DEVELOPMENT OF A PEPTIDE BASED E3 LIGASE REPORTER

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ABSTRACT

Gregery Woss: Development of Peptide Based E3 Ligase Reporter
(Under the direction of Nancy L. Allbritton)

This dissertation describes the development of a new analytical tool capable of analyzing E3 ligase activity, a critical element in the ubiquitin proteasome system. This was accomplished through the development of a novel class of peptide-based fluorescent E3 ligase reporters which are intended to be analyzed by capillary electrophoresis (CE). These E3 ligase reporters incorporate a degradation signal, or degron, which is recognized and ubiquitinated by an E3 ligase, as well as a fluorescent tag to facilitate detection by laser induced fluorescence (LIF). In order to identify potent degrons, a library of E3 ligase reporters incorporating degrons from various sources was synthesized. These reporters were characterized in cell lysates, then optimized to produce substrates that would be efficiently ubiquitinated in cells. In addition to working with minimal degron sequences, an expanded sub-library of reporters based around the degron isolated from p53 was characterized in order to identify a compact degron element and investigate its ubiquitination kinetics and specificity. In order to circumvent peptidase-mediated degradation, a traditional weakness of peptide reporters, investigations were also carried out to develop novel degradation-resistant E3 ligase reporters incorporating β-hairpin secondary structures. Serendipitously, a β-hairpin that was both peptidase-resistant and functioned as an E3 ligase reporter was identified. Finally, electrophoretic separation conditions for the analysis of E3 ligase peptide reporters were identified, and ubiquitination of E3 ligase reporters in cell

lysates was observed by CE-LIF. This dissertation lays the groundwork for future development of next generation peptide-based E3 ligase reporters.

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LIST OF ABBREVIATIONS AND SYMBOLS

6-FAM	6-Carboxyfluorescein
A	alanine
AML	acute myeloid leukemia
ATP	adenosine triphosphate
ATP-ERRS	adenosine triphosphate energy regenerating solution
°C	degrees Celcius
CD	circular dichroism
cDNA	complementary deoxyribonucleic acid
CE	capillary electrophoresis
CE-LIF	capillary electrophoresis with laser-induced fluorescence detection
cm	centimeter
CO2	carbon dioxide
C-terminus	carboxy terminus
d	diameter
D	aspartic acid
Da	Daltons
Degron	degradation signal
DIPEA	N,N-diisopropylethylamine
DMEM	Dulbecco’s modified eagle medium
DMF	dimethylformamide
DNA	deoxyribonucleic acid
dP	D-proline
DTT	dithiothreitol
DUB	deubiquitinating enzymes
E	glutamic acid
E1	E1 ubiquitin activating enzyme
E2	E2 ubiquitin conjugating enzyme

E3	E3 ubiquitin ligase
EDTA	ethylene diamine tetraacetic acid
e.g.	for example
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionization
F	phenylalanine
FBS	fetal bovine serum
FRET	Förster resonance energy transfer
g	acceleration due to gravity
G	glycine
h	hours
H	Histidine
HBTU	2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HCl	hydrochloric acid
HDM2	human double minute 2 homolog
HECT	homologous to the E6-AP carboxyl terminus
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
H2O	water
HOBt	hydroxybenzotriazole
HPLC	high performance liquid chromatography
I	Isoleucine
ID	inner diameter
ISG15	interferon stimulated gene 15
K	lysine
kcat	turnover number
KM	Michaelis-Menten constant
kV	kilovolt
L	length

LIF	laser induced fluorescence
LOD	limit of detection
μ	electrophoretic mobility
μA	microamps
μ_{eo}	electroosmotic mobility
μ_{ep}	electrophoretic mobility
μg	microgram
μJ	microJoules
μL	microliter
μM	micromolar
μm	micrometers
M	molar
M	mass
m	meter
MALDI	matrix-assisted laser desorption/ionization
MDM2	mouse double minute 2 homolog
MeUb	methylated ubiquitin
mg	milligram
$MgCl_2$	magnesium chloride
min	minutes
mL	milliliter
mm	millimeters
mM	millimolar
MM	multiple myeloma
MPER	mammalian protein extraction reagent
mRNA	messenger ribonucleic acid
MS	mass spectrometry
ms	millisecond

