of 18-21 amino acids.[80] Rap 80 has binding preferentiality for K63-linked diUb chains with a binding affinity of K_d= 22 μM[70]. Selection of these UBDs were based on their known interactions and binding affinities with monoUb, K48 and K63-linked polyUb chains.

As a means to understand the interaction of Ub with UBD, different residues were selected to incorporate pBpa. The binding surface that UBDs generally interact with is the Ile44 hydrophobic patch.[59,60,50] Therefore, the positions selected were within the Ile44 patch region and no further than 5-8 Å away from the binding region of the UBDs. It is imperative to check the efficacy of the Ub mutants with the known UBDs to confirm the incorporation of pBpa does not destroy naturally occurring interactions, pBpa is in a position of crosslinking to occur, and there is no intramolecular crosslinking. Upon validating, the effectiveness of incorporating pBpa into Ub and identifying the unique positions crosslinking occurs we foresee this molecular technique being used to help understand the Ub signaling pathways. Moreover, for identifying novel UBDs that interact with atypical linkages.

Chapter 2 focuses on capturing the transient interaction of diUb with a conjugating E2 enzyme. In collaboration with the Yin lab from Georgia State University, we are interested in understanding the role of E2 enzymes in the enzymatic cascade. One belief is E2 enzymes are responsible for the specific Ub chain formation.[81–84] Herein, we will discuss creating diUb probes that contain UAA lysine derivatives, N^{ε}-$_{L}$-Thiaprolyl-$_{L}$-lysine (ThzK) and N^{ε}-$_{L}$-Cysteinyl-$_{L}$-lysine (CysK). We rely on and explore the pyrrolysyl-tRNA synthetases for an efficient way of incorporating the lysine derivatives into Ub. These lysine derivatives were chosen because they have or react to have a thiol group that can interact with the catalytic cysteine of E2 enzyme to create a covalent interaction via a disulfide bond. The formation of the disulfide creates a stable interaction between the diUb and E2 complex, which is useful, for our ultimate goal of understanding the complex's mode of Ub signaling and chain formation through protein crystallization.

One ongoing project is discussed in **Appendix 1**. This project focuses on the nonenzymatic assembly of an atypical linkage, K6, with an affinity "handle" for immobilization. We plan to immobilize the polyUb chain, and expose it to cellular lysates, to identify potential cellular binding partners that are specific to the K6 linkage.

Chapter 2: Progress on Mapping Ubiquitin Signaling using Photo-crosslinking Mono and Di-ubiquitin Probes

2.1 Introduction

This chapter describes a molecular technique used to capture transient interactions between ubiquitin (Ub) and known ubiquitin binding domains (UBD) to understand the ubiquitin code. The chemical technology used was unnatural amino acid (UAA) mutagenesis, in which, an UAA and photo-crosslinker, *p*-Benzoyl-$_L$-phenylalanine (pBpa) was incorporated into ubiquitin at different positions. Incorporating pBpa into Ub relied on the suppressor tyrosyl tRNA ($tRNA^{tyr}$) and *Methannococcus jannaschii* tyrosyl-tRNA synthetase (MjTyrRS) pair.[50] Crosslinking experiments captured the transient interactions of Ub and its known UBDs: ubiquitin-associated (UBAy) domain of ubiquilin-1 (UQ1)[61], ubiquitin-associated 2 domain (UBA2) of human homolog of budding yeast Rad 23 (hHR23a)[61], and Rap 80 of human Rap 80 tandem ubiquitin interacting motifs (tUIM)[69]. Selection of these UBDs were based on their known binding preferentiality with monoUb, K48-linked and K63-linked dimers. The crosslinking experiments consisted of bacterial expressed monoubiquitin (monoUb) or enzymatically assembled K48-linked or K63-linked dimers (diUb) with UBDs. Our hope is to answer Ub biology's main

questions such as: i) What do these chains interact with? ii) How do these interactions mediate biological responses? Previous experiments performed through NMR verified monoUb and diUb chains interact with the aforementioned UBDs.[66,85] The binding affinities (K_d) of monoUb for UBAy, UBA2, and Rap 80 are 27 μM [72], 400-500 μM [29,78], and 520 μM[70], respectively. UBA2 favorably binds to K48-linked dimers (K_d= 8 μM)[79] and Rap 80 favorably binds to K63-linked dimers (K_d= 22 μM)[70]. In contrast to UBA2, Rap 80 has a nine-fold decrease in affinity for K48-linked dimers (K_d= 157 μM).[70] Here, the use of known UBDs helped with identifying the best positions to incorporate pBpa in ubiquitin to capture transient interactions, in expectation to be able to use this molecular technique to seize unknown binding partners.

2.1.1 Rationale for using photo-crosslinkers

One-way to answer the aforementioned questions was to develop and use a proteomic strategy to enrich and identify novel cellular Ub-binding proteins. Currently, bioinformatics[61], yeast two-hybrid[62,63], and affinity pull down technologies help identify UBDs[64,65]. Each of these systems have their advantages and drawbacks. Bioinformatics is convenient, gives accurate verifications, but it can be costly and time consuming. Yeast two-hybrid is efficient and scalable, but give high numbers of false positives. Affinity pull-down has an array of bait tag/affinity ligand systems to choose from and is scalable, but non-specific binding and false positives can occur. The use of a photo-crosslinker was our approach to enrich and identify novel cellular UBDs. Photo-crosslinkers are light sensitive unnatural amino acids that can capture transient interactions between two macromolecules when irradiated with non-damaging UV (365 nm) (**Figure 2.1**).[50] The formation of a covalent bond captures the transient interaction between Ub and UBDs. Our lab's previous experience in genetically encoding unnatural amino acids in Ub fortified the creation of Ub containing a photo-crosslinker.

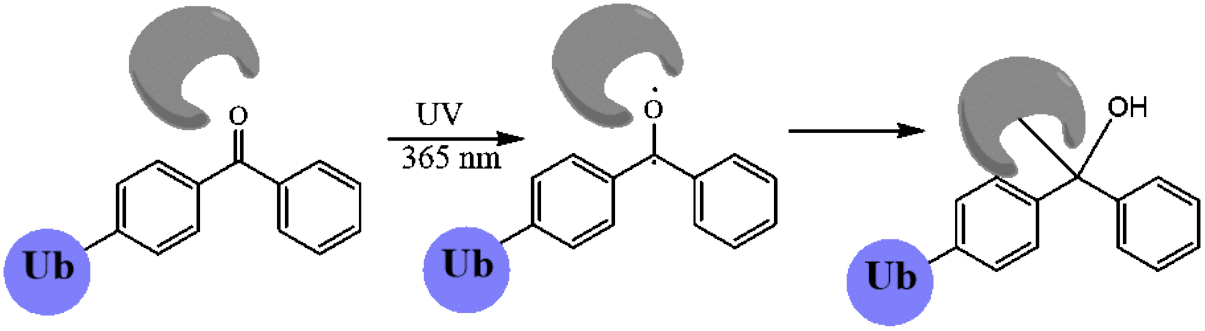


Figure 2.1. Depiction of photo-crosslinking with a benzophenone after irradiation with UV light.

2.1.2 Rationale for using p-benzoyl-$_L$-phenylalanine

There are different classes of photo-crosslinkers: diazirines[52,53], aryl azides[52,53], and benzophenones[51–54] (**Figure 2.2**). Diazirines are stable at room temperature and highly reactive, but synthesizing can be complex.[52] Aryl azides are easily synthesized and commercially available, but induce severe damage to biological systems (< 300 nm).[52] Damaging the naturally occurring interactions and structures of proteins. Of the different classes mentioned we chose to work with a benzophenone, *p*-benzoyl-L-phenylalanine (pBpa).[54] *p*-Benzoyl-$_L$-phenylalanine was selected because it is cost efficient, chemically stable, and ambient light tolerant (**Figure 2.2**).[56] Upon excitation, pBpa forms a diradical leading to an H-abstraction.[56] The H-donor can come from the C-H bond of an amino acid or the backbone of the polypeptide chain.[56] The reactivity order for the H-donation of C-H is : > NCH_x > $-SCH_x$ > methine > $C{=}CCH_2$ > $-CH_2$ > $-CH_3$.[56] Another advantage for using pBpa is its ability to be reversible if it cannot find C-H bonds. We developed our Ub moieties containing pBpa by genetically encoding pBpa at different locations in Ub, via the orthogonal $tRNA^{tyr}$/MjrTyrRS synthetase pair.[50] The desired residues of Ub were mutated to an amber stop codon, TAG, and base paired with the anti-codon of the $tRNA^{tyr}$.[50] PyMOL helped

us determine the residues of interest for the incorporation of pBpa by surveying the Ub and ubiquitin-associate (UBA) domain interaction of ubiquilin-1 (UQ1) (PDB: 2JY5) (**Figure 2.3**). Selected residues of Ub were determined based on their distance in angstroms, typically 5-8 Å, from the binding pockets of UBA. This angstrom range was chosen to ensure the transient interaction between Ub and the UBDs could be captured through the formation of a covalent bond. The residues selected were L8, T9, G10, K11, T12, S20, R42, A46, K48, Q49, E51, D52, H68, and V70. Residues, L8, H68, and V70, were of interest because they are located in Ub's hydrophobic pocket surface, Ile 44 patch, which is recognized by UBA and UBA2.[59,60,66] Residues, E51 and D52, were negative controls, in which we did not expect crosslinking to occur because they were outside of the binding regions of the UBDs.

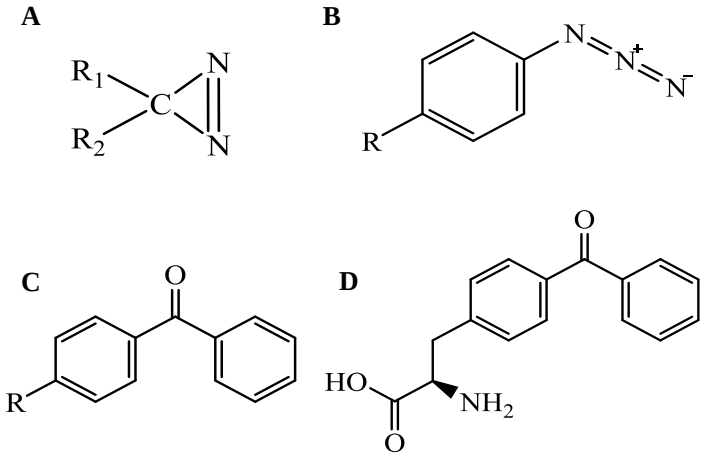


Figure 2.2. Chemical structures of the photo-crosslinkers. **A)** diazirine **B)** aryl azide **C)** benzophenone. **D)** Structure of unnatural amino acid, *p*-Benzoyl-L-phenylalanine (pBpa).

2.1.3 Rationale for selected ubiquitin binding domains

Ubiquitin binding domains are small modular domains of larger protein that interact with Ub non-covalently. As previously mentioned, the rationale for using a photo-crosslinker is to help capture the transient interaction of UBDs and Ub. To test the functionality of the Ub mutants containing pBpa, we wanted to perform photo-crosslinking experiments with the Ub mutants and known UBDs. This was important because we wanted to make sure mutations did not destroy native interaction, be too far for crosslinking to occur, or cause intramolecular crosslinking. In addition, it was important to make sure the E2 enzymes for the specific linkages still worked to create the linkage with the incorporation of pBpa. The UBDs selected were, ubiquitin associated domain (UBAy) of ubiquilin-1 (UQ1)[61], ubiquitin-associated two domain (UBA2) of human homolog of budding yeast RAD 23 (hHR23a)[61], and human receptor-associated protein (Rap 80) of tandem ubiquitin interacting motifs (tUIM)[70]. These three were chosen because of their well-studied interaction with monoUb, K48-linked, and K63-linked dimers. Can our mutants (monoUb, K48-linked, and K63-linked dimers) mimic the previously reported UBD interactions or interact differently?

2.2 Results and Discussion

2.2.1 Residue selection for the incorporation of pBpa

Our residue selection for the incorporation of pBpa was based on the interaction of Ub with UBDs. Selected residues of Ub were determined based on their distance in angstroms, typically 5-8 Å, from the binding pockets of UBA. PyMOL was used to determine the residues of interest for the incorporation of pBpa by surveying the Ub and ubiquitin-associate (UBA) domain interaction of ubiquilin-1 (UQ1) (PDB: 2JY5) (**Figure 2.3**). The residues selected were F4, T7, L8, T9, G10, K11, T12, S20, R42, A46, K48, Q49, E51, D52, H68, and V70. Residues, L8, H68,

and V70, were of interest because they are located in Ub's hydrophobic pocket surface, Ile 44 patch, which is recognized by UBA and UBA2.[59,60] Residues, E51 and D52, were negative controls, in which we did not expect crosslinking to occur because they were outside of the binding regions of the UBDs. In addition, it was important to select an array of residues in case the mutations destroyed interactions, were too far for cross-linking, and intramolecular crosslinking occurred.

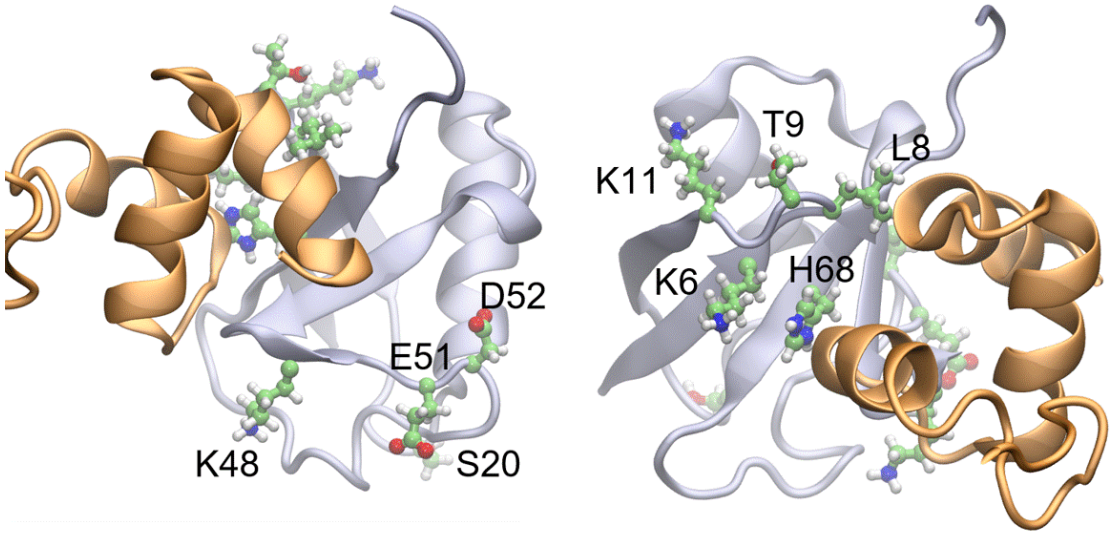


Figure 2.3. Interaction of Ub and ubiquitin-associate (UBA) domain of ubiquilin-1 (UQ1). Residue selection for the incorporation of pBpa. Residues selected were F4, T7, L8, T9, Gl0, K11, T12, S20, R42, A46, K48, Q49, E51, D52, H68, and V70. Negative controls: G51 and D52. Ub in silver and UBA in gold. (PDB: 2JY5).

2.2.1.1 Construction of ubiquitin pET11a-SynUb(AA)tag-His_6 vectors

Ubiquitin, pET11a-SynUb(AA)tag-His_6 vectors (AA=amino acid), were created by site-directed mutagenesis. The desired residues of Ub were mutated to an amber stop codon, TAG. DNA sequencing verified the site-directed mutagenesis of pET11a-SynUb(AA)tag-His_6 vectors: F4, T7,

L8, T9, G10, T12, S20, R42, A46, Q49, E51, D52, T66, H68, L69, and V70. Previously, created vectors used from our lab were pET11a-SynUbK11tag and pET11a-SynUbK48tag. Incorporation of pBpa was reliant on the pET11a-SynUb(AA)tag-His_6 vectors being co-transformed with pSup-pBpa-2. pSup-pBpa-2 contains the orthogonal $tRNA^{tyr}$/MjrTyrRS synthetase pair[50] to genetically incorporate pBpa at the TAG site. Small scale purifications were performed to identify pET11a-SynUb(AA)tag-His_6 vectors co-transformed with pSup-pBpa-2 capable to incorporate pBpa. Successful incorporation of pBpa was observed with co-transformation of pSup-pBpa-2 and pET11a-SynUb(AA)tag-His_6: L8, T9, G10, K11, T12, S20, R42, A46, K48, Q49, E51, D52, T66, H68, L69, and V70 (**Figure 2.4 and Appendix II**). After successful incorporation of pBpa, the mutants were purified by batch mode nickel purification. Successfully purified mutants were Ub8pBpa (.204/ 10 mL), Ub10pBpa, Ub11pBpa, Ub12pBpa (1.27 mg/ 10 mL), Ub20pBpa (.342 mg/ 10 mL), Ub42pBpa (.071 mg/ 10 mL), Ub46pBpa, Ub48pBpa (.576 mg/ 10 mL), Ub49pBpa (.373 mg/ 10 mL), Ub51pBpa (.541 mg/ 10 mL), Ub52pBpa (.074 mg/ 10mL), Ub68pBpa, and Ub70pBpa.

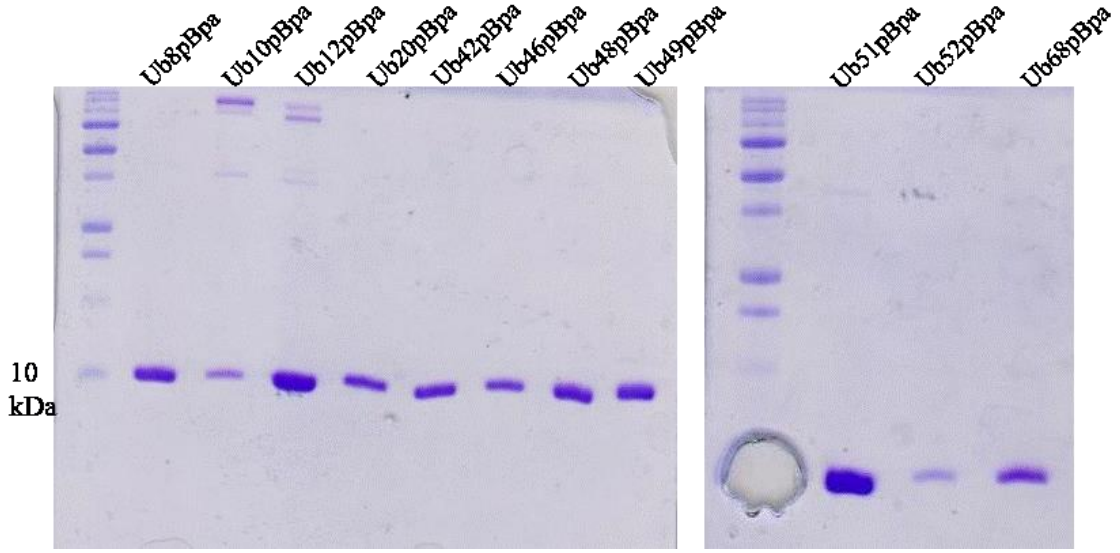


Figure 2.4. SDS-PAGE gel of UbpBpa-His_6 mutants. Other successfully purified mutants not shown were Ub9pBpa, Ub11pBpa, and Ub70pBpa. Expected size of the UbpBpa mutants are (Da): Ub8pBpa, 9525; Ub9pBpa, 9537; Ub10pBpa, 9581; Ub11pBpa, 9510; Ub12pBpa, 9537; Ub20pBpa, 9551, Ub42pBpa, 9482; Ub46Bpa, 9567; Ub48pBpa, 9510; Ub49Bpa, 9510; Ub51Bpa, 9509; Ub52pBpa, 9523; Ub68pBpa, 9501

2.2.2 Photo-crosslinking experiments with Ub and known UBD

Different ubiquitin moieties were created containing the UAA photo-crosslinker, pBpa. The next step was to analyze the Ub moieties' functionality when mixed in a reaction containing UBD and irradiated with UV light (365 nm). The UBDs selected were UBA2, UBAy, and Rap 80. These UBDs were selected due to their previously reported interactions with monoUb and K48-linked, and K63-linked dimers.[61,66,70] Ubiquitin-associated 2 domain (UBA2) has a specificity for K48-linked dimers. Ubiquitin associated domain (UBAy) has a structural specificity for K48, K63, and mixed K29/K6 tetramers.[72] In addition, UBAy has an interaction with monoUb, K_d= 27 μM.[72] Human receptor-associated protein 80 (Rap80) has structural specificity for K63-linked dimers.[70] Photo-crosslinking experiments were performed with monoUb, K48-linked, and K63-linked dimers to identify Ub moieties (or residue positions) that capture transient interactions with UBDs. Furthermore, there was an interest to see if the photo-crosslinking agrees with the expected binding preference or new binding preferences are observed.

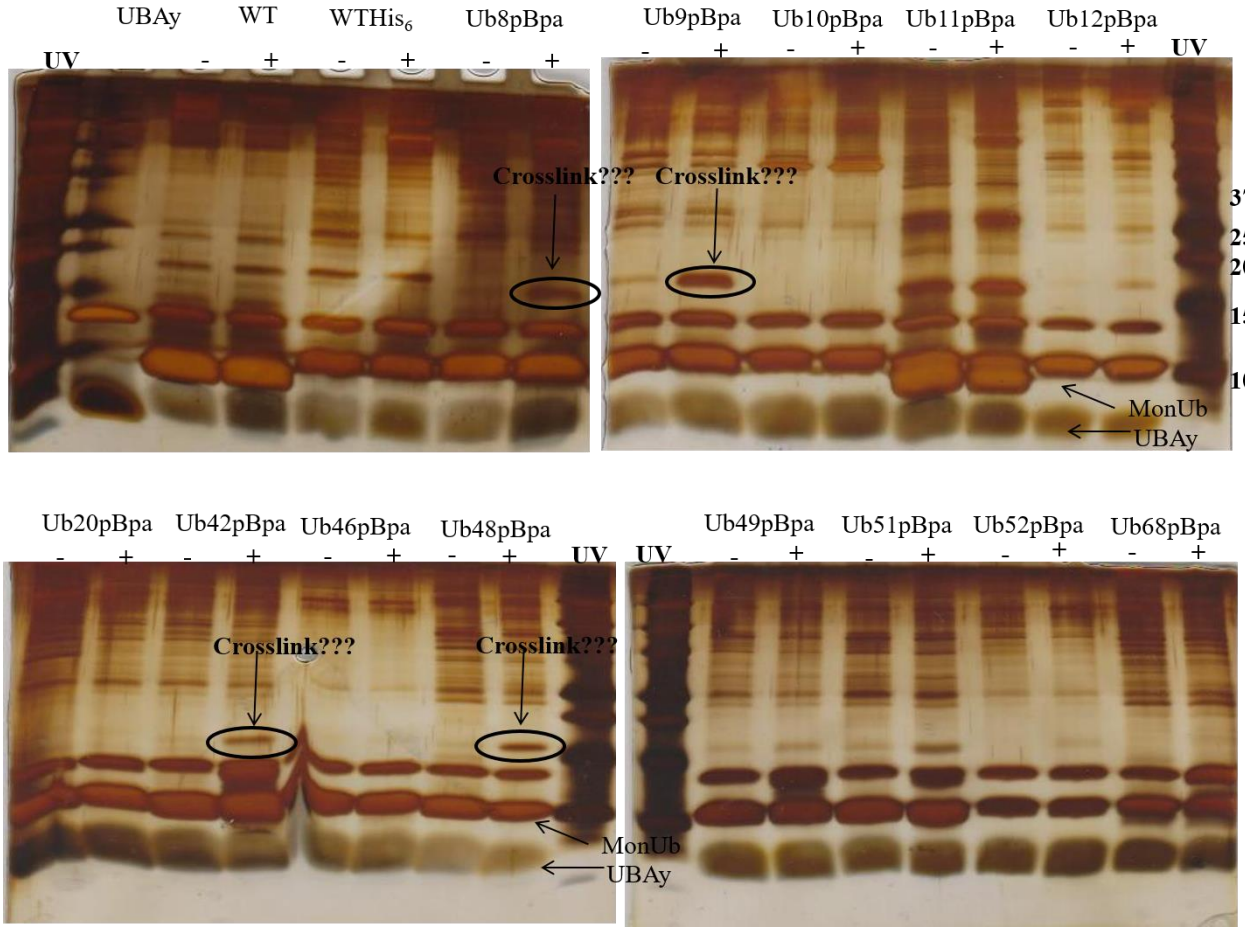


Figure 2.5. Silver stained SDS-PAGE of monoUb mutants crosslinked with UBAy. Potential crosslinking was observed for Ub8pBpa, Ub9pBpa, Ub42pBpa, andUb48pBpa. The expected crosslink is ~14,500-14,700 Da. Molecular weight (Da) of Ub moieties: WTUb,8564; WTUbHis, 9387;Ub8pBpa, 9525; Ub9pBpa, 9537; Ub10pBpa, 9581; Ub11pBpa, 9510; Ub12pBpa, 9537; Ub20pBpa, 9551, Ub42pBpa, 9482; Ub46Bpa, 9567; Ub48pBpa, 9510; Ub49Bpa, 9510; Ub51Bpa, 9509; Ub52pBpa, 9523; UbH68pBpa, 9501,UBAy, 5079.6.

2.2.2.1 MonoUb photo-crosslinking with UBAy and UBA2

Photo-crosslinking experiments were performed with the Ub moieties and UBAy. A 50 µL photo-crosslinking reaction consisted of 23 µM (10 µg) Ub mutant, 35 µM (10 µg) UBD, 1 X PBS, pH 7.4 in a 1.5 microcentrifuge tube. Half of the reaction is placed on a chilled 96 well plate and irradiated with UV light (365 nm) for 30 min. Reactions exposed and unexposed to UV light are ran on a protein gel for analyzing.

The initial set of Ub mutants tested with UBAy were: Ub8pBpa, Ub9pBpa, Ub11pBpa, Ub20pBpa, Ub48pBpa(no His_6), UbpBpa48(His_6), Ub51pBpa, Ub52pBpa in addition to WTUb(no His_6). The samples were analyzed on a Coomassie stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the expected band (~14,500-14,700 Da) cross-linked band was not observed. As previously mentioned, UBAy has an interaction with monoUb (K_d= 27 μM[72]), thus this observation seemed abnormal, leaving one to consider if crosslinking was not occurring or happening at a concentration undetectable on a Coomassie SDS-PAGE gel. The photo-crosslinking experiments were repeated and more Ub mutants were tested with UBAy. The additional mutants were Ub10pBpa, Ub12pBpa, Ub42pBpa, Ub46pBpa, Ub49pBpa, Ub68pBpa, and Ub70pBpa, in addition to WTUb(His_6). Differently, the reactions were analyzed on a silver stained SDS-PAGE gel because silver staining is 400x more sensitive than Coomassie (0.25 ng vs 100 ng). Potential crosslinked bands were observed for Ub8pBpa, Ub9pBpa, Ub42pBpa, and Ub48pBpa mutants (**Figure 2.5**). However, the crosslinking may be obscure because less than 1% is observed. Similarly, the aforementioned reaction set up and photo-crosslinking experiments were performed with UBA2 and the same mutants. Likewise, a crosslinked band was not observed on the Coomassie stained gel. Unlike, UBAy, observing no crosslinking is not abnormal because Ub has a low affinity for UBA2 (K_d= 400-500 μM[29]). In spite of UBA2's low affinity, it was still believed crosslinking may have occurred with the Ub mutants, but at a lower concentration below the detectable limits of a Coomassie stained SDS-PAGE gel. Crosslinking was observed for Ub8pBpa, Ub9pBpa, Ub11pBpa, Ub42pBpa, and Ub68pBpa (**Figure 2.6**). The monoUb crosslinking experiments laid the framework for identifying positons that crosslink to UBDs.

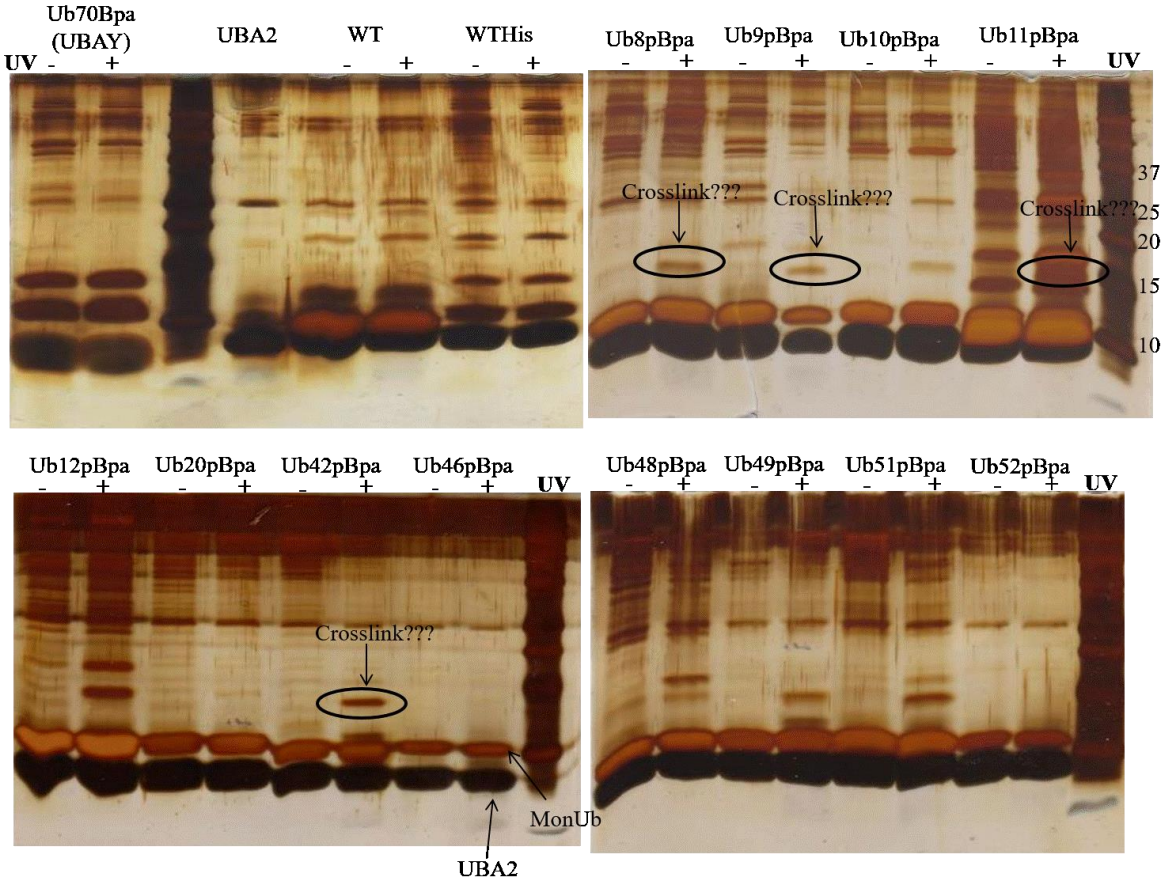


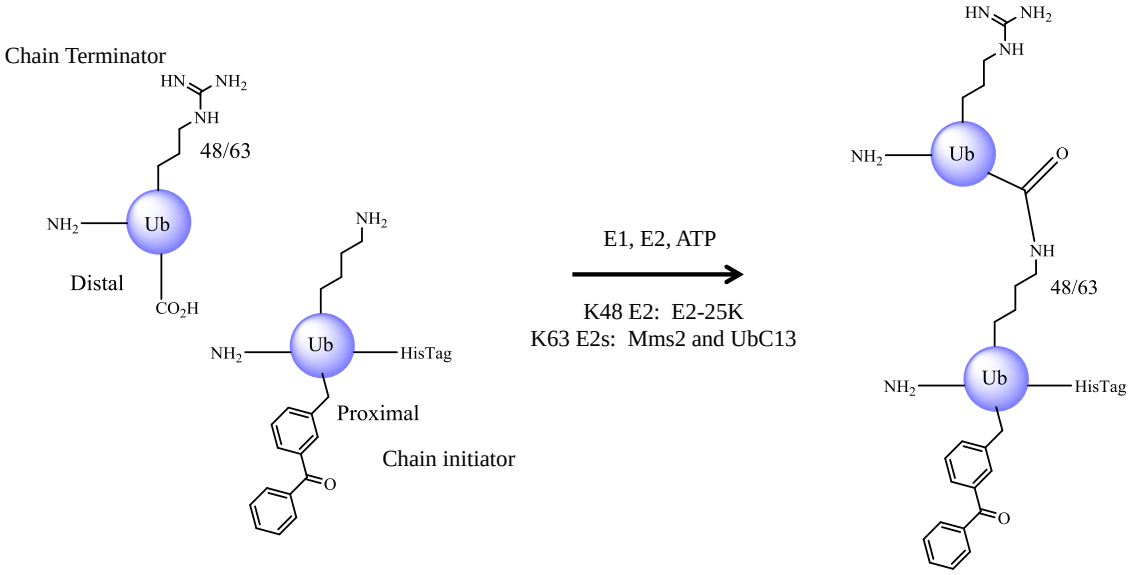
Figure 2.6. Silver stained SDS-PAGE of monoUb mutants crosslinked with UBAy and UBA2. Potential crosslinking was observed for Ub8pBpa, Ub9pBpa, Ub11pBpa, Ub42pBpa, andUb68pBpa (not shown). The expected crosslink is ~15,000-15,150 Da. Molecular weight (Da) of Ub moieties: WTUb,8564; WTUbHis, 9387;Ub8pBpa, 9525; Ub9pBpa, 9537; Ub10pBpa, 9581; Ub11pBpa, 9510; Ub12pBpa, 9537; Ub20pBpa, 9551, Ub42pBpa, 9482; Ub46Bpa, 9567; Ub48pBpa, 9510; Ub49Bpa, 9510; Ub51Bpa, 9509; Ub52pBpa, 9523; UbH68pBpa, 9501; UbV70pBpa, 9539; UBAy, 5079.6; UBA2, 5573.2.

2.2.2.2 DiUb photo-crosslinking with UBA2 and Rap 80

To expand upon our findings diUb chains of Ub9pBpa, Ub11pBpa, Ub46pBpa, Ub48pBpa, and Ub49pBpa mutants were made. Our collaborators at UMD enzymatically created two different dimer linkages, K48 and K63. Scheme 1 shows the Ub building blocks needed to create the Ub dimers. The building blocks are a proximal Ub containing pBpa (chain initiator), distal Ub containing an arginine mutation (chain terminator), and activating enzyme E1. The conjugating

enzymes used for the K48 linked and K63 linked dimer formation were, E2-25K-GST, and Ubc13-GST/Mms2-His_6, respectively. All three of these Ub building blocks needed to be purified. The experimental set up was a 50 µL photo-crosslinking reaction consisting of 15 µM Ub mutant, 60 µM UBD, 1 X PBS, pH 7.4 in a 1.5 microcentrifuge tube. Half of the reaction is placed on a chilled 96 well plate and irradiated with UV light (365 nm) for at least 30 min. Our positive control was a K48-linked dimer with no pBpa (**Figure 2.9**). We tested the ability of K48-linked-$Ub_2$9pBpa, $Ub_2$11pBpa, $Ub_2$46pBpa, and $Ub_2$49Bpa to crosslink with UBA2 and Rap 80. In addition, we tested the ability of K63-linked-$Ub_2$9pBpa, $Ub_2$11pBpa, $Ub_2$48pBpa, and $Ub_2$49pBpa with UBA2 and Rap 80.

Scheme 1: Enzymatic formation of K48 and K63 linked dimers



As previously stated, UBA2 has a weak binding affinity for monoUb, however, UBA2 has a greater binding affinity for K48-linked diUb chains (K_d = 8 µM)[79]. A previous report[59,60] stated the increase in the binding affinity for K48-linked diUb is due to an extended hydrophobic patch created by the dimer. Based on these reports, we expected to see crosslinking between UBA2 and the K48-linked diUb mutants. Crosslinking was observed for UBA2 and K48-linked $Ub_2$9pBpa

(**Figure 2.7**) and $Ub_2$49pBpa (**Figure 2.8B**). No crosslinking was shown for UBA2 with $Ub_2$46pBpa (**Figure 2.8B**). No crosslinking was observed for UBA2 K63-linked $Ub_2$9pBpa, $Ub_2$11pBpa, $Ub_2$48pBpa, and $Ub_2$49pBpa (**Figure 2.9B and Figure 2.9A**). These results were expected and support the established finding that UBA2 is selective for K48-linked polyUb chains.[79]

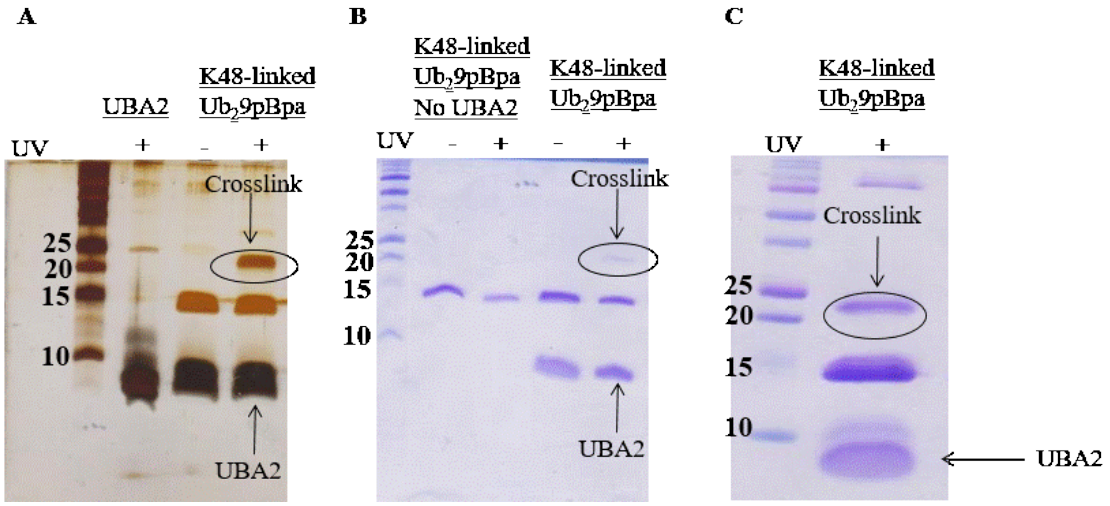


Figure 2.7. SDS-PAGE gels of K48 linked $Ub_2$9pBpa with UBA2 **A)** Silver stained gel showing crosslinking with $Ub_2$9pBpa and UBA2 (24,497 Da) ($Ub_2$9pBpa: 23 µM and UBA2: 36 µM). **B)** Coomassie stained gel showing a control $Ub_2$9pBpa with no UBA2 and $Ub_2$9pBpa crosslinking with UBA2. ($Ub_2$9pBpa: 23 µM and UBA2: 36 µM) **C**) Coomassie stained gel showing the crosslinking of $Ub_2$9pBpa with UBA2 (10X concentration of: $Ub_2$9pBp =15 µM and UBA2= 60 µM). Crosslinking was observed for $Ub_2$49pBpa with RAP80 (24,552 Da). Molecular weight (Da) of diUb: $Ub_2$9pBpa, 18,924. Molecular weight (Da) of UBA2 5573.2. Ladder is in kDa.

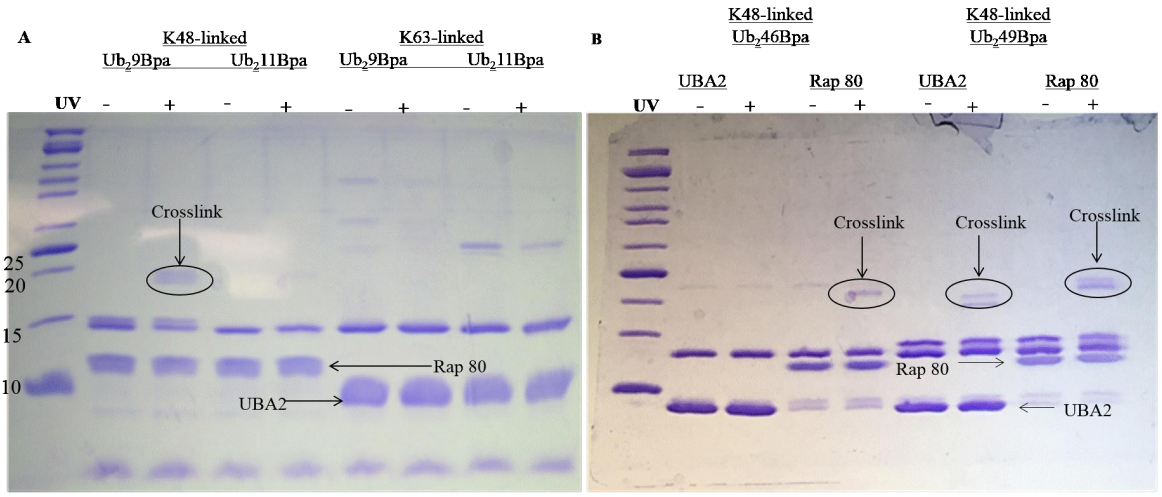


Figure 2.8. SDS-PAGE of K48 and K63 linked diUb with UBA2 or RAP80. **A)** Crosslinking was observed for K48 linked $Ub_2$9pBpa with RAP80 (24,579 Da). **B)** Crosslinking was observed for K48 linked $Ub_2$46pBpa and with $Ub_2$49pBpa with RAP80 (24,609; 24,552 Da, respectively). Crosslinking was observed for K48 linked $Ub_2$49pBpa with UBA2 (24,470 Da) Molecular weights (Da) of diUb mutants: $Ub_2$9pBpa, 18,924; $Ub_2$11pBpa, 18,897; $Ub_2$48pBpa, 18,897; $Ub_2$49pBpa, 18,897. Rap 80 and UBA2, 5655 and 5573.2, respectively. Ladder is in kDa

Rap 80 has binding preferentiality for K63-linked diUb chains with a binding affinity of K_d = 22 μM.[70] Photo-crosslinking experiments showed crosslinking with Rap 80 and K63-linked $Ub_2$9pBpa and $Ub_2$49pBpa (**Figure 2.9A and Figure 2.9B**). No crosslinking was observed with Rap 80 and K63-linked $Ub_2$11pBpa and $Ub_2$48pBpa (**Figure 2.9A and Figure 2.9B**). Good positions for capturing transient interactions are 9 and 49. Unexpected crosslinking happened between Rap80 and K48-linked $Ub_2$9pBpa, but did not occur with $Ub_2$11pBpa, further proving, position 9 is good for capturing transient interactions (**Figure 2.8A**). In addition, crosslinking occurred between Rap 80 and K48-linked $Ub_2$46pBpa and $Ub_2$49pBpa (**Figure 2.8B**). In consideration, Rap 80 has a lower binding affinity for K48-linked dimers (K_d =157 μM[70]), so possibility of crosslinking to occur should not be nullified. Both, $Ub_2$11pBpa and $Ub_2$48pBpa, did not crosslink with UBA2 and Rap 80, which can suggest lysine residue positions are not good

positions to incorporate pBpa for photo-crosslinking. Furthermore, K48-linked dimers crosslinking with Rap 80, a UBD atypical of interacting with K48 linked diUbs, shows incorporating a PCL in diUb chains may interact with UBDs not typical for its specific linkage. For both linkages, crosslinking occurred with pBpa in the proximal position of a dimer in positions 9, 46, and 49, suggesting that these positions may be a universal position for capturing transient interactions of UBDs and other linkages. The crosslinking data is summarized in **Table 1** and crosslinking positions are visualized in **Figure 2.10**.