η	viscosity
N	asparagine
NaCl	sodium chloride
NaH_2PO_4	sodium phosphate
NaOH	sodium hydroxide
NEDD8	neural precursor cell expressed developmentally down regulated protein 8
nL	nanoliter
nM	nanomolar
nm	nanometer
NMP	N-methyl-2-pyrrolidone
NMR	nuclear magnetic resonance spectroscopy
No K Ub	no lysine ubiquitin
N-terminus	amino terminus
O	ornithine
OD	outer diameter
π	pi
P	proline
PBS	phosphate-buffered saline
PCV	packed cell volume
PEG	polyethylene glycol
PMT	post-translational modification
PolyUb	polyubiquitin
PyBOP	benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
Q	glutamate
Q-PCR	quantitative polymerase chain reaction
R	Arginine
RFU	relative fluorescence units
RING	really interesting new enzyme

RPMI	Roswell Park Memorial Institute
RT-PCR	reverse transcription polymerase chain reaction
s	seconds
S	serine
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
SILAC	stable isotope labeling with amino acids in cell culture
SPPS	solid phase peptide synthesis
SUMO	small ubiquitin-like modifier
t	time
T	threonine
t1/2	half-life
TFA	trifluoroacetic acid
TIPS	triisopropylsilyl
TUBE	tandem Ubiquitin Binding Entity
Ub	ubiquitin
UbL	ubiquitin like
UPS	ubiquitin proteasome system
V	Valine
V	volts
v	velocity
W	tryptophan
x	times
Y	tyrosine

Chapter 1: Introduction

1.1 The Ubiquitin Proteasome System

The ubiquitin proteasome system (UPS) is an essential enzymatic pathway that is crucial to cell homeostasis, migration, and proliferation of eukaryotic cells.[1] The UPS is comprised of several families of enzymes and collectively functions to degrade damaged, misfolded, or excess proteins, to modulate the abundance of key regulatory proteins, and to post-translationally modify proteins via the conjugation of the small protein ubiquitin (ubiquitination).[2-4] Ubiquitination is at the heart of the UPS's tight regulation, and is carried out by a cavalcade of progressively specific enzyme families (Figure 1.1).

The process begins when ubiquitin, a 76-amino acid protein found throughout the cell, is activated by an E1 activating enzyme (E1) in an ATP-driven process. This results in the formation of a high-energy thioester bond between ubiquitin and the E1. This complex then interacts with a second family of enzymes, an E2 ubiquitin-conjugating enzyme (E2), and the activated ubiquitin is transferred to form a new ubiquitin-E2 complex. A third class of enzymes, the E3 ubiquitin ligases (E3), mediate the transfer of ubiquitin directly (RING family of E3 ligases) or indirectly (HECT family of E3 ligases) to a targeted protein.[2] Ubiquitin forms an isopeptide bond via the amine group on a proximal lysine (K) or ornithine (O) residue on the targeted protein. Following the initial ubiquitination event, additional ubiquitin proteins can be conjugated to one of the original ubiquitin moieties via one of seven lysine residues (e.g. K11, K48, or K63) to form a poly-ubiquitin chain.[5] The structure and length of the poly-ubiquitin chain dictate the effect ubiquitination has on a protein; for example, poly-ubiquitin chains

formed on the K48 residue is recognized by regulatory elements of the proteasome, while poly-ubiquitin chains formed on the K63 residue play a role in cell signaling, endocytic trafficking, and DNA damage repair.[5] While the UPS is comprised of a conglomeration of enzymes, E3 ligases are largely responsible for the UPS's high level of specificity. E3 ligases recognize targeted proteins through a degradation signal, or a degron. Degrons can be simple amino acid sequences or more complex, requiring post-translational modifications such as phosphorylation or oxidation.[3] The role that E3 ligases play as the ultimate arbitrators of ubiquitination is reflected in their expansive diversity, with over 600 distinct E3 ligases documented in humans.[6]