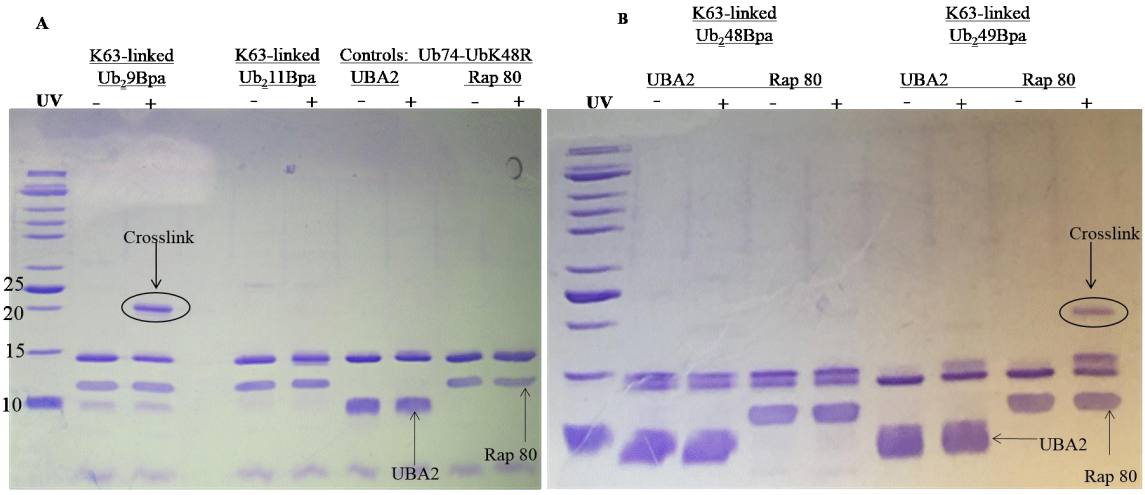


Figure 2.9. SDS-PAGE of K63 linked diUb with UBA2 or Rap 80. **A)** Crosslinking was observed for $Ub_2$9pBpa with Rap 80 (24,579 Da). **B)** Crosslinking was observed for $Ub_2$49pBpa with RAP80 (24,552 Da). Molecular weights (Da) of diUb: $Ub_2$9pBpa, 18,924; $Ub_2$11pBpa, 18,897; $Ub_2$48pBpa, 18,897; $Ub_2$49pBpa, 18,897. Rap 80 and UBA2, 5655 and 5573.2, respectively. Ladder is in kDa.

Table 1. Crosslinking summary of K48 and K63 linked dimers with UBA2 and Rap 80

	UBDs	
	UBA2	Rap 80
Control		
Ub74-Ub48R	–	–
K48-linked		
$Ub_2$9pBpa	+	+**
$Ub_2$11pBpa	–	–
$Ub_2$46pBpa	–	+**
$Ub_2$49pBpa	+	+**
K63-linked		
$Ub_2$9pBpa	–	+
$Ub_2$11pBpa	–	–
$Ub_2$48pBpa	–	–
$Ub_2$49pBpa	–	+

+ crosslinking, +** unexpected crosslinking
– no crosslinking

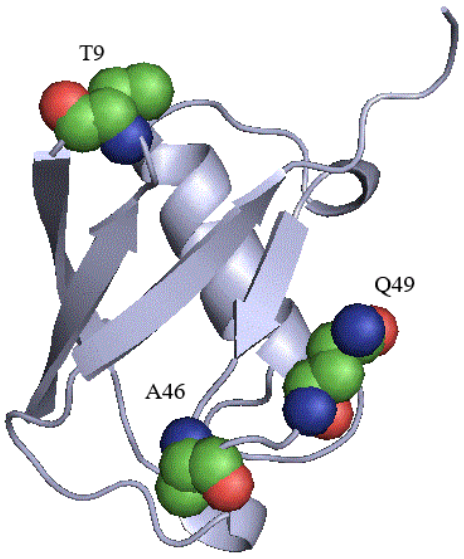


Figure 2.10. A space-filling structure of Ub showing the crosslinking positions.

2.2.2.3 LC MS/MS of crosslinked band of K48 linked $Ub_2$9pBpa/UBA2 and K63 linked $Ub_2$9pBpa/Rap 80

We wanted to identify the residue or the point of crosslinking between our Ub dimers and UBDs. Our school's chemical and proteomic masspectrometry facility performed a tryptic digest of our crosslinked bands for K63 linked $Ub_2$9pBpa/Rap 80 (**Figure 2.9A**) and K48 linked $Ub_2$9pBpa/UBA2 (**Figure 2.7C**). The digested samples were ran on the facility's Orbitrap Velos mass spectrometer. We were unsuccessful with identifying the point of crosslinking. However, we were able to verify by Scaffold that the peptides detected were that of Ub and Rap 80 for the K63 linked $Ub_2$9pBpa/Rap 80 band (**Appendix III**). Additionally, we were able to verify peptides for Ub for the K48 linked Ub $Ub_2$9pBpa/UBA2 band (**Appendix III**).

2.2.3 UbK27BocLys and Arginine mutation building blocks

In further exploration, our collaborators will make K48 and K63-linked diUb probes with pBpa in the distal position. To make the diUb probes the distal Ub needs to contain an Arg at the K48 or K63 position and pBpa at the desired crosslinking position. We were able to purify Arg mutants for Ub9pBpa with an Arg at the K48 (9 mg/L) and K63 (4 mg/L) position via a chitin purification (**Figure 2.11 and Appendix II**). In addition, we purified UbK27BocLys (86 mg/L) (**Figure 2.12**) to be used in future studies to make K27-linked diUb probes containing the photo-crosslinkers in the distal and proximal positions. These different diUb probes will help with understanding the Ub signaling of Ub with UBDs.

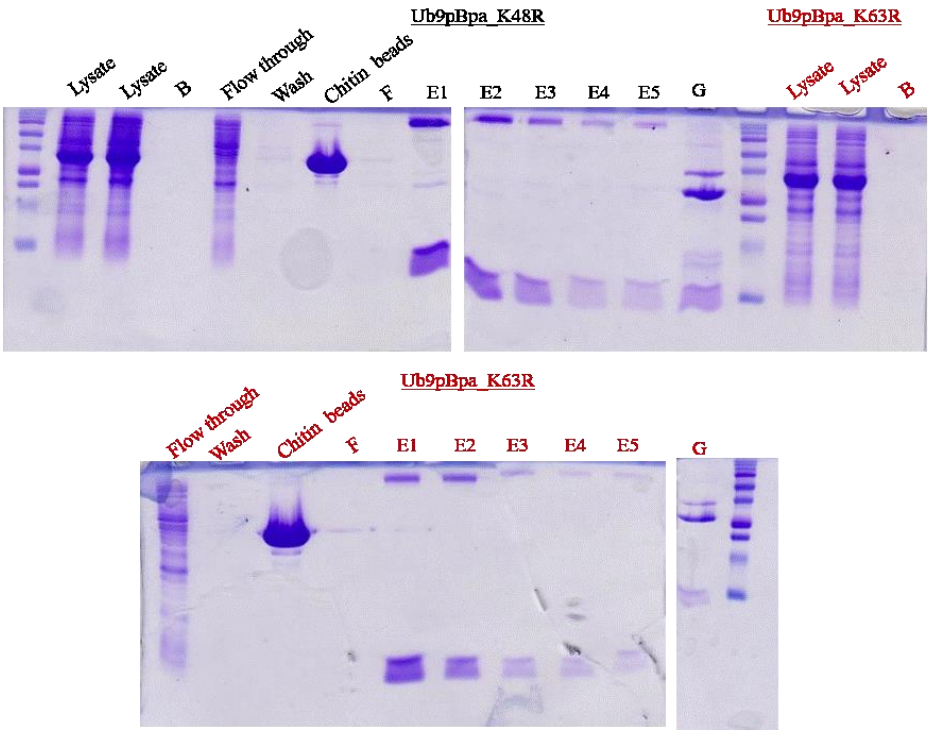


Figure 2.11. SDS-PAGE gels showing the chitin purification steps for Ub9pBpa Arg mutants. Expression is observed in the chitin bead lane and these beads contain the Ub-inteinCBD. Ub-inteinCBD size for Arg mutants is 36, 483 Da. The eluted protein for Arg mutants is 8742 Da. B= regenerated chitin beads, E=elution, and G= chitin beads after elution

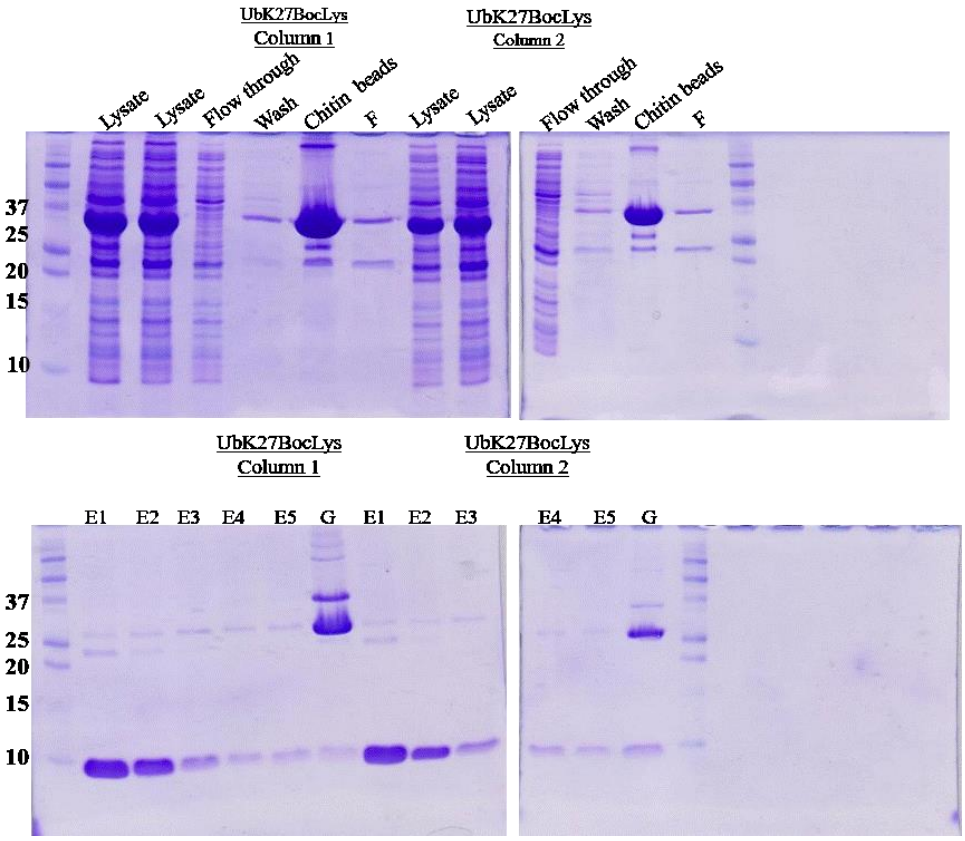


Figure 2.12. SDS-PAGE gels showing the chitin purification steps for UbK27BocLys. Expression is observed in the chitin bead lane and these beads contain the Ub-inteinCBD. Ub-inteinCBD size for UbK27BocLys is 36, 405 Da. The eluted protein for UbK27BocLys is 8664 Da. E=elution and G= chitin beads after elution

2.3 Conclusion and Future Application

We successfully identified different positions to incorporate pBpa that result in intermolecular crosslinks, specifically, with the proximal position in the dimer capturing the transient interactions. The proximal incorporation of pBpa in positions 9, 46, and 49 of diUb are positions that successfully crosslinked (**Figure 2.10**). Furthermore, photo-crosslinking of diUbs agree with the expected binding preference and captures low affinity interactions, which, can possibly be

controlled with irradiation time. In addition, known E1 and E2 enzymes can process our Ub mutants to yield diUbs.

Our current findings have sparked interest in continuing to understand Ub's cell signaling communication with UBDs. One approach is creating K48 and K63-linked dimers with pBpa in the distal position. Ubiquitin building blocks were created containing pBpa at the ninth position and an Arg mutation at K48 or K63 to begin the process of developing dimers with pBpa in the distal position. Similar experiments will be performed to validate the effectiveness of crosslinking. Furthermore, we can compare if the different location of pBpa is effective, less effective, or equal for capturing transient interactions. This same concept can be considered for creating dimers where pBpa incorporated at both the distal and proximal positions. Additionally, it is important to identify crosslinked residues(s) on the UBD/UB interface via LC/MS/MS. Identifying the point of crosslink for known UBDs can potentially help with identifying points of covalent attachment for unknown UBDs or help identify common trends of interaction with Ub and UBDs. The established and developing framework for our molecular technique sets the foundation for identifying UBDs of atypical linkages.

An atypical linkage of interest is K27. Innately, K27-linked polyUb chains are not well studied and it is due to the inability to create the chains in pure form. As previously mentioned, our lab has an advantage for studying K27-linked polyUb chains because we have the tools to create these chains.[31] We are interested in contributing to the biological understanding[86–88] of K27-linked polyUb chains and their role in the cell signaling pathway. One way is going beyond the scope of dimers and creating K27 linked tetramers to contain a photo-crosslinker. Creating a K27 linked tetramer creates a larger binding surface for UBDs, and incorporating the photo-crosslinker will help with capturing the tetramer's transient interaction. Instead of testing with known UBDs we

will go a step further by performing crosslinking experiments with HEK293-T and yeast cells. Depending on the success rate of the aforementioned cells, the cell line would be broadened to neuronal-specific or disease-state cells lines. Similarly, identification of the UBDs would be done by performing LC/MS/MS and MASCOT. By establishing our molecular technique to K27 polyUb chains, we hope it sets the stage to apply our technique to other atypical linkages and topologies, thus bringing forth more understanding to the ubiquitin code.

2.4 Materials and Methods

2.4.1 Site-directed mutagenesis for pET11-SynUb(AA)tag-His_6 vectors

2.4.1.1 PCR setup for pET11a-SynUbT9Tag, SynUbS20Tag, SynUbN25Tag, SynUbD52Tag, and SynUbT66Tag

The vectors pET11a- SynUbT9Tag, SynUbS20Tag, SynUbN25Tag, SynUbD52Tag, SynUbT66tag, and were created by site directed mutagenesis. The oligos used for pET11a-SynUbT9Tag, SynUbS20Tag, SynUbN25Tag, SynUbD52Tag, SynUbT66Tag were T9FWD/T9REV, S20FWD/S20REV, N25FWD/N25REV, D52FWD/D52REV, and T66FWD/T66REV, respectively (**Appendix IV**). The PCRs were performed using *Phusion* polymerase and the following PCR conditions for a 50 µL reaction were: initial duration at 98°C for 30 sec, 30 cycles of 98°C for 10 sec, annealing at 65°C for 20 sec, 72°C for 3 min, and a final extension at 72°C for 5 min.

2.4.1.2 PCR setup for pET11a-SynUbF4Tag, SynUbD32Tag, SynUbL69Tag

The vectors pET11a-SynUbF4Tag, SynUbD32Tag, and SynUbL69Tag were created by site directed mutagenesis. The oligos used for pET11a-SynUbF4TAG, SynUbD32TAG, and SynUbL69Tag were F4FWD(CL1747)/F4REV(CL1748), D32FWD(CL1761)/D32REV(CL1762), and L69FWD(CL1840)/L69REV(CL1841) (**Appendix**

IV). The PCRs were performed using *Phusion* polymerase and the following PCR conditions for a 50 µL reaction were : initial duration at 98°C for 30 sec, 30 cycles of 98°C for 10 sec, annealing at 60°C for 20 sec, 72°C for 3 min, and a final extension at 72°C for 5 min.

2.4.1.3 PCR setup for pET11a-SynUbL8Tag and pET11a-SynUbD52Tag

The vectors pET11a-SynUbL8Tag and pET11-SynUbD52Tag were created by site directed mutagenesis. The oligos used for pET11a-SynUbL8Tag and pET11a-SynUbD52Tag were L8FWD(CL1784)/L8REV(CL1785) and D52FWD(CL1788)/D52REV(CL1789), respectively (**Appendix III**). The PCRs were performed using *Phusion* polymerase. The gradient PCR conditions for a 50 µL reaction were: initial duration at 98°C for 30 sec, 30 cycles of 98°C for 10 sec, annealing at 72°C-50°C (72.0°C, 71.1°C, 69.6°C, 67.2°C, 64.5°C, 62.5°C, 60.9°C, and 60.0°C) for 20 sec, 72°C for 3 min, and a final extension at 72°C for 5 min.

2.4.1.4 PCR setup for pET11a-SynUbE51Tag

The vector pET11a-SynUbE51Tag was created by site directed mutagenesis. The oligos used for pET11a-SynUbE51Tag were E51FWD(CL1786)/E51REV(CL1787) (**Appendix IV**). The PCR was performed using *Phusion* polymerase and the following gradient PCR conditions for a 50 µL reaction was : initial duration at 98°C for 30 sec, 30 cycles of 98°C for 10 sec, annealing at 72.5°C-55.5°C (72.5°C, 71.2°C, 69.0°C, 65.5°C, 61.6°C, 58.4°C, 57.3°C, and 55.0°C) for 20 sec, 72°C for 3 min, and a final extension at 72°C for 5 min.

2.4.1.5 PCR setup for pET11a-SynUbH68Tag

The vector pET11a-SynUbH68Tag was created by site directed mutagenesis. The oligos used for pET11a-SynUb68Tag were H68FWD(CL1790)/H68REV(CL1791) (**Appendix IV**). The PCR was performed using *Phusion* polymerase and the following touchdown PCR conditions for a 50 µL reaction was: initial duration at 98°C for 30 sec, 6 cycles of 98°C for 10 sec, annealing at

72°C (-2 per cycle) for 30 sec, 72°C for 3 min, 20 cycles 98°C for 15 sec, annealing temperature not recorded, 72°C for 3 min., and final extension 72°C for 10min.

2.4.1.6 PCR setup for pET11a-SynUbG10TAG and pET11a-SynUbV70Tag

Vectors pET11a-SynUbG10 and pET11a-SynUbV70Tag were created by site directed mutagenesis. The oligos used for pET11a-SynUbG10Tag and pET11a-SynUBV70Tag were G10FWD (CL1828)/G10REV(CL1829) and V70FWD(CL1842)/V70REV(CL1843), respectively (**Appendix IV**). The PCR was performed using *Phusion* polymerase and the following touchdown PCR conditions for a 50 µL reaction was: initial duration at 98°C for 30 sec, 6 cycles of 98°C for 10 sec, annealing at 68°C (-2 per cycle) for 30 sec, 72°C for 3 min, 20 cycles 98°C for 15 sec, annealing at 65°C for 30 sec, 72°C for 3 min., and final extension 72°C for 10min.

2.4.1.7 PCR setup for pET11a-SynUbT7Tag and pET11a-SynUbR42Tag

Vectors pET11a-SynUbT7Tag and pET11a-SynUbR42Tag were created by site directed mutagenesis. The oligos used for pET11a-SynUbE51Tag and pET11a-SynUbR42Tag were T7FWD(CL1826)/T7REV(CL1827) and R42FWD(CL1832)/R42REV(CL1833) (**Appendix IV**). The PCR was performed using *Phusion* polymerase and the following gradient PCR conditions for a 50 µL reaction was: initial duration at 98°C for 30 sec, 30 cycles of 98°C for 10 sec, annealing at 65°C-55°C (65.0°C, 64.3°C, 63.0°C, 61.1°C, 58.8°C, 56.9°C, 55.7°C, and 55.0°C) for 20 sec, 72°C for 3 min, and a final extension at 72°C for 5 min.

2.4.1.8 PCR setup for pET11a-SynUbT12Tag, SynUbA46Tag, and SynUbQ49Tag

Vectors pET11a-SynUbT12Tag, SynUbA46Tag, and SynUbQ49Tag were created by site directed mutagenesis. The oligos used for pET11a-SynUbT12Tag, SynUBA46Tag, and SynUbQ49Tag were T12FWD(CL1830)/T12REV(CL1831), A46FWD(CL1836)/A46REV(CL1837), and Q49FWD(CL1838)/Q49REV(CL1839) respectively (**Appendix IV**). The PCR was performed

using *Phusion* polymerase and the following touchdown PCR conditions for a 50 µL reaction was: initial duration at 98°C for 30 sec, 6 cycles of 98°C for 15 sec, annealing at 65°C (-2 per cycle) for 30 sec, 72°C for 3 min, 20 cycles 98°C for 15 sec, annealing at 65°C for 30 sec, 72°C for 3 min., and final extension 72°C for 10min. Samples were heated before the polymerase was added.

2.4.1.9 Transformation and mini prep of pET11a mutant DNA

Gel electrophoresis and DNA sequencing verified pET11a vectors. Upon verification, the vectors were transformed. Vectors were transformed into chemically competent *E. coli* NEBturbo cells or electrocompetent *E. coli* genehogs and DH10B cell (cells used for the transformation were based on availability). Chemically competent transformations consisted of 1 µL of DNA added to 100 µL of cells, incubated on ice for 30 min, heat shocked for 90 sec, incubated on ice for 2 min, recovered with 1 mL of SOC or LB broth, incubated for 1 hr 37°C, and plated on LB agar plate (Amp 100). Electrocompetent transformations consisted of 1 µL of DNA added to 100 µL of cells, electroporated cells, recovered with 1 mL of SOC or LB broth, incubated for 1 hr 37°C, and plated on LB agar plate (Amp 100). DNA was extracted following the QIAprep Spin Miniprep Kit (Qiagen).

2.4.1.10 Expression plasmids pET11a-SynUbK11tag-His$_6$ and pSup-pBpa-2

Plasmid pET11a-SynUbK11tag[89], encoding a c-terminal his-tag and amber stop codon (TAG), at residue 11, was created by site directed mutagenesis. The pET11a-SynUbK11tag mutant was co-transformed with, pSup-pBpa-2[89], containing cellular machinery to incorporate, *p*-Benzoyl-$_L$-phenylalanine (pBpa), as a genetically encoded unnatural amino acid at the TAG codon into BL21 (DE3) *E. coli* cells. To 100 µL of thawed BL21 (DE3) *E. coli* cells, 1 µL of each plasmid was added. The cells were electroporated, recovered with 1 mL SOC, and incubated for 1 hr at 37ºC. Once the incubation was completed, the cells were plated on a LB agar (Amp 100/ Chl 35) plate

and incubated overnight at 37ºC. (general experimental protocol used for co-expression of pET11a vectors with pSup-pBpa)

2.4.1.11 Protein expression and purification of UbK11pBpa-His_6

Plasmid pET11-SynUbK11tag was co-transformed with pSup-BocLys into EC BL21 (DE3) *E. coli* cells. A 5 mL overnight starter culture of LB broth (Amp 100Chl 35) was inoculated with a single colony from the transformed plate. A 100 mL flask of LB broth (Amp 100/Chl 35) was inoculated with 1. 5mL of starter culture. The cells grew to an $OD600_{nm}$=0.6, induced with 1 mM of IPTG, 1 mM pBpa, and incubated overnight at 37ºC. The expression was harvested at 5, 000 rpm for 10 min at 4ºC. The cells were resuspended in 2.5 mL of binding buffer (50 mM NaH_2PO_4, pH 8.00, 300 mM NaCl, 10 mM imidazole, and 1 mM PMSF). The cells were lysed by lysozyme and incubated on ice for 30 min. The cells were sonicated on ice for 10 sec six times with 10 sec resting periods. The cells were centrifuged at 10,000 g for 20 min at 4ºC to obtain the soluble protein. Then a batch mode nickel purification was performed. The cellular lysate was added to 200 µL of washed (50 mM NaH_2PO_4, pH 8.00, 300 mM NaCl, and 10 mM imidazole) Promega HisLink resin (Promega, Madison, WI) and incubated 1 hr on ice shaking. The resin was washed four times with ~6 mL of bind buffer and then eluted two times with 500 µL of elution buffer (50 mM NaH_2PO_4, pH 8.00, 300 mM NaCl, and 500mM imidazole). (general procedure used for the purification of UbpBpa mutants).

2.4.1.12 Expression plasmid pET11a-SynUbWT

Plasmid pET11a-SynUbWT[89] ,encoding WTUb with a c-terminal his-tag, was transformed into EC BL21 (DE3) *E. coli* cells. To 100 µL of thawed EC BL21 (DE3) cells, 1 µL of plasmid was added. The cells were electroporated and recovered in 1 mL of SOC. The cells were incubated at

37ºC for 1 hr. About 100 µL of cells were plated on a LB agar (Amp 100) plate and incubate overnight at 37ºC.

2.4.1.13 Protein expression and purification of UbWT-His_6

Plasmid pET11a-SynUbWT was transformed into EC BL21 (DE3) *E. coli* cells. A 5 mL overnight starter culture of LB broth (Amp 100) was inoculated with a single colony from the transformed plate. A 100 mL flask of LB broth (Amp 100) was inoculated with 1. 5mL of starter culture. The cells grew to an $OD600_{nm}$=0.6, induced with 1 mM of IPTG, and incubated overnight at 37ºC. The expression was harvested at 5, 000 rpm for 10 min at 4ºC. The cells were resuspended in 2.5 mL of binding buffer (50 mM NaH_2PO_4, pH 8.00, 300 mM NaCl, 10 mM imidazole, and 1 mM PMSF). The cells were lysed by lysozyme and incubated on ice for 30 min. The cells were sonicated on ice for 10 sec six times with 10 sec resting periods.. The cells were centrifuged at 10,000 g for 20 min at 4ºC to obtain the soluble protein. Then a batch mode nickel purification was performed. The cellular lysate was added to 200 µL of washed (50 mM NaH_2PO_4, pH 8.00, 300 mM NaCl, and 10mM imidazole) Promega HisLink resin (Promega, Madison, WI) and incubated 1 hr on ice shaking. The resin was washed four times with ~13 mL of bind buffer and then eluted five times with 200 µL of elution buffer (50 mM NaH_2PO_4, pH 8.00, 300 mM NaCl, and 500mM imidazole).

2.4.2 Non-tagged WTUb and Ub48pBpa

2.4.2.1 Expression plasmid pET3a-SynUbWT

Plasmid pET3a-SynUbWT[89], encoding WTUb, was transformed into BL21 (DE3) *E. coli* cells. To 100 µL of thawed BL21 (DE3) cells, 1 µL of plasmid was added. The cells were electroporated and recovered in 1 mL of SOC. The cells were incubated at 37ºC for 1 hr. About 100 µL of cells were plated on a LB agar (Amp 100) plate and incubate overnight at 37ºC.

2.4.2.2 Expression and purification of UbWT

Plasmid pET3a-SynUbWT was transformed into BL21 (DE3) *E. coli* cells. A 1L flask of LB broth (Amp 100/Chl 35) was inoculated with an overnight culture. The cells grew to an $OD600_{nm}$=0.6, induced with 1 mM of IPTG, and incubated over night at 37°C. The cells were harvested at 5,000 rpm resuspended in 25 mL of lysis buffer (50 mM tris buffer, pH 7.6, 0.02% triton X-100 (v/v), 0.4 mg/mL lysozyme, and 1 mM PMSF). Cells were sonicated on ice 3 times for 2.5 min with 2.5 min rest. The cells were harvested at 12,100 rpm for 25 min and the lysate was transferred to a clean 50 mL conical tube. Seventy percent perchloric acid was added dropwise to the supernatant while stirring. After a white precipitate formed, the protein was centrifuged at 12, 100 rpm for 25 min. The protein was dialyzed (3500 MWCO) in 2 L 50 mM NH_4Ac, pH 4.5. Protein was verified by SDS-PAGE gel.

2.4.2.3 Expression plasmids pET3a-SynUbK48tag and pSup-pBpa-2

Plasmid pET3a-SynUbK48tag[89]

, encoding an amber stop codon (TAG), at residue 48, was created by site directed mutagenesis. The pET3a-SynUbK48tag mutant was co-transformed with, pSup-pBpa-2, containing cellular machinery to incorporate, *p*-Benzoyl-$_L$-phenylalanine (pBpa), as a genetically encoded unnatural amino acid at the TAG codon into EC BL21 (DE3) *E. coli* cells. To 100 µL of thawed EC BL21 (DE3) *E. coli* cells, 1 µL of each plasmid was added. The cells were electroporated, recovered with 1 mL SOC, and incubated for 1 hr at 37ºC. Once the incubation was completed, the cells were plated on a LB agar (Amp 100/ Chl 35) plate and incubated overnight at 37ºC.

2.4.2.4 Expression and purification of UbK48Bpa

Plasmids pET3a-SynUbK48tag and pSUP-pBpa-2 were double transformed into transformed into EC BL21 (DE3) *E. coli* cells. A 1L flask of LB broth (Amp 100/Chl 35) was inoculated with an overnight culture. The cells grew to an $OD600_{nm}$=0.6, induced with 1 mM of IPTG, 1 mM of pBpa, and incubated over night at 37°C. The cells were harvested at 5,000 rpm resuspended in 25 mL of lysis buffer (50 mM tris buffer, pH 7.6, 0.02% triton X-100 (v/v), 0.4 mg/mL lysozyme, and 1 mM PMSF). Cells were sonicated on ice 3 times for 2.5 min with 2.5 min rest. The cells were harvested at 12,100 rpm for 25 min and the lysate was transferred to a clean 50 mL conical tube. Seventy percent perchloric acid was added dropwise to the supernatant while stirring. After a white precipitate formed, the protein was centrifuged at 12, 100 rpm for 25 min. The protein was dialyzed (3500 MWCO) in 2 L 50 mM NH_4Ac, pH 4.5.

2.4.2.5 Ion exchange of UbK48pBpa

The UbK48pBpa was further purified using an FPLC ion exchange column. The protein (25 mL) was added directly to a HiTrap SPFF cation exchange column. The column was washed (50 mM NH_4Ac, pH 4.5) and eluted by a 70% gradient (50 mM NH_4Ac, pH 4.5, 1 M NaCl). Collected protein was verified via SDS-PAGE gel and MS.

2.4.3 Purification of UBA2 and UBAy

2.4.3.1 Expression plasmid pGex-hHR23a-UBA2

Plasmid pGex-hHR23a-UBA2, encoding UBA2, was transformed into EC BL21 (DE3) *E. coli* cells. To 100 µL of thawed EC BL21 (DE3) cells, 1 µL of plasmid was added. The cells were electroporated and recovered in 1 mL of SOC. The cells were incubated at 37ºC for 1 hr. About 85 µL of cells were plated on a LB agar (Amp 100) plate and incubate overnight at 37ºC.

2.4.3.2 Expression plasmid pGex-UQ1-UBAy

Plasmid pGex-UQ1-UBAy, encoding UBAy, was transformed into EC BL21 (DE3) *E. coli* cells. To 100 µL of thawed EC BL21 (DE3) cells, 1 µL of plasmid was added. The cells were electroporated and recovered in 1 mL of SOC. The cells were incubated at 37ºC for 1 hr. About 85 µL of cells were plated on a LB agar (Amp 100) plate and incubate overnight at 37ºC.

2.4.3.3 Protein expression and purification of pGex-hHR23a-UBA2

Plasmid pGex-hHR23a-UBA2 was transformed into EC BL21 (DE3) *E. coli* cells. An overnight starter culture of LB broth (Amp 100) was inoculated with a single colony from the transformed plate. A 1 L flask of LB broth (Amp 100) was inoculated with starter culture. The cells grew to an $OD600_{nm}$=0.6, induced with 1 mM of IPTG, and incubated overnight at 37ºC. The expression was harvested at 5, 000 rpm for 10 min at 4ºC. The cells were resuspended in 25 mL of lysis buffer (1X PBS, pH 7.4, 0.02% Triton X-100, 0.4 mg/mL lysozyme, and protease inhibitor). The cells were sonicated on ice for 3 times for 2.5 min with 2.5 min rest in between. The cells were centrifuged at 20,000 rpm for 25 min to obtain the soluble protein. Protein purification was performed on the FPLC using a glutathione S-transferase (GST)-column (10 mL) (Pierce glutathione agarose). The column was equilibrated with 3-5 column volumes of 1 X PBS, pH 7.4. After equilibration, the lysate was loaded onto the column 1 mL/min. The column was washed with 3-5 column volumes of 1 X PBS, pH 7.4 to remove unbound protein. The protein was eluted with fresh 10 mM glutathione in 50 mM Tris, pH 8. The collected protein was dialyzed overnight using 3,500 MWCO dialysis tubing. Then the protein was concentrated using a 3,000 MWCO Vivaspin 20 concentrator. To cleave off the GST-tag 50 U of thrombin was added to the concentrated protein and incubated at room temperature for 5 hours. Size-exclusion chromatography was performed to separate the UBA2 from the GST tag (20 mM sodium

phosphate, pH 6.8, 130 mM NaCl, and 3 mM DTT). FPLC GST-column methods: fraction collect all (5 mL), 0-90 min (1 mL/min) Buffer A (1 X PBS, pH 7.4), 90-180 min (1 mL/min) Buffer B (10 mM glutathione in 50 mM Tris, pH 8), 180 min-210 Buffer A. FPLC SEC methods: fraction collect all (4.5 mL), 0-700 min (0.3 mL/min) Buffer A (20 mM sodium phosphate. pH 6.8, 130 mM NaCl, and 3 mM DTT). Thrombin reactions and UBA2 was verified by SDS-PAGE. (general purification used for the purification of pGEX-UQ1-Ubay).

2.4.4 Ub building blocks for distal pBpa dimers

2.4.4.1 Expression plasmids pTXB1-SynUbT9Tag and pSup-pBpa-2

Plasmid pTXB1-SynUbT9Tag[89], encoding a tagged chitin binding domain (CBD) Ub and amber stop codon (TAG), at residue 9, was created by site directed mutagenesis. The pTXB1-SynUbT9Tag mutant was co-transformed with, pSup-pBpa-2, containing cellular machinery to incorporate, *p*-Benzoyl-$_L$-phenylalanine (pBpa), as a genetically encoded unnatural amino acid at the TAG codon into BL21 (DE3) *E. coli* cells. To 100 µL of thawed BL21 (DE3) *E. coli* cells, 1 µL of each plasmid was added. The cells were electroporated, recovered with 1 mL SOC, and incubated for 1 hr at 37ºC. Once the incubation was completed, the cells were plated on a LB agar (Amp 100/ Chl 35) plate and incubated overnight at 37ºC.

2.4.4.2 PCR setup for pTXB1-SynUbT9Tag_K48R and pTXB1-SynUbT9_K63R

Vectors pTXB1-SynUbT9TAG_K48R and pTXB1-SynUbT9_K63R were created by site directed mutagenesis. The oligos used for pTXB1-SynUbT9Tag_K48R and SynUbT9Tag_K63R were K48R3 FWD(CL2109)/K48R3 REV(CL2110) and K63R FWD(CL1982)/K63R REV(CL1983), respectively (**Appendix IV**). The PCR was performed using *Phusion* polymerase and the following touchdown PCR conditions for a 20 µL reaction was: initial duration at 98°C for 1 min, 10 cycles of 98°C for 30 sec, annealing at 72°C (-1 per cycle) for 45 sec, 72°C for 3:30 min, 20

cycles 98°C for 15 sec, annealing at 62°C for 45 sec, 72°C for 3:30 min., and final extension 72°C for 5 min. Samples were heated before the polymerase was added.

2.4.4.3 Transformation and mini prep of pTXB1-SynUbT9Tag_K48R and SynUbT9Tag_K63R

Gel electrophoresis and DNA sequencing verified pTXB1-SynUbT9Tag_K48R and SynUbT9Tag_K63R vectors. Upon verification, the vectors were transformed into EC DH10-β *E. coli* cells. Electrocompetent transformations consisted of 2 µL of DNA added to 50 µL of cells, electroporated cells, recovered with 500 µL of LB broth, incubated for 1 hr 37°C, and plated on LB agar plate (Amp 100). DNA was extracted following the QIAprep Spin Miniprep Kit (Qiagen).

2.4.4.4 Protein expression and purification of UbT9pBpa

Plasmid pTXB1-SynUbT9tag was co-transformed with pSup-pBpa-2 into BL21 (DE3) *E. coli* cells. A single colony was picked from its respective plate to grow a 10 mL overnight starter culture in LB media (Amp 100/Chl 35). Four 1 L flasks of LB media (Amp 100/ Chl 35) were inoculated with 2 mL of starter culture. The cells grew to an $OD600_{nm}$= 0.6, induced with 1 mM of IPTG, inoculated with 1 mM pBpa, and then grown at 37° C overnight. Cells were harvested at 4,000 rpm for 10 min and then resuspended in 60 mL of lysis buffer (20 mM Hepes, pH 8.5, 0.5 M NaCl, 1 mM EDTA, 0.02% NaN_3, 100 mM PMSF). The cells were freeze/thawed three times with liquid nitrogen, sonicated on ice for a 5 min pulse, and cooled down on ice. The cells were centrifuged at 12,000 rpm for 20 min to isolate the soluble proteins. The lysate was added to an equilibrated chitin bead column (2L per column). The binding flow through was added two the column two additional times. The chitin beads were washed with 600 mL of wash buffer (20 mM Hepes, pH 8.5, 0.5 M NaCl, 1 mM EDTA, 0.02% NaN_3). On column cleavage was induced by flushing the

beads with 100 mL of elution buffer (20 mM Hepes, pH 8.5, 0.5 M NaCl, 1 mM EDTA, 0.02% NaN_3, and 50 mM DTT). The column was incubated at 37°C for 48 hrs. Protein was eluted with 100 mL of fresh elution buffer and collected in 20 mL fractions (5 fractions). After the initial elution, the column was incubated at 37°C for an additional cleavage of protein. Collected fractions were dialyzed in water using dialysis tubing with a MWCO of 3,500. (Purification set up for Ub9Bpa_K48R and Ub9Bpa_K63R)

2.4.5 Intein CBD UbK27pBpa

2.4.5.1 Expression plasmids pTXB1-SynUbK27Tag and pSup-pBpa-2

Plasmid pTXB1-SynUbK27Tag[89], encoding a tagged chitin binding domain (CBD) Ub and amber stop codon (TAG), at residue 27, was created by site directed mutagenesis. The pTXB1-SynUbK27Tag mutant was co-transformed with, pSup-pBpa-2, containing cellular machinery to incorporate, 1 mM *p*-Benzoyl-$_L$-phenylalanine (pBpa), as a genetically encoded unnatural amino acid at the TAG codon into BL21 (DE3) *E. coli* cells. To 100 µL of thawed BL21 (DE3) *E. coli* cells, 1 µL of each plasmid was added. The cells were electroporated, recovered with 1 mL SOC, and incubated for 1 hr at 37ºC. Once the incubation was completed, the cells were plated on a LB agar (Amp 100/ Chl 35) plate and incubated overnight at 37ºC.

2.4.5.2 Protein expression and purification of UbK27pBpa

Plasmid pTXB1-SynUbK27tag was co-transformed with pSup-pBpa-2 into BL21 (DE3) *E. coli* cells. A single colony was picked from its respective plate to grow a 10 mL overnight starter culture in LB media (Amp 100/Chl 35). Four 1 L flasks of LB media (Amp 100/ Chl 35) were inoculated with 2 mL of starter culture. The cells grew to an $OD600_{nm}$= 0.6, induced with 1 mM of IPTG, inoculated with 1 mM pBpa, and then grown at 37° C overnight. Cells were harvested at 4,000 rpm for 10 min and then resuspended in 60 mL of lysis buffer (20 mM Hepes, pH 8.5, 0.5 M NaCl,

1 mM EDTA, 0.02% NaN_3, 100 mM PMSF). The cells were freeze/thawed three times with liquid nitrogen, sonicated on ice for a 5 min pulse, and cooled down on ice. The cells were centrifuged at 12,000 rpm for 20 min to isolate the soluble proteins. The lysate was added to an equilibrated chitin bead column (2L per column). The binding flow through was added two the column two additional times. The chitin beads were washed with 600 mL of wash buffer (20 mM Hepes, pH 8.5, 0.5 M NaCl, 1 mM EDTA, 0.02% NaN_3). On column cleavage was induced by flushing the beads with 100 mL of elution buffer (20 mM Hepes, pH 8.5, 0.5 M NaCl, 1 mM EDTA, 0.02% NaN_3, and 50 mM DTT). The column was incubated at 37°C for 48 hrs. Protein was eluted with 100 mL of fresh elution buffer and collected in 20 mL fractions (5 fractions). After the initial elution, the column was incubated at 37°C for an additional cleavage of protein. Collected fractions were dialyzed in water using dialysis tubing with a MWCO of 3,500.

Chapter 3: Increasing the Efficiency of Incorporating ThzK and CysK into Ubiquitin

3.1 Introduction

This chapter focuses on our collaborative work with Yin's lab from Georgia State University to develop technology to covalently capture E2 enzymes with diUbs. As previously mentioned, ubiquitination occurs in a concerted three step enzymatic cascade, consisting of an activating enzyme (E1), conjugating enzyme (E2), and ligating enzyme (E3).[6,90] Furthermore, Ub contains seven lysine residues and a methionine group that contribute to Ub's ubiquitination variability, and its variability can be observed in chain length, topology and linkage type.[9,10] Previous research suggests Ub chain formation occurs at step two of the enzymatic cascade by the conjugating enzyme.[82–84,91] This can be validated by the different linkage specific enzymes E2-25K[32], Ubc13/Mms2[33,34], and UBE2s[35], for K48, K63, and K11 linkages, respectively. Even though insight has been established in understanding Ub chain formation, there is more to be understood. Therefore, our collaborators and we are interested in creating a crystal structure that shows the interaction of diUb conjugated to the catalytic cysteine residue of an E2 enzyme to understand the catalytic mechanism of E2 enzyme. To crystalize the interaction of diUb with E2 enzyme, diUb probes need to be created to contain a functional group with a thiol to form a disulfide bond with the catalytic cysteine of E2 (**Figure 3.1A**). The disulfide bond between diUb and E2 enzyme will generate a stable complex that can be crystalized. Creating the diUb relies

on creating an Ub building block that, contain an UAA, ThzK or CysK. Collaboratively, we will use our UAA expertise to establish a system to efficiently incorporate and express ThzK and CysK into Ub.

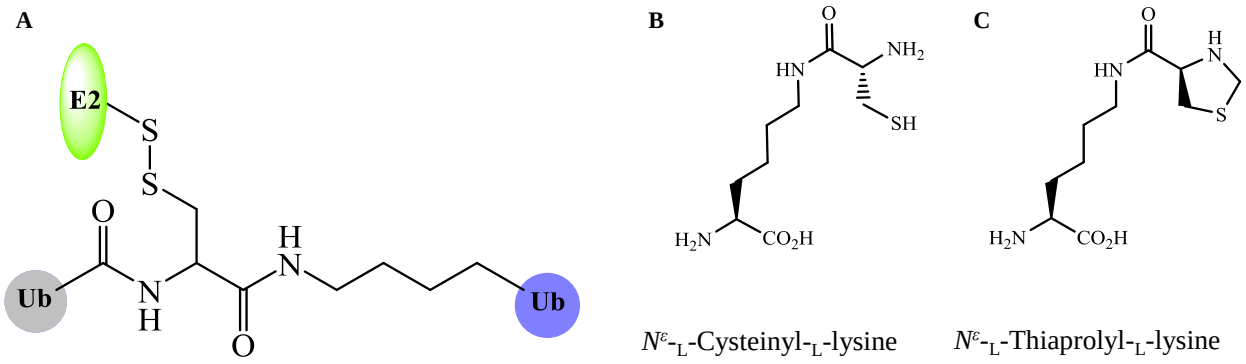


Figure 3.1. Structures for the diUb-E2 complex. **A)** Disulfide bond formation between E2 enzyme and diUb probe. **B)** N^{ε}-$_L$-Cysteinyl-$_L$-lysine an unnatural amino to be incorporated into Ub. **C)** N^{ε}-$_L$-Thiaprolyl-$_L$-lysine an unnatural amino acid to be incorporated into Ub.

3.1.1 Rational for enhancing UbK11ThzK and UbK11CysK expression

Previously, known pyrrolysyl-tRNA synthetases (PylRS) from the *Methanosarcina* species were used to incorporate synthesized UAAs, ThzK and CysK into Ub. One system was the use of engineered tRNA synthetase variants of *Methanosarcina barkeri (Mb)* PylRS established by the Chin lab.[92] The PylRS variants used were thiazolidinyl-lysyl-tRNA synthetase (ThzKRS) and cysteinyl-lysyl-tRNA synthetase (CysKRS) to incorporate N^{ε}-$_L$-Thiaprolyl-$_L$-lysine (ThzK) and N^{ε}-$_L$-Cysteinyl-$_L$-lysine (CysK) into Ub, respectively (**Figure 3.1B and Figure 3.1C**).[92] ThzK is a protected amino acid and can be unprotected to form a cysteinyl lysine. Another synthetase used was wild-type PylRS (wtPylRS) from the Chan lab to incorporate synthesized CysK.[93] Both approaches gave low yields of Ub and a mixture of Ub containing the lysine variants and native

lysine. To help circumvent these issues, collectively, we will explore other avenues such has creating a new PylT vector to contain the ThzKRS, screen available PylRS, use RF1KNO cells[94], and condense culture expressions.[95]

3.2 Results and Discussion

3.2.1 Incorporation of ThzK into UbK11Tag

3.2.1.1 The construction of pSup-ThzKRS

Previously, our lab created a pSup plasmid system that incorporates different lysine derivatives and contains both the tRNA and aaRS genes.[96] The unique advantages of using the pSup plasmid system is its proK-pylT promotor that enhances the incorporation of the lysine derivatives and its compatibility with many *E.coli* expression vectors.[96] Recognizing, the benefits of this system we wanted to create a pSup plasmid containing the MbPylRS from pBK-ThzKRS in hopes of forming a vector that will enhance the expression yield of ThzK. The MbPylRS was PCR amplified from the pBK expression plasmid. The MbPylRS insert (1460 bp) was successfully amplified (**Figure 3.2A**) from pBK-ThzKRS and observed by gel electrophoresis. Next, the pSup vector was PCR amplified from pSup-BocLys[89]. The appropriate size fragment of 2785 bp was observed by gel electrophoresis, implying that the pSup fragment was created (**Figure 3.2B**). After obtaining the appropriate fragments, Gibson cloning was performed creating the plasmid pSup-ThzKRS. Upon successful creation of pSup-ThzKRS, we tested its efficiency for incorporating ThzK.