Degradation of proteins via the UPS is catalyzed by the proteasome. The 26S proteasome is a large, multi-unit protein consisting of a 19S regulatory cap and a 20S core particle.[4] The 19S cap has multiple functions: it recognizes proteins targeted for degradation through poly-ubiquitin chains, cleaves ubiquitin from proteins prior to degradation, and unfolds and threads the protein into the 20S core for degradation. The 20S core of the proteasome is barrel shaped and contains catalytic sites with trypsin-like, chymotrypsin-like, and peptidylglutamyl-peptide hydrolyzing proteolytic activities. These catalytic sites are responsible for the cleavage of proteins into short peptide fragments. Adding a further level of refinement to the UPS are deubiquitinating enzymes (DUBs), which selectively cleave ubiquitin from substrates. DUBs work in opposition to E3 ligases, rescuing targeted proteins from being degraded by the proteasome and allowing ubiquitin to be efficiently recycled by the cell.

While not the focus of the research presented here, it is worth noting that the UPS is paralleled by several ubiquitin-like systems (UbL). UbLs make use of proteins analogous to ubiquitin, E1, E2, E3, and DUB proteins. UbLs have been shown to play a role in many important cellular processes, including DNA repair and transcriptional regulation.[7] Prominent

examples of UbLs include the small ubiquitin-like modifier (SUMO), neural precursor cell expressed developmentally downregulated protein 8 (NEDD8), and interferon stimulated gene 15 (ISG15).

1.2 Importance of the Ubiquitin Proteasome System in Biomedical Research

Dysregulation of UPS components, such as E3 ligases and the proteasome, have been observed in several disorders, including multiple cancers and neurodegenerative diseases.[8] These observations, paired with the central role the UPS plays in cell homeostasis though ubiquitination and though the degradation of proteins, have made components of the UPS the foci of intense research and interest.[9] This interest rapidly increased following the clinical success of bortezomib (Velcade®), a small molecule inhibitor of the proteasome, which demonstrated the potential benefits of developing novel therapeutics targeting the UPS.[10, 11]

Bortezomib was approved in 2007 for the treatment of multiple myeloma, and later for acute myeloid leukemia (AML).[10] AML is a cancer of the myeloid line of blood cells found in bone marrow. These cancerous cells quickly crowd out normal cells, which interferes with production of healthy blood cells, in turn resulting in the development of anemia, infection, and eventual death. AML predominantly afflicts older patients, accounting for 14,590 new cases (30%) of newly diagnosed leukemia among elderly patients in 2013, according to the American Cancer Society. The majority of patients with AML are precluded from undergoing conventional intensive chemotherapy due to the high risk of treatment-related mortality.[12] This results in a grim prognosis for this group of patients, with a five-year survival rate of 25%. The development of Bortezomib and its successor Carfilzomib (Kyprolis®) proved a major breakthrough in the treatment of AML; a number of studies have since demonstrated the efficacy of Bortezomib in treatment of AML, both as a standalone therapeutic and in combination with

other drugs.[13, 14] Dysregulation of protein production in AML creates an overreliance on the proteasome to degrade these proteins. As a result, incomplete inhibition of the proteasome proves too stressful for cancer cells to overcome, while simultaneously being less cytotoxic to healthy cells than traditional chemotherapeutics.

In addition to multiple myeloma and AML, members of the UPS are suspected to play a direct role in other cancers and neurodegenerative diseases.[10, 15, 16] Though there are conflicting reports, decreased proteasome activity is suspected to play a key role in conformational diseases such as Parkinson's and Huntington's disease.[17] These diseases are characterized by the accumulation of misfolded proteins, such as amyloid plaques, with decreased proteasome activity suggesting it may be an attractive target for inhibition. Additionally, dysregulation of other UPS components has been observed in neurodegenerative diseases, such as the frame-shifted ubiquitin UBB^{+1}.[18] This mutated form of ubiquitin contains an extra 19 amino acids on the Ub C-terminus preventing it from being activated by E1 enzymes, potentially putting additional stress on the UPS. Furthermore, the tumor suppressor p53 and its E3 ligase Hdm2 are well known to play a role in cancers, highlighting an additional role for the UPS in disease.[19]