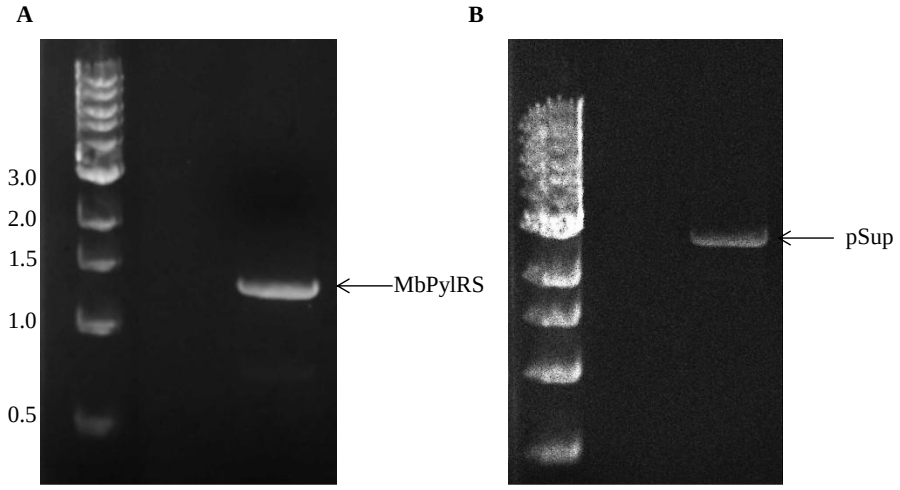


Figure 3.2. Gel-electrophoresis of PCR product. **A)** The MbPylRS fragment amplified from pBK plasmid, 1,460 bp. **B)** The pSup fragment amplified form pSup-BocLys, 2785 bp. 1 kb ladder used.

3.2.1.2 Testing the efficacy of pSup-ThzKRS for incorporating BocLys and ThzLys

Two different successful co-transformations of pTXB1-SynUbK11Tag with pSup-ThzKRS plasmids (#1 and #2) were performed. As a control, we tested the efficacy of the plasmids incorporating BocLys into ubiquitin. It was previously reported that the ThzKRS could incorporate 1 mM Boclys into a protein, so we performed expressions using 1 mM of BocLys.[92] We performed a small scale (5 mL) expression of Ub11BocLys. The expression ran overnight and the expressed cells were analyzed by SDS-PAGE gel. There was no signs of the incorporation of BocLys into Ub on our initial try. This was concerning because BocLys is a known lysine variant that has been successfully incorporated using the pyrrolysyl-tRNA synthetase.[97–99] A protein band was not distinctly visible at the 36 kDa marker position.

In response, we performed parallel 25 mL expressions of Ub11BocLys and Ub11ThzLys. For these expressions, we increased the concentration of BocLys (5 mM) and used 10- mM ThzK. We increased the concentration of BocLys because we considered that our vector may incorporate

BocLys at a higher concentration and the concentration of ThzK was 10 mM, which was previously used for successful incorporation of ThzK into a protein using the ThzKRS.[92] The expressions ran overnight and the expressed cells were analyzed by SDS-PAGE gel. Similarly, to the initial expression, it was hard to determine the incorporation of the amino acids into Ub because there was not a distinguishable protein band at the expected molecular weight. However, we decided to perform a chitin purification of the UbK11Thz purification in hopes to identify the amino acid was incorporated into Ub. There was a visible protein band observed by SDS-PAGE gel after purification, but the band was faint and it was not sufficient to conclude that ThzK was incorporated. It could have been a false positive in that native lysine was incorporated instead of ThzK. Running an ESI-MS would have been useful to support this claim. After several unsuccessful attempts of getting UbK11Thz in high yield, we decided to screen a selection of PylRS variants to identify PylRS variants that would improve the incorporation of ThzK and CysK into Ub.

3.2.1.3 Screening of different PylRS variants to incorporate BocLys, ThzLys, and CysLys into sfGFP

Different PylRS have been used to for incorporating UAA, and we wanted to screen them to identify synthetases that incorporate ThzK and CysK in greater yield than what was experienced. These synthetases are from *Methanosarcina mazei (Mm)* and *Methanosarcina bakeri* (*Mb*). The *Mm*PylRS was in the expression vector pBK-BocLys and *Mb*PylRSs were found in pBK-CysKRS, EV 16.5, EV 17, PCC2, EV20, and azide expression vectors. These synthetases were co-transformed with, pPY1T-sfGFPY151tag (sfGFP). sfGFP is a fluorescent protein, so we screened the ability of the synthetases to incorporate BocLys, ThzK, and CysK into sfGFP by fluorescence. We performed 1 mL expressions in triplicate and grew them to an OD 600_{nm}=.6, inoculated with

20% arabinose (arb), and inoculated with their respective amino acid, 2mM BocLys, 10 mM ThzK, and 10 mM CysK.[92] The synthetase that exhibited the greatest fluorescence for the incorporation of the three amino acids was CysKRS. From this observation, we constructed a new Ub plasmid that would be orthogonal to pBK-CysKRS.

3.2.1.4 The construction of pCNB-SynUbK11Tag

We constructed a PylT-Ub expression vector to be co-transformed with pBK-CysKRS. Our lab had a Py1T expression vector called pCATUPP that we used to incorporate an Ub intein chitin binding domain fragment (Ub-InteinCBD). First, we PCR amplified pCATUPP from the pCATUPP_PylT_D111TAG vector. The pCATUPP fragment was (5396 bp) was successfully amplified from pCATUPP_PylT_D111TAG and observed by gel electrophoresis (**Figure 3.3**). Next, the Ub-InteinCBD fragment from pTXB1-SynUbK11Tag was PCR amplified and the appropriate size fragment of the Ub-InteinCBD (1,162 bp) was observed by gel electrophoresis (**Figure 3.3**). After obtaining the appropriate fragments, a NEBuilder® HiFi DNA Assembly was performed, creating the pPylT_Ub plasmids (#3). A restriction digest and sequencing confirmed the cloning of pPy1T_Ub, which is named, pCNB-SynUbK11tag.

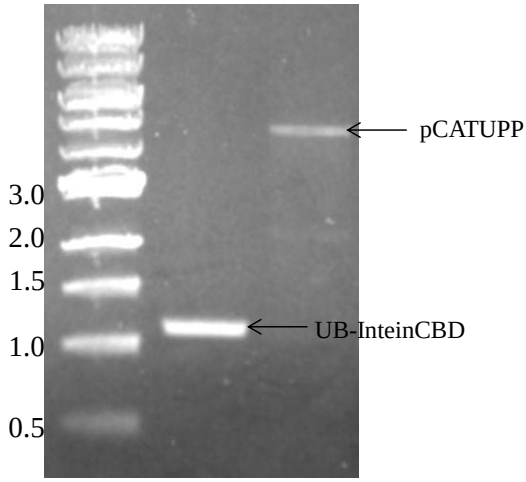


Figure 3.3. Amplified PCR fragments of Ub-Intein CBD (1,162 bp) from pTXB1-SynUbK11TAG and pCATUPP (5,396 bp) from pCATUPP_PylT_D111TAG.

3.2.1.5 Testing the efficacy of pCNB-SynUbK11tag for incorporating BocLys, ThzK, and CysK

A successful co-transformation of pCNB-SynUbK11Tag with pBK-CysK plasmids was performed. We tested the efficacy of pCNB-SynUbK11Tag incorporating BocLys, CysK, and ThzK into ubiquitin. We performed a 100mL expression to obtain Ub11BocLys, Ub11CysK, and Ub11ThzK. The concentrations of the amino acids were 2 mM BocLys, 2.5 mM CysK, and 10mM ThzK. BocLys was used as a positive control because it is a known lysine variant for PylRS.[97–99] The concentration for CysK was selected based on a previous report that the CyKRS can incorporate CysK more efficiently than the parent MbPylRS. The concentration for ThzK was 10 mM because it was previously reported that there was an increase in protein production using this concentration with the ThzKRS.[92] Therefore, we felt it would be beneficial to us 10 mM ThzK for the CysKRS to yield a high protein expression.

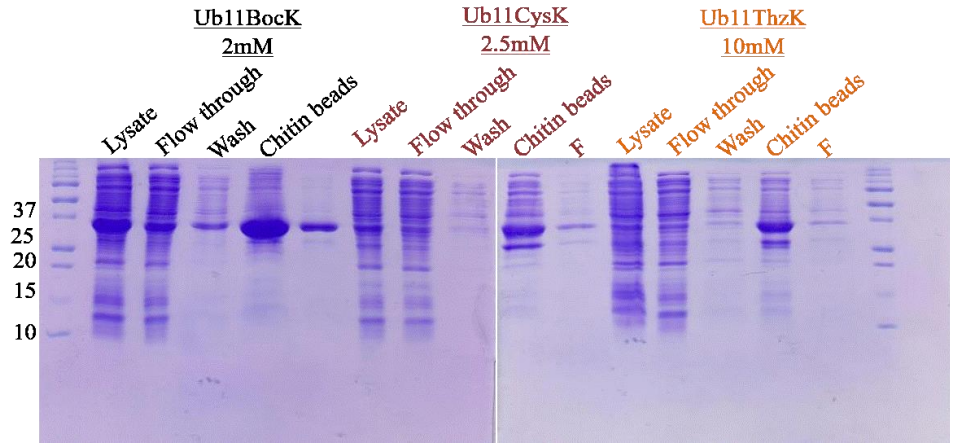


Figure 3.4. SDS-PAGE gels showing the chitin purification steps to confirm the incorporation of the different lysine derivatives into Ub. Expression is observed in the chitin bead lane and these beads contain the bound Ub-inteinCBD. Ub-inteinCBD size for Ub11BocK, Ub11CysK, and Ub11ThzK, 36, 405, 36,409, and 36,421 Da, respectively. Ladder is in kDa. F= flow through after induced cleavage.

We performed a chitin purification to see if we successfully incorporate BocLys, CysK, and ThzK into Ub (**Figure 3.4**). Differently, than what we observed with the pSup-ThzKRS/pTXB1_SynUBK11Tag expression system, we had a greater yield in protein expression for Ub11BocLys, Ub11CysK, and Ub11ThzK (**Figure 3.5**). To confirm our findings we ran an ESI-MS of the Ub11CysK and Ub11ThzK proteins. The Ub11CysK mass spectrum showed lysine incorporation occurred instead of CysK incorporation (**Appendix II**). The Ub11ThzK showed a mixture of lysine and ThzK incorporation, with it being approximately 75% ThzK incorporation (**Appendix II**). The protein concentration for Ub11ThzK was approximately 0.5 mg/100 mL. These results showed that CysK does not work well with CysKRS, but ThzK works with CysKRS. For crystallization purposes, we knew the concentration of 10 mM was too high, so we considered other alternatives to allow for a lower concentration of ThzK, but still give a higher protein yield. These alternatives were using a different cell line for protein production and performing a condensed expression

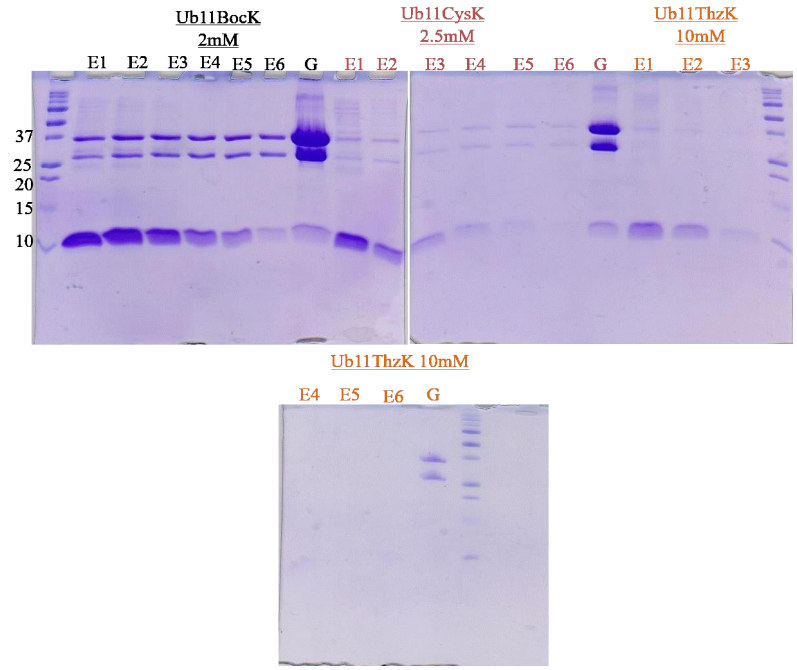


Figure 3.5. SDS-PAGE gels showing the eluted protein of Ub11BocK (8664 Da), Ub11CysK (8667 Da), and Ub11ThzK (8679 Da). E= elution and G=chitin beads after elution

3.2.1.6 The incorporation of ThzK using RF1KNO E. coli cells and condense expression.

We decided to perform an Ub11ThzK expression with a different strand of *E.coli* cells to lower the ThzK concentration and increase the yield of protein production. The strand of *E.coli* cells we used were release factor 1 knock out cells (RF1KNO).[94] Release factor 1 is a protein that recognizes the amber stop codon UAA (not the abbreviation for unnatural amino acid) and UAG and terminates translation.[100] The RF1KNO cells were made to circumvent the low efficiency of orthogonal synthetase pairs competing with endogenous release factors to incorporate an unnatural amino acid. Removal of the RF1 increased the unnatural amino acid incorporation efficiency at a one TAG site by 200% in comparison to the RF1 containing cells.[94]

With this knowledge, we created RF1KNO cells to contain the pCNB-SynUBK11Tag vector. We then transformed the pBK-CysKRS into the cells. We performed a 100 mL expression to obtain Ub11ThzKand inoculated with 2 mM ThzLys. The concentration of ThzK was lower because we felt the RF1KNO system would efficiently incorporate ThzLys, leading to an increased yield in UbThzK11. We performed a chitin purification and saw that protein was expressed. In comparison, to the DH-10β cell line the RF1KNO cell line seemed to have a better expression (**Figure 3.6**). This confirmed that the use of RF1KNO cells might be one useful approach to help with increasing the Ub production containing the lysine variants.

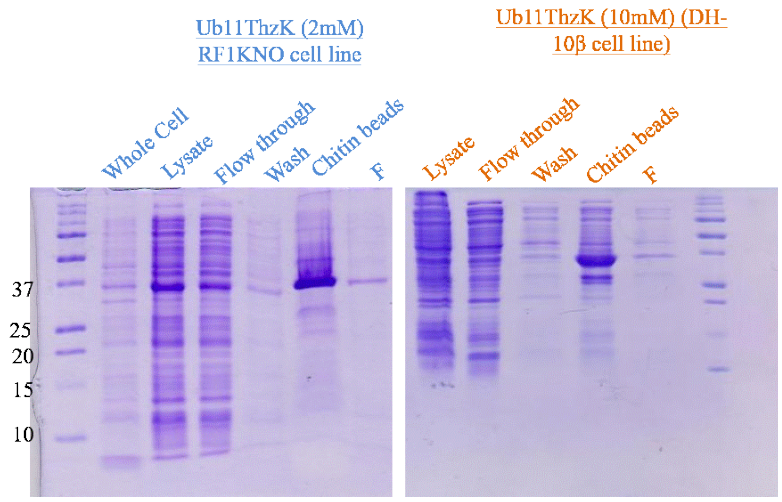


Figure 3.6. SDS-PAGE gels comparing the expression of the incorporation of ThzK with RF1KNO and DH-10β cell lines. The RF1KNO cells show a better expression.

During protein expression with UAAs, specifically synthesized (non-commercially available) UAA, > 99% of the UAA is not used and found in spent media.[95] To prevent the wasting of the UAA our lab previously developed a condense expression method.[95] This method condenses a larger culture by harvesting the cells after the appropriate $OD600_{nm}$ is reached and then the cells are resuspended in a smaller culture, allowing for a higher protein expression. We performed a 10 X condensed expression using our RF1KNO(pCNB-SynUbK11Tag)/pBK-CysKRS system and inoculated the expression with. 2 mM ThzLys. The protein was purified using the chitin purification system. After purification, we realized the protein expression was less than what we expected. We were expecting to see an increase in protein expression because of the robust RF1KNO cell line in combination with the efficiency of incorporating UAA using the condensed expression. There was no change in comparison to the non-condensed expression.

3.3 Conclusion and Future Application

In all, to enhance the expression systems for the incorporation of ThzK and CysK into Ub, new vectors were made and different alternatives for protein expression were explored. We created a

new pSup-ThzKRS vector and found it was not effective for incorporating ThzK into Ub. This was not what we expected and felt there was a potential mutation in the DNA sequence of ThzKRS that could be affecting its functionality. The sequencing results revealed there was a mutation, confirming our concerns. We were able to screen our PylRS systems and found that pBK-CysKRS was effective for incorporating ThzK into Ub, but not for CysK. In addition, a new PylR vector was created to be able to incorporate UAA into Ub. Additionally, we were able to see an increase in expression using RF1KNO cells. More recently, our interest have shifted to creating a photocaged cysteine with a methyl ester because it has the potential to be incorporated into Ub efficiently.[101,102] However, our work discussed here is a stepping-stone for future experiments in our lab or other labs that are interested in incorporating UAA into Ub or other protein system and for those continually wanting to expand the genetic code.

3.4 Materials and Methods

3.4.1 Creation of pSup-ThzKRS

3.4.1.1 Cloning of pSup from pSup-BocLys vector

pSup was cloned from pSup-BocLys vector to perform a Gibson cloning with the ThzKRS insert from pBK-ThzKRS and the pSup vector. The oligos used for obtaining the pSup vector without MmPylRS were 5'-ATGCAGTTTCAAACGCTAAATTGCCTGATGCGC-3' (FWD) and 5'-ATGGGATTCCTCAAAGCGTAAACAACGTATAAC- 3' (REV). The PCRs were performed using *Phusion* polymerase and the following PCR conditions for a 50 µL reaction were: initial duration at 98°C for 30 sec, 30 cycles of 98°C for 10 sec, annealing at 63°C for 20 sec, 72°C for 3 min, and a final extension at 72°C for 5 min. Expected size of pSup-boclys, MmPylRS, and pSup without MmPylRS are 4150 bp, 1365 bp, and 2785, respectively. The pSup vector product was verified by gel electrophoresis.

3.4.1.2 Cloning of MbPylRS from pBK-ThzKRS

The MbPylRS was cloned from the pBK vector to perform a Gibson cloning with the pSup vector. The oligos used for obtaining MbPylRS from pBK-ThzKRS were 5'-GTTATACGTTGTTTACGCTTTGAGGAATCCCATATGATGGATAAAAAACCGCTGGATGTG-3' (FWD) and 5'-GCGCATCAGGCAATTTAGCGTTTGAAACTGCATTTACAGGTTCGTGCTAATGCC-3' (REV). The PCRs were performed using *Phusion* polymerase and the following PCR conditions for a 50 µL reaction were: initial duration at 98°C for 30 sec, 34 cycles of 98°C for 10 sec, annealing at 65°C for 20 sec, 72°C for 3 min, and a final extension at 72°C for 5 min. Expected size of MbPylRS is 1460 bp.

3.4.1.3 The cloning of pSup-ThzKRS

The pSup-ThzKRS vector was created by Gibson cloning (Gibson Assembly® Master Mix, New England Biolab) MbPylRS and the pSUP vector together. On ice, the Gibson reaction was assembled: 3.5 µL pSup vector (0.045 pmols), 1 µL MbPylRS (0.24 pmols), 10 µL Gibson assembly master mix and 5.5 µL deionized water. The sample was incubated at 50°C for 15 min. Following, incubation the sample was stored on ice. A positive control reaction was setup and consisted of 10 µL of positive control and 10 µL of Gibson assembly master mix. Incubation time and storage was the same for the positive control.

3.4.1.4 Expression plasmid pSup-ThzKRS

Gibson cloning created plasmid pSup-ThzKRS. The pSup-ThzKRS was transformed into chemically competent NEBturbo cell. To 50 µL of thawed NEBturbo cells, 1 µL of plasmid was add. The cells were incubated on ice for 30 min. After incubation, the cells were heat shocked at

42ºC for 90 sec. Then the cells were recovered on ice for 2 min. Next 950 µL of SOC media was added to the cells. The cells were incubated for 1 hr at 37ºC. Once the incubation was completed, the cells were plated on a LB agar (Amp 100/ Chl 35) plate and incubated overnight at 37ºC. The same procedure was done for Gibson's positive control transformation. Colonies formed on the transformed plate for pSup-ThzKRS. Ten colonies were selected and DNA was extracted following the QIAprep Spin Miniprep Kit (Qiagen). The DNA was restricted digested as one way to identify if the plasmid was properly made. The plasmid was cut with restriction enzyme, EcoR1 (New England BioLabs), the expected cuts for pSup-ThzKRS were 2583, 1393, and 7.2 bp. The positive control was pSup-BocLys and the expected cuts were 2294 and 1856 bp. The restriction digest setup for 20 µL was 5 µL DNA, 2 µL CutSmart® (NEB) buffer, 1 µL EcoR1, 0.2 µL BSA, and 11.8 µL sterile deionized water. The reaction incubated for at least 1 hr. The tested by gel electrophoresis and ran on a 1% agarose gel. Of the 10 colonies, DNA from colonies 1 (pSup-ThzKRS #1), 2 (pSup-ThzKRS #2), and 8 (pSup-ThzKRS #8) were sent out for sequencing. Plasmids pSup-ThzKRS #1 and pSup-ThzKRS #2 were used for double transformations and expressions.

3.4.1.5 Expression plasmids pTXB1-SynUbK11Tag and pSup-ThzKRS

Plasmid pTXB1-SynUbK11Tag[89], encoding a tagged chitin binding domain (CBD) Ub and amber stop codon (TAG), at residue 11, was created by site directed mutagenesis. The pTXB1-SynUbK11Tag mutant was co-transformed with, pSup-ThzKRS, containing cellular machinery to incorporate, ThzLys (ThzK), as a genetically encoded unnatural amino acid at the TAG codon into EC BL21 (DE3) *E. coli* cells. To 50 µL of thawed BL21 (DE3) *E. coli* cells, .5 µL of each plasmid was added. The cells were electroporated, recovered with 500 µL SOC, and incubated for 1 hr at

37ºC. Once the incubation was completed, the cells were plated on a LB agar (Amp 100/ Chl 35) plate and incubated overnight at 37ºC.

3.4.1.6 Purification of SynUbK11ThzLys

Harvested cell from a 25 mL expression of SynUbK11ThzLys were lysed with 750 µL of lysis buffer (20 mM Hepes, pH 8.5, 0.5 M NaCl, 1 mM EDTA, 0.02% NaN_3, 100 mM PMSF). The cells were freeze/thawed three times with liquid nitrogen, sonicated on ice for a 2 min pulse, and cooled down on ice. The cells were centrifuged at 12,000 rpm for 20 min to isolate the soluble proteins. The lysate was added to 200 µL of chitin beads and incubated (rotating) at room temperature for 1 hr. The chitin beads were washed (20 mM Hepes, pH 8.5, 0.5 M NaCl, 1 mM EDTA, 0.02% NaN_3) and the protein was eluted with 1 mL of elution buffer (50 mM DTT). Protein expression was verified by SDS-PAGE gel.

3.4.2 Screening of PylRS

3.4.2.1 Testing of different synthetases to incorporate BocLys, ThzLys, and CysLys into sfGFP

Different synthetases pBK-ThzKRS, CysKRS, BocLys, EV 16.5, EV 17, PCC2, EV20, and azide were tested to identify if BocLys, ThzLys, and CysLys can be incorporated into sfGFP. The synthetases were co-transformed with, pPY1T-sfGFPY151tag (sfGFP). Mini expressions of 1 mL (3 per synthetase). LB Broth (Amp 100/ Tet 15 or Kan 50/Tet 15) were inoculated with 50 µL of overnight culture (overnight culture inoculated from glycerol stocks from previous expressions). The cells grew to an $OD600_{nm}$=0.6, induced with 20% arabinose (arb), inoculated with 2mM BocLys , 10 mM ThzLys, or 10 mM CysK. The expressions incubated over night at 37 ºC. The incorporation of the amino acids into sfGFP were verified by fluorescence.

3.4.2.2 Cloning of pCATUPP from pCATUPP_PylT_D111TAG

The pCATUPP was cloned from the pCATUPP_Pylt_D111TAG vector to perform a NEBuilder® HiFi DNA Assembly with the Ub-inteinCBD insert from pTXB1-SynUbK11tag. The oligos used for cloning pCATUPP from the vector were 5'-AATCGACCATATGGGAATTCGAAGCTTGGGCCC- 3' (FWD) and 5'-GGTTAATTCCTCCTGTTAGCCCAAAAAACGGT- 3' (REV). The PCRs were performed using *Phusion* polymerase and the following PCR conditions for a 20 µL reaction were: initial duration at 98°C for 30 sec, 25 cycles of 98°C for 10 sec, annealing at 60°C for 20 sec, 72°C for 3 min, and a final extension at 72°C for 5 min. Expected size of pCATUPP is 5396 bp. The pCATUPP vector product was verified by gel electrophoresis.

3.4.2.3 Cloning of Ub intein chitin binding domain (CBD) from pTXB1-SynUbK11Tag

The tagged Ub-inteinCBD insert was cloned from pTXB1-SynUbK11Tag to perform a NEBuilder® HiFi DNA Assembly with the pCATUPP vector. The oligos used for obtaining Ub-inteinCBD from pTXB1-SynUbK11Tag were 5'-ACCCGTTTTTTGGGCTAACAGGAGGAATTAACCCATATGCAGATTTTTGTGAAAACC CTG- 3' (FWD) and 5'-GGGCCCAAGCTTCGAATTCCCATATGGTCGATTATCCGGATATAGTTCCTC- 3' (REV). The PCRs was performed using *Phusion* polymerase and the following PCR conditions for a 20 µL touchdown reaction was: initial duration at 98°C for 30 sec, 10 cycles of 98°C for 10 sec, annealing at 70°C (-1 per cycle) for 30 sec, 72°C for 3 min, 15 cycles 98°C for 15 sec, annealing

at 65°C for 30 sec, 72°C for 3 min., and final extension 72°C for 30 sec. Expected size of Ub-inteinCBD is 1,162 bp. The Ub-inteinCBD product was verified by gel electrophoresis.

3.4.2.4 The cloning of pCNB-SynUbK11Tag

The pCNB-SynUbK11Tag vector was created by NEBuilder® HiFi DNA Assembly (New England Biolabs) Ub-inteinCBD and the pCATUPP vector together. On ice, the reaction was assembled: 10 µL pCATUPP vector (0.060 pmols), 1 µL Ub-inteinCBD (0.033 pmols), 15 µL NEBuilder® HiFi DNA Assembly master mix and 4 µL deionized water. The sample was incubated at 50°C for 15 min. Following, incubation the sample was stored on ice. A positive control reaction was setup and consisted of 10 µL of positive control and 10 µL of NEBuilder® HiFi DNA Assembly master mix. Incubation time and storage was the same for the positive control.

3.4.2.5 Protein expression and purification of UbK11BcoLys, UbK11CysK, UbK11ThzK

Plasmid pCNB-SynUbK11tag was co-transformed with pBK-CysKRS into DH10-β *E. coli* cells. A single colony was picked from its respective plate to grow a 5 mL overnight starter culture in LB media (Tet 15/Kan 50). A 100 mL flask of LB media (Tet 15/ Kan 50) was inoculated with 1.5 mL of starter culture. The cells grew to an $OD600_{nm}$= 0.6, induced with 20% arb, inoculated with 1 mM of respective unnatural amino acid (BocLys, CysLys, or ThzLys), and grew 37° C overnight. Cells were harvested at 5,000 rpm for 10 min and then resuspended in 3 mL of lysis buffer (20 mM Hepes, pH 8.5, 0.5 M NaCl, 1 mM EDTA, 0.02% NaN_3, 100 mM PMSF). The cells were freeze/thawed three times, sonicated on ice for a 5 min pulse, and cooled down on ice. The cells were centrifuged at 12,000 rpm for 35 min to isolate the soluble proteins. The lysate was added to equilibrate chitin beads. The chitin beads were washed with 8 mL of wash buffer (20 mM Hepes, pH 8.5, 0.5 M NaCl, 1 mM EDTA, 0.02% NaN_3). On column cleavage was induced by

flushing the beads with 1.2 mL of elution buffer (20 mM Hepes, pH 8.5, 0.5 M NaCl, 1 mM EDTA, 0.02% NaN_3, and 50 mM DTT). The beads were incubated at 37°C for 48 hrs. Protein was eluted with 200 µL of fresh elution buffer (6 fractions).

3.4.3 Alternative methods for increasing the efficiency of the incorporation of UAA

3.4.3.1 Electrocompetent RF1KNO cells containing pCNB-SynUbK11Tag

A 100 mL culture of LB broth (Tet 15) was inoculated with 1 mL of starter culture. The cells grew to an $OD600_{nm}$=0.600. Cells were incubated on ice for 15 min. Centrifuged at 5,000 rpm for 10min at 4°C. Supernatant was discarded, the cells were resuspended 50 mL of cold glycerol, and centrifuged at 5,000 rpm for 1 0min at 4°C. This was repeated three times. After the final wash, the cells were resuspended in the residual glycerol and 500 µL of glycerol was added. The cells were distributed to cold centrifuge tubes and frozen by liquid nitrogen and store at -80°C.

3.4.3.2 Protein expression and purification of UbK11ThzK

Plasmid pBK-CysKRS was transformed into EC RF1KNO cells containing pCNB-SynUbK11tag. A 100 mL culture of LB media (Tet 15/ Kan 50) was inoculated with 2 mL of starter culture. The cells grew to an $OD600_{nm}$= 0.6, induced with 20% arb, inoculated with 1 mM of ThzLys, and grew at 37°C overnight. Cells were harvested at 5,000 rpm for 10 min and then resuspended in 3 mL of lysis buffer (20 mM Hepes, pH 8.5, 0.5 M NaCl, 1 mM EDTA, 0.02% NaN_3, 100 mM PMSF). The cells were freeze/thawed three times, sonicated on ice for a 5 min pulse, and cooled down on ice. The cells were centrifuged at 12,000 rpm for 20 min to isolate the soluble proteins. The lysate was added to chitin beads. The chitin beads incubated for 1 hr at 4°C, rotating. The chitin beads were washed with 8 mL of wash buffer (20 mM Hepes, pH 8.5, 0.5 M NaCl, 1 mM EDTA, 0.02% NaN_3). On column cleavage was induced by flushing the beads with 1.2 mL of elution buffer (20

mM Hepes, pH 8.5, 0.5 M NaCl, 1 mM EDTA, 0.02% NaN_3, and 50 mM DTT). The beads were incubated at 37°C for 48 hrs. Protein was eluted with 240 µL (5 fractions) and 500 µL (2 fractions) of fresh elution buffer (50 mM DTT).

3.4.3.3 Condensed protein expression and purification of UbK11ThzK

Plasmid pBK-CysKRS was transformed into RF1KNO cells containing pTXB1-SynUbK11Tag. A 100 mL culture of LB media (Tet 15/ Kan 50) was inoculated with 2 mL of starter culture. The cells grew to an $OD600_{nm}$= 0.6, induced with 20% arb, inoculated and incubated on ice for at least 15 min. The cells were harvested at 5, 000 rpm for 10 min, resuspended 10 mL LB broth (Tet 15/Kan 50), and inoculated with 2 mM ThzyLys. The expression grew overnight at 37°C. Cells were harvested at 5,000 rpm for 10 min and then resuspended in 300 µL of lysis buffer (20 mM Hepes, pH 8.5, 0.5 M NaCl, 1 mM EDTA, 0.02% NaN_3, 100 mM PMSF). The cells were freeze/thawed three times, sonicated in a water bath for 5 min, and cooled down on ice. The cells were centrifuged at 12,000 rpm for 20 min to isolate the soluble proteins. The lysate was added to chitin beads. The chitin beads incubated for 1 hr at 4°C, rotating. The chitin beads were washed with 3 mL of wash buffer (20 mM Hepes, pH 8.5, 0.5 M NaCl, 1 mM EDTA, 0.02% NaN_3). On column cleavage was induced by flushing the beads with 500 µL of elution buffer (20 mM Hepes, pH 8.5, 0.5 M NaCl, 1 mM EDTA, 0.02% NaN_3, and 50 mM DTT). The beads were incubated at 37°C for 48 hrs. Protein was eluted with 50 µL (1 fraction) and 100 µL of fresh elution buffer (50 mM).

Appendix I

Understanding Ubiquitin Signaling using Immobilized DiUb Probes

I-1 Introduction

In a continual effort to answer Ub biology's main questions of concern, what do polyUb chains interact with in the cell and how do these interaction mediate a biological response?, we are interested in non-enzymatically[31,48] synthesizing atypical K6 linked dimers with an affinity "handle" for immobilization. An immobilized polyUb will help with identifying potential cellular binding partners, specific to this linkage.

I-2 Rationale

As an initial step for determining the protein-protein interaction of the K6 linked chain, the chains will be immobilized on streptavidin coated plates. To facilitate the immobilization there needs to be an incorporation of biotin, preferably on the distal Ub domain of the poly-Ub chains. Ubiquitin does not have sulfhydryl groups, so we plan to incorporate a cysteine mutation by site-directed mutagenesis. Maintaining the overall functionality of Ub and the poly-Ub chains is important, so we plan to mutate amino acids that are not close to the linkage sites. An alternative approach is to take advantage of our intein-CBD fushion tag during the purification process. Using cysteine during the cleaving process (in place of DTT) will lead to a final product containing an addition

C-terminal cysteine mutation. These mutant building blocks can be carried through the synthesis sequence as normal and we do not anticipate a cysteine mutation to interfere with the chemistries. The assembled chains will be reacted with EZ-Link Iodoacetyl-LC-Biotin (Pierce), and the reaction verified by ESI-MS. The final products can be captured on neutravidin or streptavidin coated plates to generate "chain affinity reagents." As an alternative strategy if biotinylations are problematic, we will append N-terminal affinity tags such as Strep-tag or FLAG tags that can be captured on the appropriate surfaces. Finally, "natural" chains that have no mutations could be non-selectively immobilized on chemically reactive beads such as UltraLink resin (Pierce).

After immobilization of the K6 dimer, we will begin the process of identifying cellular binding partners. Cellular lysates that we are interested in using are HEK293-T cells and yeast. The cellular lysate will be added to the wells containing the K6 diUb, and the cellular debris will be washed with phosphate buffered saline (PBS). Different elution methods will be explored to remove the proteins that created protein-protein interactions. One method is eluting with a low pH glycine solution. Another elution method is using free K6 diUb to prevent nonspecific binding interactions. To lower false positives from non-specific binding, we will alternate immobilization techniques between streptavidin and neutravidin. The eluted proteins will be separated by running a SDS-PAGE gel. Individual bands will be excised from the gel and undergo a tryptic digest as previously described.[103] Since we are specifically interested in the selectivity of K6 diUb, it is important to establish a control, in which, WTUb is immobilized and undergoes the same process as K6. This is important because it is common for many proteins to bind to any Ub, establishing this control will help us compare and identify bands that show intensity differences specific for K6 diUb. The tryptic digested samples will be resolved through capillary LC and later analyzed by tandem MS/MS. The MASCOT search engine will be used to identify peptides from the

digestion.[104] Our labs Agilent 6340 Ion Trap LC/MS will be used to start; however, if the results are not conducive we will use the VCU proteomics facility for its LTQ orbitrap instrument.

I-3 Preliminary Results

I-3-1 K6 polyUb chain building blocks.

In order to assemble the K6 dimer we had to create the building blocks needed to form the chain, as mentioned in **Chapter 1** and shown in **Figure 1.5**. Our first building block was purifying UbK6BocLys (Figure I-I). We co-transformed pTXB1-SybUbK6tag and pSup-BocLys into T7 express *E.coli* cells. Then the UbK6BocLys was expressed and purified using the IMPACT™ purification system (**Figure I-1 and I-2**). After purification, UbK6BocLys was further purified using HiLoad 16/60 Superdex^{TM} 75 pg size-exclusion chromatography. Our next building block we created was an Ub thioester. We performed a MESNA reaction on WTUb to create a thioester on the C-terminus. Our reaction was verified by ESI-MS (**Figure I-3**).

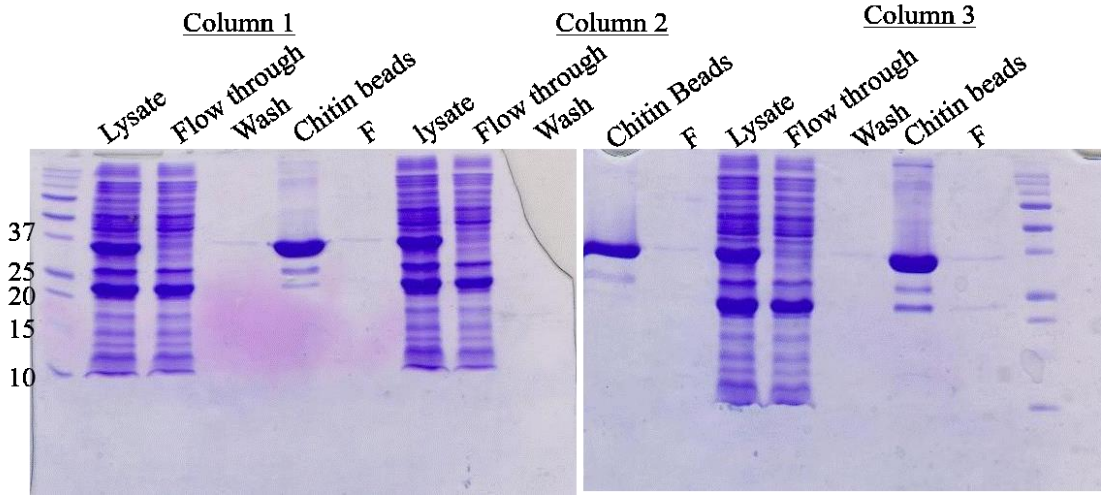


Figure I-1. SDS-PAGE gels showing the chitin purification of UbK6. The expressed band of UbK6 with the inteinCBD is 36, 405 Da. Chitin beads contain the bound UbK6-inteinCBD. F= flow through after elution. Ladder is in kDa.

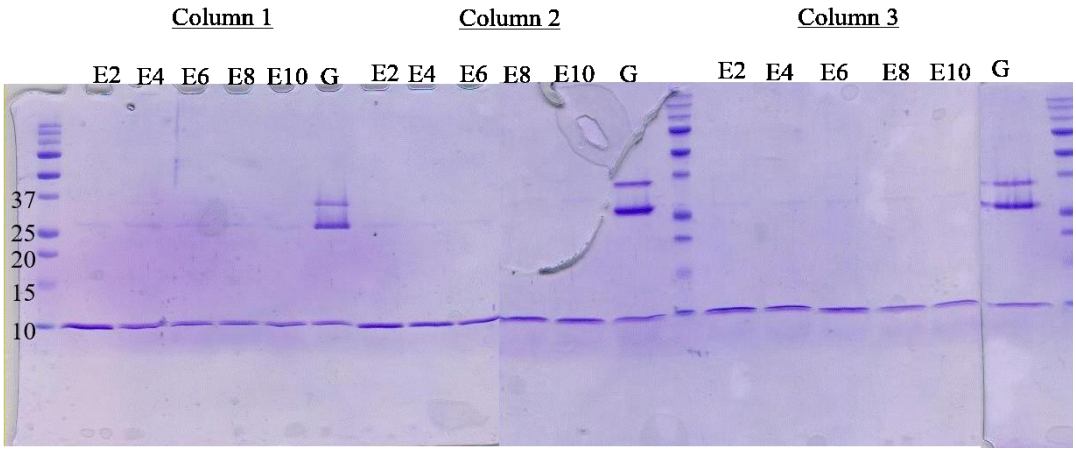


Figure I-2. SDS-PAGE gels showing the elution of UbK6 from a chitin purification. UbK6 is 8665 Da. G= chitin beads after elution. Ladder is in kDa.

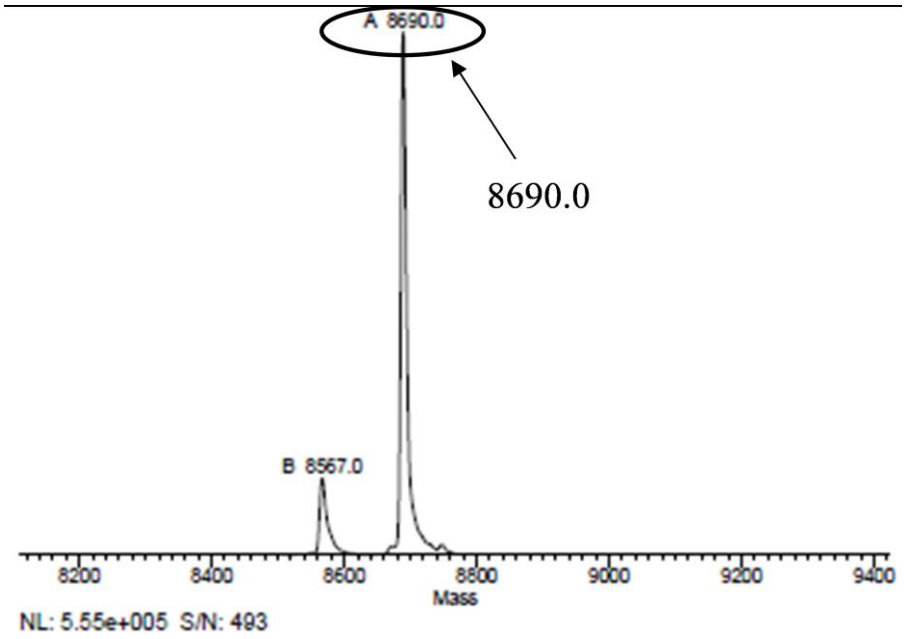


Figure I-3. ESI-MS of the thioesterfication of WTUb.

I-4 Future Experiments

1. Synthesize a K6 linked dimer containing and affinity "handle for immobilization
2. Identify K6 linked dimer binding partners

I-5 Materials and Methods

I-5-1 PolyUb chains K6

I-5-1-1 Expression plasmids pTXB1-SynUbK6Tag and pSup-BocLys

Plasmid pTXB1-SynUbK6tag[89] encoding an amber stop codon (TAG), at residue position 6, was created by site directed mutagenesis. The pTXB1-SynUbK6tag mutant was co-transformed with, pSup-BocLys, containing cellular machinery to incorporate N^{ε}-Boc-L-lysine as a genetically encoded unnatural amino acid at the TAG codon into chemically competent T7 express (T7) cells. To 75 µL of thawed T7 express cells, 1 µL of each plasmid was added. The cells were incubated on ice for 30 min. After incubation, the cells were heat shocked at 42ºC for 90 sec. Then the cells were recovered on ice for 2 min. Next 1 mL of SOC media was added to the cells. The cells were incubated for 1 hr at 37ºC. Once the incubation was completed, the cells were plated on a LB agar (Amp 100/ Chl 35) plate and incubated overnight at *37ºC*.