In healthy cells, regulation of p53 is maintained by constant degradation; however, when cells are subjected to genotoxic stress, p53 degradation is suppressed.[20] This allows p53 to accumulate in cells, which in turn leads to an upregulation of genes associated with apoptosis or growth. Because of p53's dysregulation in many cancers, Hdm2 has become the target of intense interest. Finally, the dysregulation of DUBs such as CYLD and USP9 have been observed in cancers, suggesting they likely play an important role as oncoproteins.[14, 21]

These observations precipitated a push to develop new therapeutics targeting specific dysregulated enzymes upstream of the proteasome, such as E3 ligases and deubiquitinating

enzymes (DUBs) and many such inhibitors are currently in clinical trials.[9] Despite the drive to develop these novel drugs targeting the UPS, current analytical tools used to investigate the enzymatic activity of these components leave much to be desired, most conspicuously there is no assay currently capable of directly measuring the activity of UPS components, such as E3 ligases, in clinical samples.

1.3 Survey of Current Techniques for Measuring E3 Ligase Activity in the UPS

Several technologies including Western blots, qPCR (quantitative polymerase chain reaction), and FRET (Förster resonance energy transfer) sensors, are represented widely in the literature for measuring E3 ligase activity. Each technique has advantages and drawbacks, requiring them to be employed thoughtfully and under controlled conditions.

Western blots are among the most popular techniques for investigating specific enzymes, and are considered the gold standard of ubiquitination experiments. This workhorse technique is highly selective, does not require genetic engineering, and does not require specialized instrumentation. In a typical Western blot analysis, cells are lysed and their contents separated based on molecular size using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins are then transferred to a nitrocellulose membrane where they are incubated with antibodies specific for the protein of interest. Unbound antibodies are washed away and the sample is incubated with a second antibody conjugated to a fluorescent handle. The sample is imaged and the amount of targeted protein is quantified. While very common, Western blots suffer from several limitations. Western blots measure total amount of an enzyme, such as an E3 ligase; however, this may not necessarily be a good proxy for that enzyme's activity, because it does not account for modifications such as phosphorylation or small mutations that may affect functionality. Additionally, traditional Western blots require relatively large sample sizes, which

limits their usefulness in analyzing samples from patients, where total cell numbers are low. This bulk analysis only returns information on the average amount of enzyme in a sample, potentially masking important subpopulations within the sample. Finally, while many antibodies are commercially available, Western blot analysis is restricted by the lack of appropriate antibodies.

Reverse transcription polymerase chain reaction (RT-PCR) is a powerful analytical technique that overcomes the size limitations of Western blots, allowing for measurement of mRNA in samples as small as single cells. The technique utilizes reverse transcriptase to convert mRNA into DNA and makes use of real time PCR (qPCR) to quantify the amount of the newly produced complementary DNA (cDNA). The technique is compatible with very small sample sizes (≥ 1 cell) and does not require genetic engineering approaches, so it is capable of analyzing patient samples. However, this technique does not directly measure protein activity or protein abundance, instead measuring mRNA levels for a protein, an indicator twice removed from the desired metric.

Förster resonance energy transfer (FRET) sensors allow for sensitive and high throughput screens of E3 ligase activity in cells.[22] E3 ligase FRET sensors work by genetically engineering cells to produce an E3 ligase substrate with a fluorescent donor molecule and a ubiquitin with an acceptor molecule. The activity of the E3 ligase then brings the acceptor and donor molecule into close proximity, allowing for the detection of a fluorescent signal when interrogated with the appropriate wavelength of light. This system is particularly ideal for screening large numbers of compounds for inhibitory activity. FRET sensors have the benefit of directly measuring E3 ligase activity, and are highly sensitive, allowing for use with small numbers of engineered cells. The major drawback of the technique is that it requires genetic engineering approaches, making

for long system development times and making the technique impractical for investigation of primary cells.