I-5-1-2 Protein expression and purification of UbK6BocLys

Plasmid pTXB1-SynUbK6tag was co-transformed with pSup-BocLys into T7 express *E. coli* cells. A single colony was picked from its respective plate to grow a 5 mL overnight starter culture in 2xYT media (Amp 100/Chl 35). Two 1 L flasks of 2xYT media (Amp 100/ Chl 35) was inoculated with 1 mL of starter culture. The cells grew to an $OD600_{nm}$= 0.6, induced with 1 mM of IPTG, inoculated with 1 mM N^{ε}-Boc-L-Lysine (BocLys), and then grown at 37° C overnight. Cells were harvested at 4,000 rpm for 10 min and then resuspended in 60 mL of lysis buffer (20 mM Hepes,

pH 8.5, 0.5 M NaCl, 1 mM EDTA, 0.02% NaN_3, 100 mM PMSF). The cells were freeze/thawed three times with liquid nitrogen, sonicated on ice for a 2 min pulse, and cooled down on ice. The cells were centrifuged at 12,000 rpm for 20 min to isolate the soluble proteins. The lysate was added to an equilibrated chitin bead column. The binding flow through was added two the column two additional times. The chitin beads were washed with 300 mL of wash buffer (20 mM Hepes, pH 8.5, 0.5 M NaCl, 1 mM EDTA, 0.02% NaN_3). On column cleavage was induced by flushing the beads with 50 mL of elution buffer (20 mM Hepes, pH 8.5, 0.5 M NaCl, 1 mM EDTA, 0.02% NaN_3, and 50 mM DTT). The column was incubated at 37°C for 24 hrs. Protein was eluted with 50 mL of fresh elution buffer and collected in you 5 mL fractions. After the initial elution, an additional 50 mL of fresh elution buffer was added to the column, and the column was incubated at 37°C for 24hrs. Collected fractions were dialyzed in water using dialysis tubing with a MWCO of 3,500. UbK6BocLys was further purified using HiLoad 16/60 SuperdexTM 75 pg size-exclusion chromatography.

I-5-2 Thioesterfication Ub building block

I-5-2-1 Expression plasmid pET21d-Ube1

Plasmid pET21d-Ube1 (purchased from Addgene), encoding for activating enzyme E1, was transformed into electrocompetent BL21 (DE3) *E. coli* cells. To 100 µL of thawed EC BL21 (DE3) cells, 1 µL of plasmid was added. The cells were electroporated and recovered in 1 mL of SOC. The cells were incubated at 37ºC for 1 hr. About 100 µL of cells were plated on a LB agar (Amp 100) plate and incubate overnight at 37ºC.

I-5-2-2 Protein expression and purification of Ube1

Plasmid pET21d-Ube1 was transformed into EC BL21 (DE3) *E. coli* cells. A 25 mL overnight starter culture of LB broth (Amp 100) was inoculated with a single colony from the transformed plate. A 1 L flask of LB broth (Amp 100) was inoculated with 5 mL of starter culture. The cells grew to an $OD600_{nm}$=0.6, induced with 1 mM of IPTG, and incubated at room temperature for 8 hrs. The expression was harvested at 5, 000 rpm for 10min at 4ºC. The cells were resuspended in 25 mL of binding buffer (50 mM NaH_2PO_4, pH 8.00, 300 mM NaCl, 10mM imidazole, and 1 mM PMSF). The cells were lysed by lysozyme and incubated for 20 min at 4ºC rocking. The cells were further lysed by freeze/thawing the cells three times with liquid nitrogen. The cells were centrifuged at 12, 100 rpm for 20 min at 4ºC to obtain the soluble protein. A batch mode nickel purification was performed. The cellular lysate was added to 3 mL of washed (50 mM NaH_2PO_4, pH 8.00, 300 mM NaCl, and10mM imidazole) Promega HisLink resin (Promega, Madison, WI) and incubated 1 hr on ice shaking. The resin was washed four times with ~50 mL of bind buffer and then eluted five times with 7.5 mL of elution buffer (50 mM NaH_2PO_4, pH 8.00, and 300 mM NaCl, and 500mM imidazole). The eluted protein was concentrated and buffer exchanged into 50 mM Tris, pH 8.0 using a Spin-X UF concentrator (5, 000 MWCO). The concentration was determined using nanodrop.

I-5-2-3 Thioesterfication of Ub

Wild-type ubiquitin (WTUb) was used to create an Ub-SR. Previously, purified WTUb (ion exchanged in NH_4Ac, pH 4.50) was dialyzed into 20 mM sodium phosphate buffer, pH 8. The 2 mL thioesterfication reaction consisted of WTUb, 1 M MESNA (20 mM sodium phosphate), and 0.1 M ATP (20 mM sodium phosphate), 1 M MgCl, and 200 µM Ube1. The reaction was

incubated at 37ºC overnight. The reaction was stopped by adding drops off glacial acetic acid. The thioesterfication of WTUb was verified by ESI-MS.

I-5-3 Ubiquitin containing a cysteine

I-5-3-1 Incorporation of a cysteine to UbK6BocLys

Followed the same procedure as expression and purification of UbK6BocLys. On-column cleavage was different and induced by flushing the column with 50 mL of wash buffer (20 mM HEPES, pH 8.5, 0.5 M NaCl, 1 mM EDTA, 0.02% NaN3) containing 50 mM cysteine and column was kept at 37°C for 48 hours. Cleaved UbK6Boclys was eluted from the column using 50 mL of wash buffer containing 50 mM cysteine. UbK6BocLys was further purified using HiLoad 16/60 SuperdexTM 75 pg size-exclusion chromatography.

Appendix II

ESI-MS Spectra of Ub Mutants

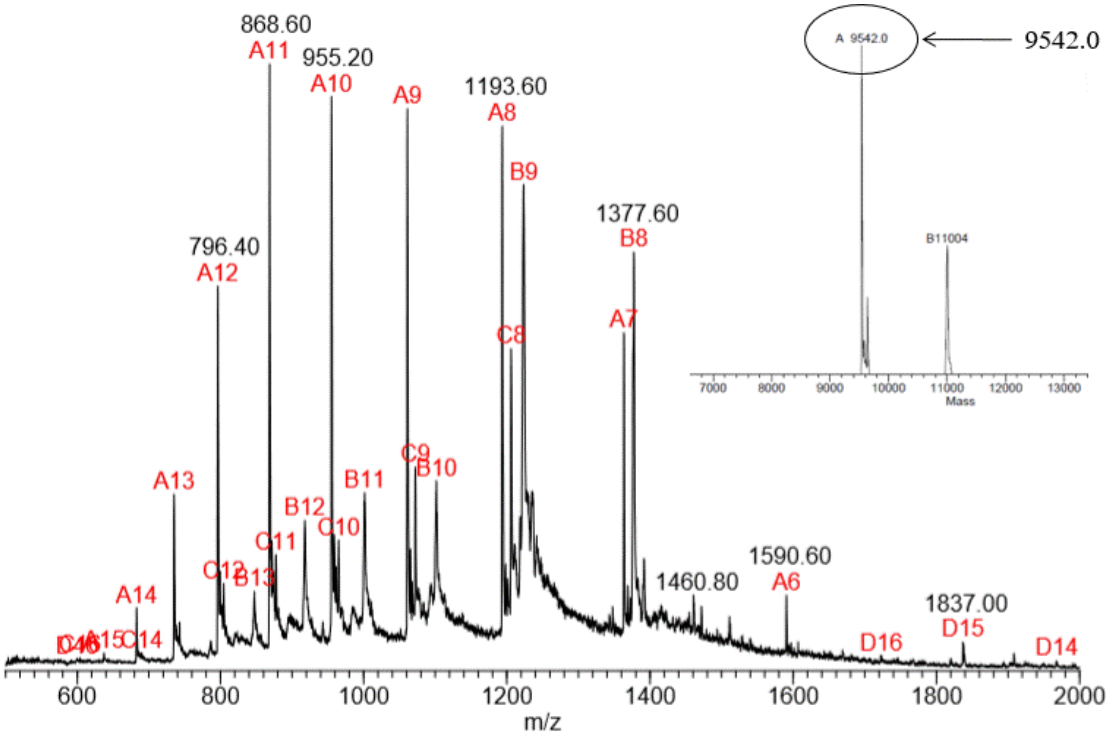


Figure II-1. ESI-MS of Ub9pBpa. Expected mass: 9537 Da. Observed mass: 9542 Da

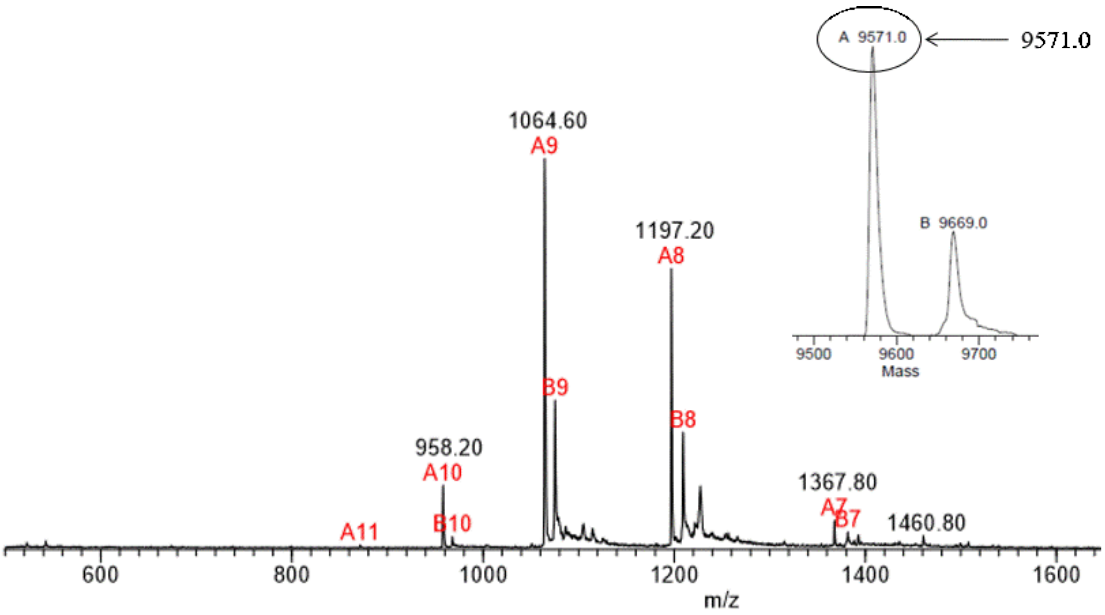


Figure II-2. ESI-MS of Ub46pBpa. Expected mass: 9567 Da. Observed mass: 9571 Da

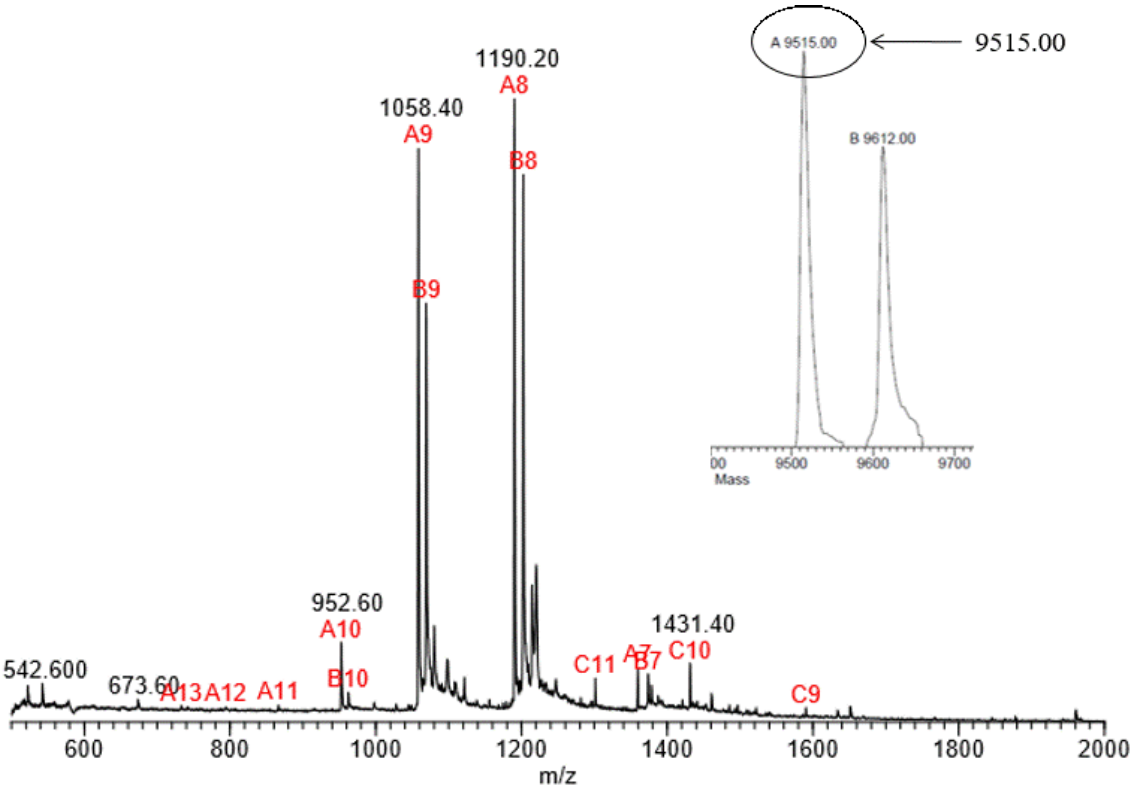


Figure II-3. ESI-MS of Ub48pBpa. Expected mass: 9510 Da. Observed mass: 9515 Da

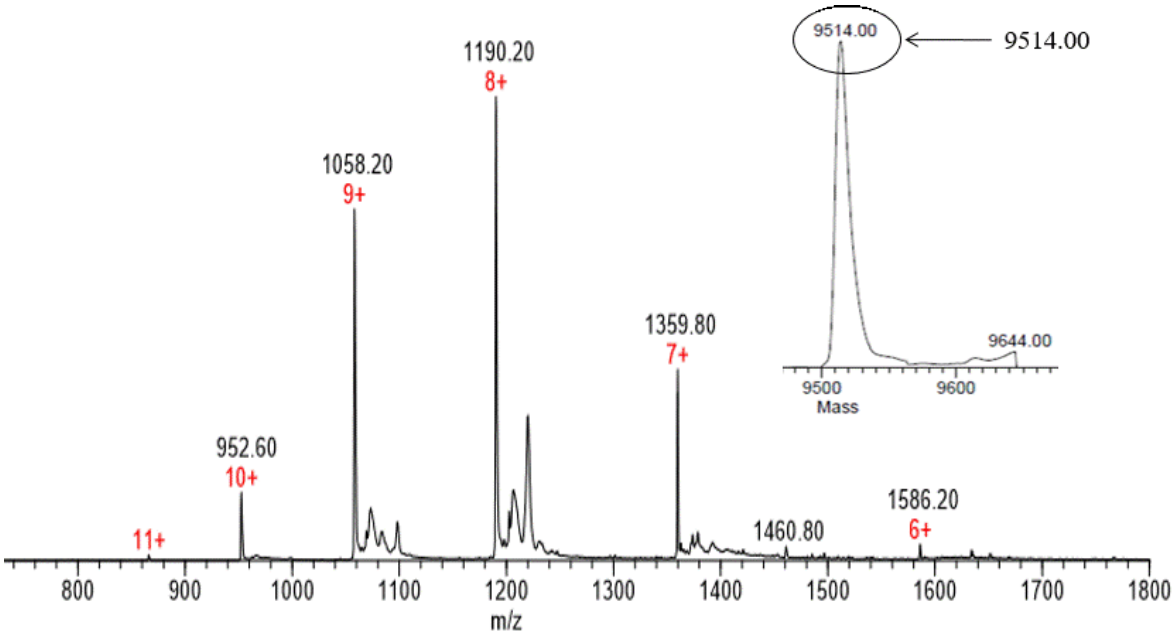


Figure II-4. ESI-MS of Ub49pBpa. Expected mass: 9510 Da. Observed mass: 9514 Da

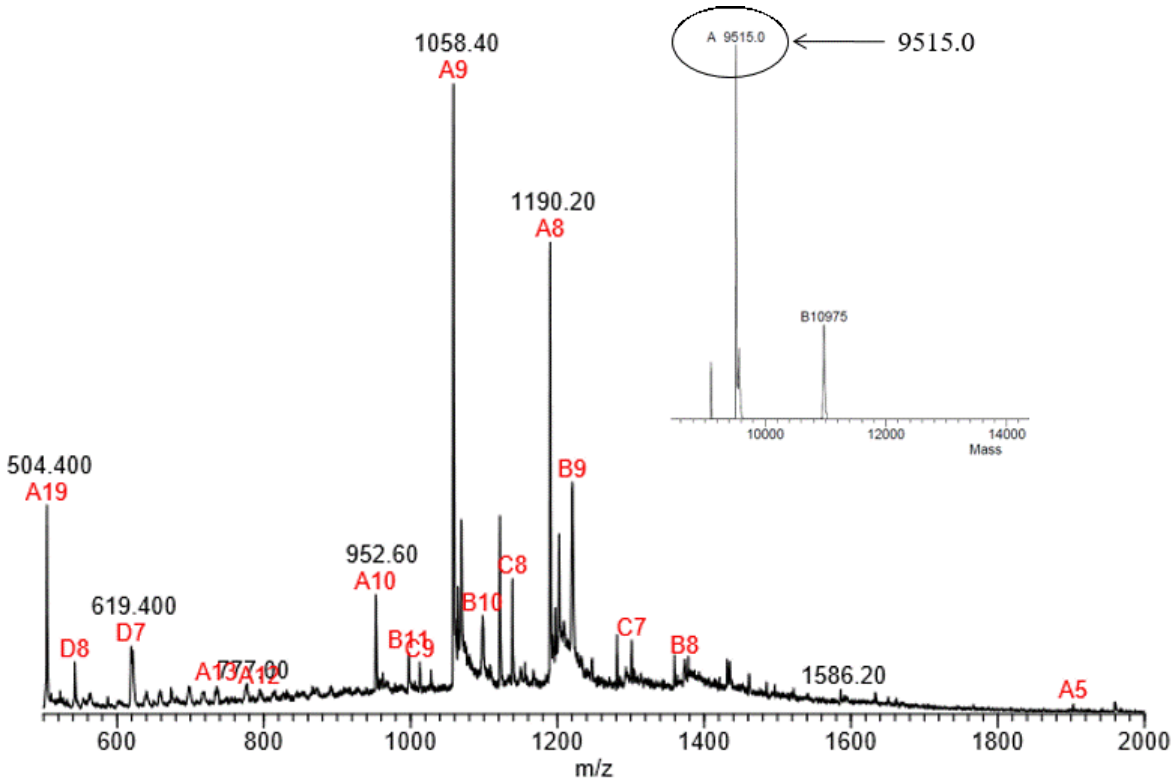


Figure II-5. ESI-MS of Ub51pBpa. Expected mass: 9509 Da. Observed mass: 9515 Da

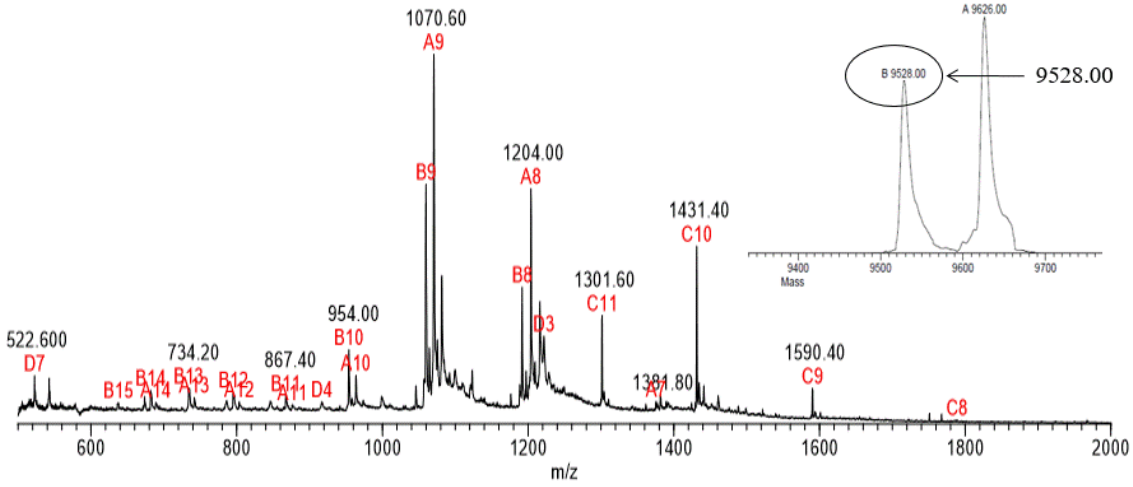


Figure II-6. ESI-MS of Ub52pBpa. Expected mass: 9523 Da. Observed mass: 9528 Da

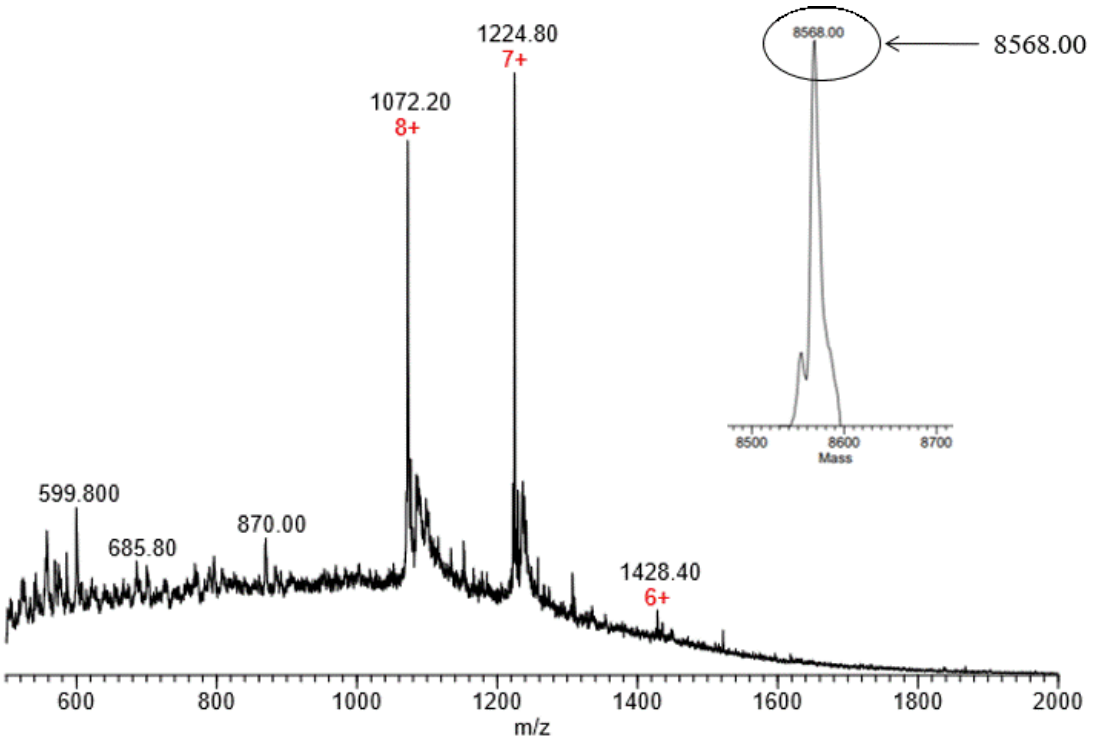


Figure II-7. ESI-MS of Ub11CysK. Expected mass: 8667 Da. Observed mass: 8568 Da

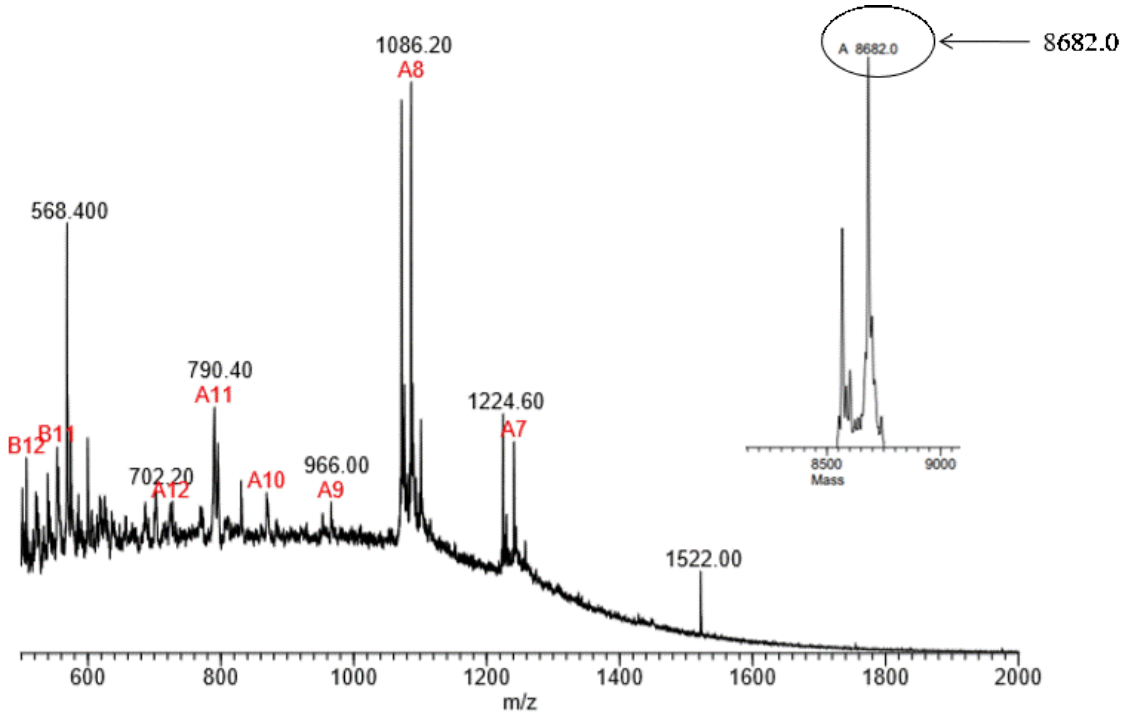


Figure II-8. ESI-MS of UbThzK. Expected mass: 8679 Da. Observed mass: 8682 Da

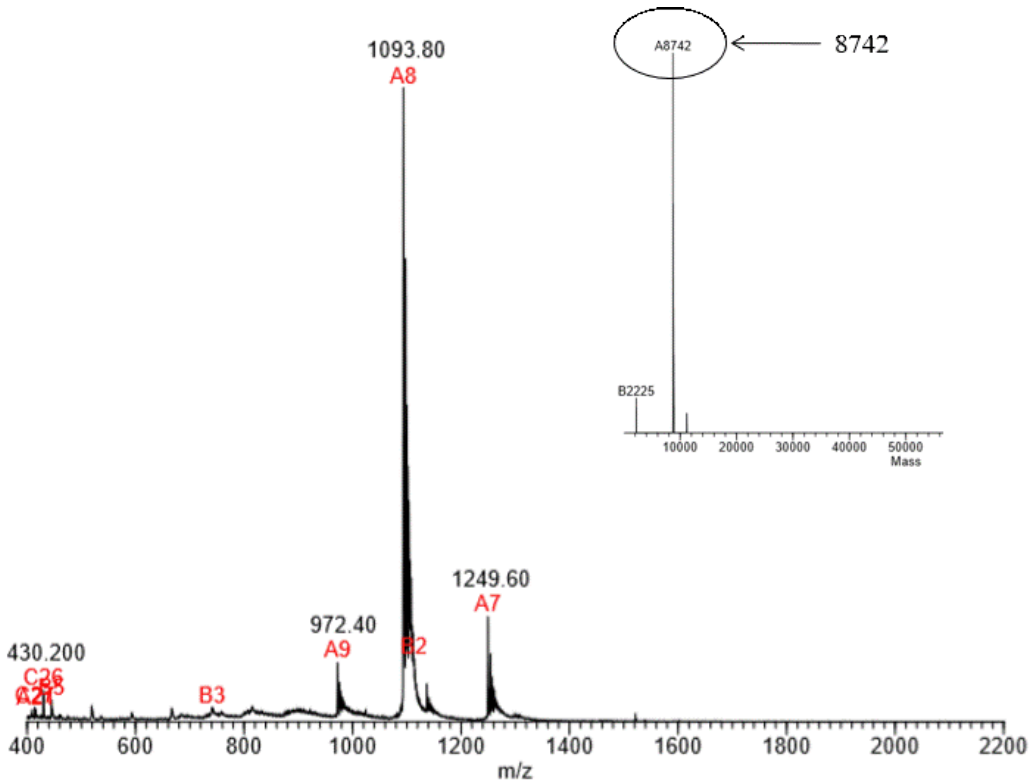


Figure II-9. ESI-MS of Ub9pBpa_K48R. Expected mass: 8742 Da. Observed mass: 8742 Da

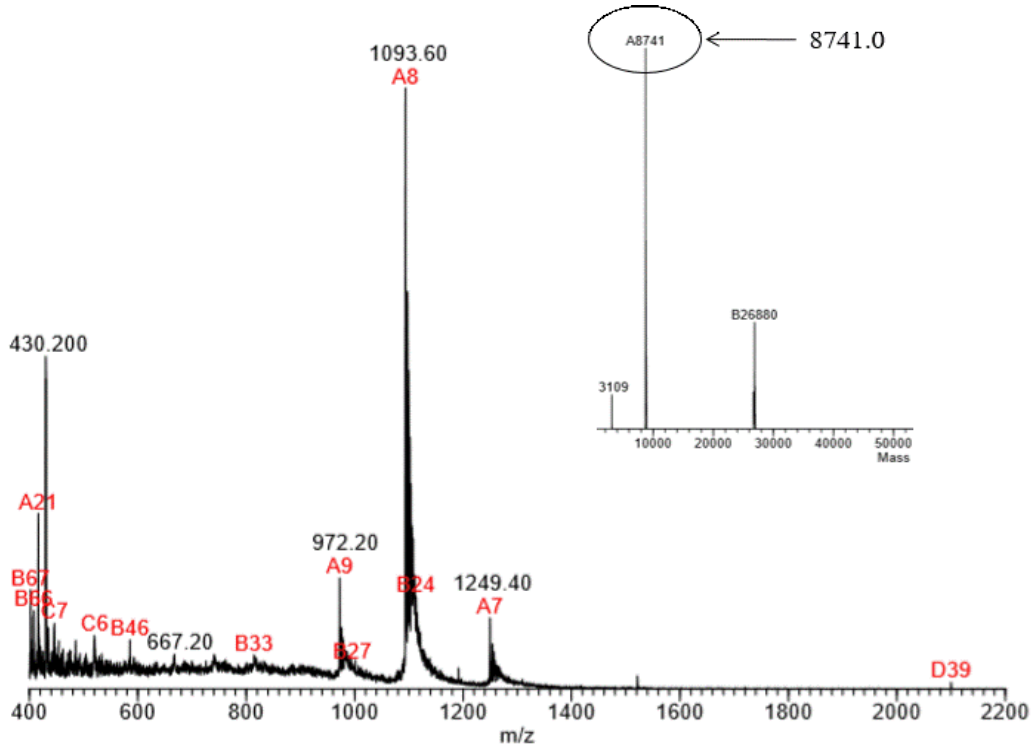


Figure II-10. ESI-MS of Ub9pBpa_K63R. Expected mass: 8742 Da. Observed mass: 8741

Appendix III

Scaffold Sequences

K63 linked $Ub_2$9pBpa with Rap 80

Rap 80

MPRRKKKVKEVSESRNLEKKDVETTSSVSVKRKRRLEDAFIVISDSDGEEPKEENGLQK
TKTKQSNRAKCLAKRKIAQMTEEEQFALALKMSEQEAREVNSQEEEEEELLRKAIAESL
NSCRPSDASATRSRPLATGPSSQSHQEKTTDSGLTEGIW

Ub

MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNI
QKESTLHLVLRLRGGMQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFA
GKQLEDGRTLSDYNIQKESTLHLVLRLRGGMQIFVKTLTGKTITLEVEPSDTIENVKAKI
QDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGGMQIFVKTLTGKTIT
LEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRG
GMQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYN
IQKESTLHLVLRLRGGMQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFA
GKQLEDGRTLSDYNIQKESTLHLVLRLRGGMQIFVKTLTGKTITLEVEPSATIENVKAKI
QDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGGMQIFVKTLTGKTIT
LEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRG
GV

K48 linked $Ub_2$9pBpa with UBA2

Ub

MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNI
QKESTLHLVLRLRGGIIEPSLRILAQKYNCDKMICRKCYARLHPRATNCRKKKCGHTNN
LRPKKKLK

Appendix IV

Primer Sequences

Quick change, TAG stop codons, into different residues in Ub (pET11a -synUb construct)

F4_FWD (CL1747) CATATGCAGATTtagGTGAAAACCCTG

F4_REV (CL1748) CAGGGTTTTCACCTAAATCTGCATATG

T7_FWD (CL1826) CATATGCAGATTTTTGTGAAAtagCTGACCGGCAAAACC

T7_REV (CL1827) GGTTTTGCCGGTCAGCTATTTCACAAAAATCTGCATATG

L8_FWD (CL1784) CAGATTTTTGTGAAAACCtagACCGGCAAAACCATTAC

L8_REV (CL1785) GTAATGGTTTTGCCGGTCTAGGTTTTCACAAAAATCTG

T9_FWD (CL1774) GTGAAAACCCTGtagGGCAAAACCATTACC

T9_REV (CL1775) GGTAATGGTTTTGCCCTACAGGGTTTTCAC

G10_FWD (CL1828) GTGAAAACCCTGACCtagAAAACCATTACCCTG

G10_REV (CL1829) CAGGGTAATGGTTTTCTAGGTCAGGGTTTTCA

T12_FWD (CL1830)

GTGAAAACCCTGACCGGCAAAtagATTACCCTGGAAGTGGAACCG

T12_REV (Cl1831)

CGGTTCCACTTCCAGGGTAATCTATTTGCCGGTCAGGGTTTTCAC

S20_FWD (CL1757) ACCCTGGAAGTGGAACCGtagGATACCATTGAAAATGTG

S20_REV (CL1758) CACATTTTCAATGGTATCCTACGGTTCCACTTCCAGGGT

N25_FWD (CL1759) ACCGAGCGATACCATTGAAtagGTGAAAGCGAAAATTCAG

N25_REV (CL1760) CTGAATTTTCGCTTTCACCTATTCAATGGTATCGCTCGGT

D32_FWD (CL1761) AAAGCGAAAATTCAGtagAAAGAAGGCATTCCG

D32_REV (CL1762) CGGAATGCCTTCTTTCTACTGAATTTTCGCTTT

R42_FWD (CL1832) CCGGATCAGCAGtagCTGATTTTTGCG

R42_REV(CL1833) CGCAAAAATCAGCTACTGCTGATCCGG

I44_FWD (CL1834) CCGGATCAGCAGCGTCTGtagTTTGCGGGCAAACAGCTG

I44_REV (CL1835) CAGCTGTTTGCCCGCAAACTACAGACGCTGCTGATCCGG

A46_FWD (CL1836) CAGCAGCGTCTGATTTTTtagGGCAAACAGCTGGAAGAT

A46_REV (CL1837) ATCTTCCAGCTGTTTGCCCTAAAAAATCAGACGCTGCTG

Q49_FWD (CL1838) CGTCTGATTTTTGCGGGCAAAtagCTGGAAGATGGTCGTACCCTG

Q49_REV (CL1839)

CAGGGTACGACCATCTTCCAGCTATTTGCCCGCAAAAATCAGACG

E51_FWD (CL1786) GGCAAACAGCTGtagGATGGTCGTACCCTGAGCG

E51_REV (CL1787) CGCTCAGGGTACGACCATCCTACAGCTGTTTGCC

D52_FWD (CL1788) GGCAAACAGCTGGAAtagGGTCGTACCCTGAGCG

D52_REV (CL1789) CGCTCAGGGTACGACCCTATTCCAGCTGTTTGCC

T66_FWD (CL1753) ATTCAGAAAGAAAGCtagCTGCATCTGGTGCTG

T66_REV (CL1754) CAGCACCAGATGCAGCTAGCTTTCTTTCTGAAT

H68_FWD (CL1790) GAAAGAAAGCACCCTGtagCTGGTGCTGCGTCTGCGTG

H68_REV (CL1791) CACGCAGACGCAGCACCAGCTACAGGGTGCTTTCTTTC

L69_FWD (CL1840)

CAGAAAGAAAGCACCCTGCATtagGTGCTGCGTCTGCGTGGCGGC

L69_REV (CL1841)

GCCGCCACGCAGACGCAGCACCTAATGCAGGGTGCTTTCTTTCTG

V70_FWD (CL1842) GAAAGCACCCTGCATCTGtagCTGCGTCTGCGTGGCGGC

V70_REV (CL1843) GCCGCCACGCAGACGCAGCTACAGATGCAGGGTGCTTTC

Gibson cloning of Mb_PylRS gene into pSup vector (pSup-ThzKRS)

Mb_PylRS_FWD (CL1884)

GTTATACGTTGTTTACGCTTTGAGGAATCCCATATGATGGATAAAAAACCGCTGGAT

GTG

Mb_PylRS_REV (CL1885)

GCGCATCAGGCAATTTAGCGTTTGAAACTGCATTTACAGGTTCGTGCTAATGCC

pSup_FWD (CL1887)

ATGCAGTTTCAAACGCTAAATTGCCTGATGCGC

pSup_REV (CL1886)

ATGGGATTCCTCAAAGCGTAAACAACGTATAAC

Gibson cloning of Ub-inteinCBD into pCATUPP vector (pCNB-UbK11TAG)

pCATUPP_FWD (CL1926) aatcgaccatatgggaattcgaagcttgggccc

pCATUPP_REV (CL1927) ggttaattcctcctgttagcccaaaaaacgggt

Ub-inteinCBD_FWD (CL1928)

acccgttttttgggctaacaggaggaattaaccCATATGCAGATTTTTGTGAAAACCCTG

Ub-inteinCBD_REV (CL1929)

gggcccaagcttcgaattcccatatggtcgattATCCGGATATAGTTCCTC

Arg Mutation (pTXB1 constructs)

K48R FWD (CL2109)

AGCAGCGTCTGATTTTTGCGGGCCGTCAGCTGGAAGAT

K48R REV (CL2110)

ATCTTCCAGCTGACGGCCCGCAAAAATCAGACGCTGCT

K63R FWD (CL1982)

AATATTCAGCGTGAAAGCACCCTGCAT

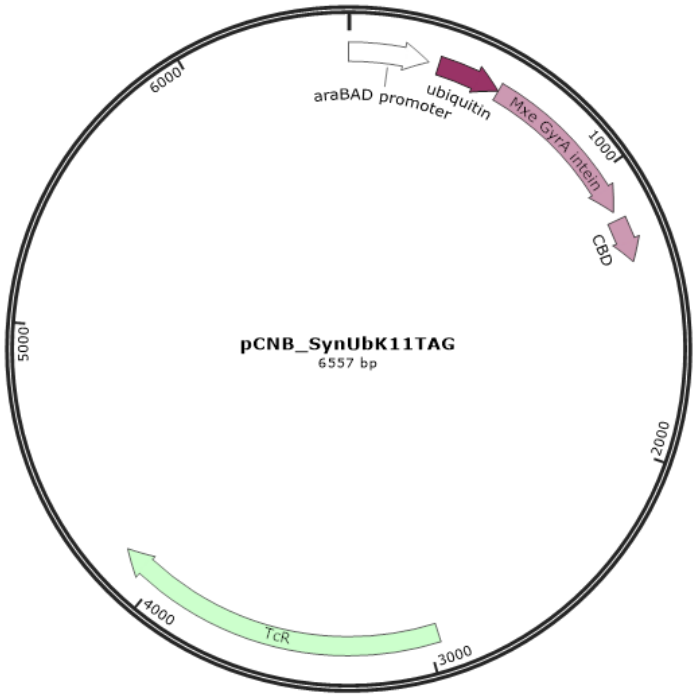
K63R REV (CL1983)

ATGCAGGGTGCTTTCACGCTGAATATT

Appendix IV

DNA Sequences and Plasmid Maps

pCNB-SynUbK11TAG



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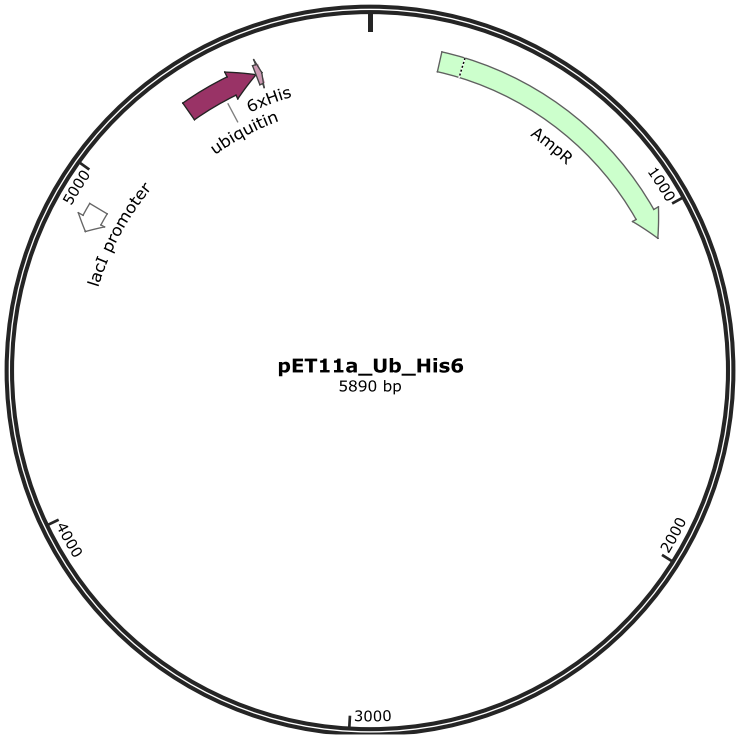

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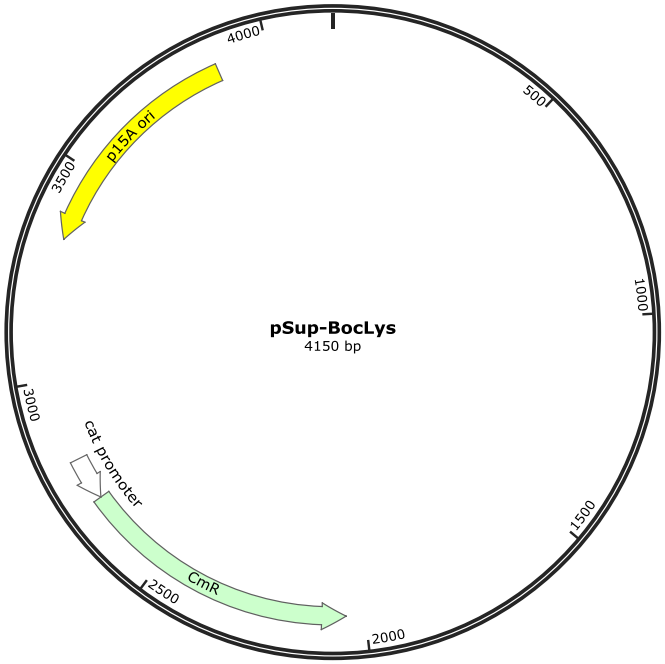

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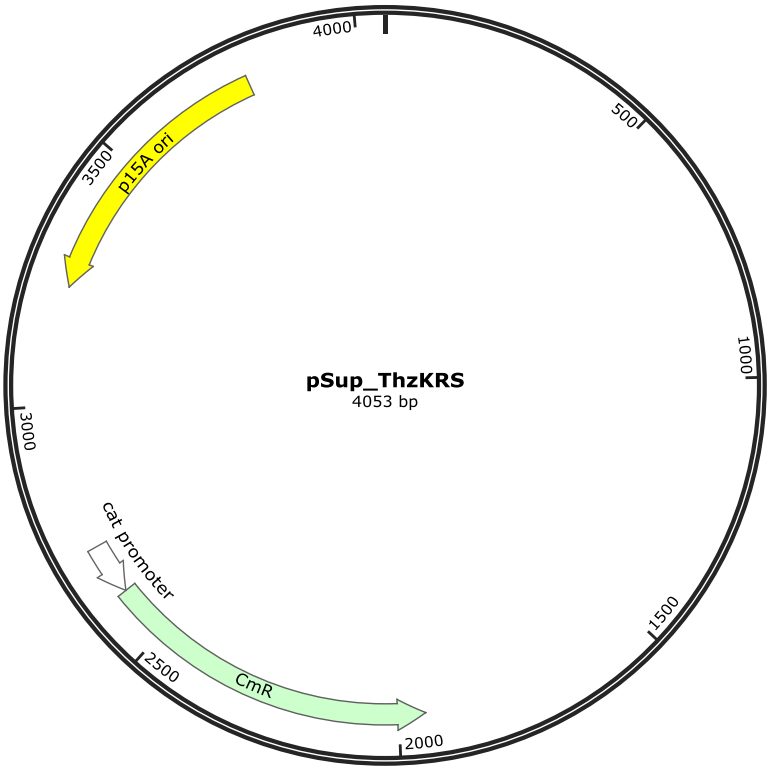
pSup-ThzKRS



M. Bakari ThzKRS

*proK-Mb*PylT sequence highlighted in blue (core MbPylT sequence shaded)

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