1.4 E3 Ligase Peptide Reporters for Chemical Cytometry

While each of the previously described techniques is powerful in the proper context, none meet the high requirements required to analyze patient samples (Figure 1.2). Chemical cytometry overcomes these limitations by directly measuring enzyme activity in small samples without the need for genetic engineering. This strategy revolves around using externally synthesized probes, such as peptide reporters labeled with fluorescein, and introducing them into a cellular environment, where they act as a substrate for an enzyme of interest. The reporters are directly modified by the enzyme of interest and substrate and product can readily be quantified by chemical cytometry techniques. Post-translational modifications, (*e.g.*, phosphorylation) can be quantified in samples as small as single cells by separating analytes via capillary electrophoresis (CE) and detecting them with highly sensitive laser induced fluorescence (LIF).[23-26]

Peptide-based reporters can be synthesized using the well-established technique of solid phase peptide synthesis (SPPS), allowing for rapid prototyping and bypassing genetic engineering requirements. Reporters are directly modified by enzymes, yielding a direct measurement of enzyme activity. And finally, the extremely small sample size requirements, exceptional separation efficiency, and peak capacity of CE-LIF allow for the detection of enzymatic activity in samples as small as single cells (~1 pL) with limits of detection as low as 10^{-21} mol.[23]

1.5 β-Hairpins Protectides

One drawback to traditional peptide reporters is they are often rapidly degraded by intracellular peptidases. It has been reported that this degradation can be diminished through the incorporation of unnatural amino acids, but this process must be carefully tailored to individual peptide reporters, requiring careful characterization following each modification.[25] Secondary structures, such as β-hairpins have been shown to increase peptide lifetimes in the presence of intracellular peptidases.[27] In collaboration with and building upon work done by members of the Waters laboratory, initial plans were to append such a structure, termed a protectide, to a peptide reporter as a means of increasing the reporter's lifetime in the face of peptidases.[28] Serendipitously, however, previous group members in the Waters and Allbritton group discovered the β-hairpin peptide that the Waters group designed served as an independent degron. Chapter four of this work describes the characterization of this highly robust peptide reporter.

1.6 Capillary Electrophoresis

Capillary electrophoresis is a separations technique first described by Jorgenson and Lukacs.[29] CE separation occurs in a fused silica capillary with a small inner diameter that is filled with a current-carrying buffer solution. Following introduction of sample, both ends of the capillary are immersed in buffer and a high electric voltage is applied across the capillary. Analytes migrate within the capillary based on their electrophoretic mobility (a combination of their charge, size, and shape) and electroosmotic flow of the buffer. The electrophoretic mobility of a sphere can be approximated using equations 1.1 and 1.2, while the velocity of a species in CE is defined by equation 1.3.[29]

Equation 1.1:

$$\mu = \frac{q}{6\pi\eta r}$$

μ = electrophoretic mobility

q = particle charge

η = buffer viscosity

r = Stoke's radius (Equation 1.2)

Equation 1.2

$$M = \frac{4\pi r^3 V}{3}$$

M = mass

r = Stoke's radius

V = partial specific volume

Equation 1.3

$$v = (\mu_{ep} + \mu_{eo})E$$

v = velocity

μ_{ep} = electrophoretic mobility

μ_{eo} = electroosmotic mobility

E = applied electric field

The electroosmotic flow of buffer is a phenomenon that results from the small internal diameter of the capillary and the charged inner surface of that capillary. For a fused silica capillary, this charge is dependent on the buffer utilized as well as any surface modifications. Capillaries without chemical surface modifications, a neutral or basic buffer will result in a more highly negatively charged capillary surface, attracting a large number of positively charged ions

to associate with the inner walls. This causes negative ions in solution to create an electric double layer. When voltage is applied across the capillary, the positively charged ions migrate towards the negative pole. These cations associated with the electric double layer interact with other components of the buffer through hydrogen-bonding and electrostatic interactions, and within the narrow confines of the capillary these interactions are strong enough to cause bulk migration of the buffer flow. This results in consistent flow towards the detector with a nearly flat fluidic profile that sweeps all analytes towards the detector. Depending on its strength, this can allow the detection of neutral or even negatively charged species. CE can be coupled with laser-induced fluorescence detection (LIF), allowing for detection of fluorescent analytes at extremely low concentrations (10^{-21} moles).[23] The high resolving power and small sample requirements (< 10 nL) of CE, paired with the high sensitivity of LIF, makes it an ideal technique for the analysis of small numbers of cells, including single cells.[23, 25, 26]

1.7 Scope of the Research

This dissertation details the development of novel peptide reporters for the measurement of E3 ligase activity via chemical cytometry. Chapter two focuses on the early development of a library of E3 ligase reporters incorporating degrons from various sources in order to identify, characterize, and optimize substrates that would be efficiently ubiquitinated in cells. This work has been published in both Cell Biochemistry and Biophysics and PLOS ONE.[30, 31] Chapter three details the creation of a sub-library of reporters around the degron isolated from p53. This work both identified a minimal degron element and investigated its ubiquitination kinetics and specificity. The results of this work were reported on in the Analyst.[32] Chapter four focuses on the characterization of novel degradation-resistant E3 ligase reporters with β-hairpin secondary structures. This work has been submitted to ACS Chemical Biology for publication. Finally,

chapter five presents unpublished work that lays the groundwork for analysis of E3 ligase peptide reporters by CE.

1.8 Figures

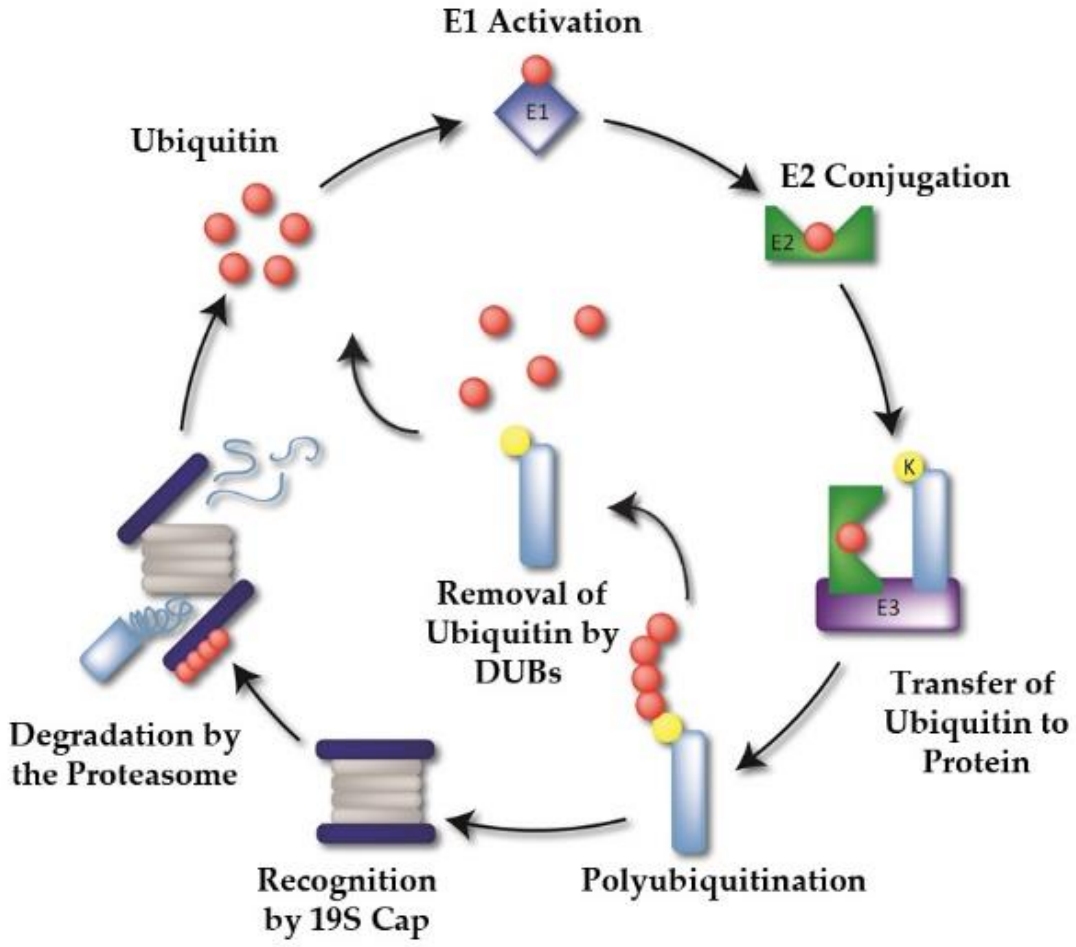

Figure 1.1 A simplified overview of the ubiquitin proteasome system (UPS). Ubiquitin is activated by an E1 activating enzyme and transferred to an E2 conjugating enzyme. This complex then interacts with an E3 ligase, which recognizes targeted proteins and facilitates the conjugation of ubiquitin to a proximal lysine. The conjugated ubiquitin can have further ubiquitin molecules added to, forming a poly-ubiquitin chain. This poly-ubiquitin chain is recognized by the 19S regulatory element of the proteasome, which unfolds the protein into the catalytic core, resulting in the degradation of the protein. Deubiquitinating enzymes (DUBs), including those on the 19S regulatory element of the proteasome, can cleave ubiquitin from a protein, allowing it to be recycled by the cell.

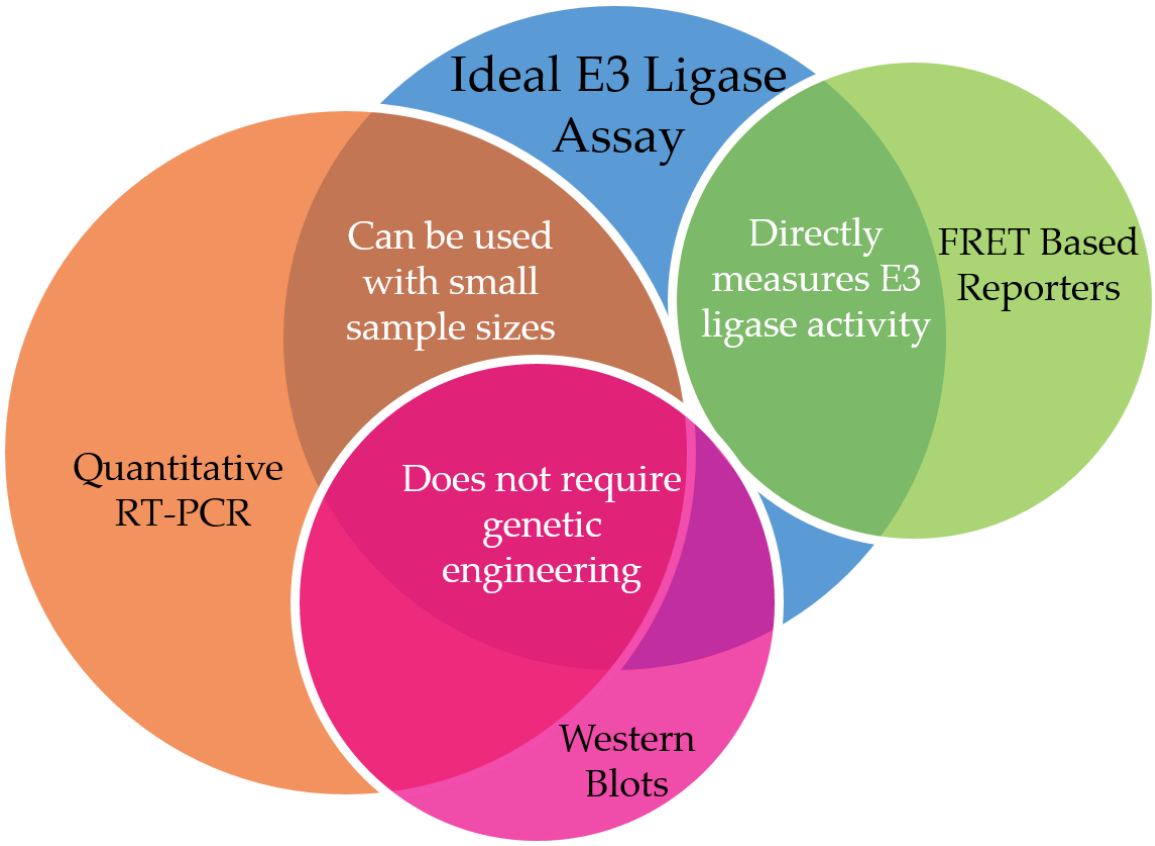

Figure 1.2 Limitations of current analytical techniques for measuring E3 ligase activity. Current popular techniques do not encompass the requirements for the analysis of patient samples. Western blots are the most popular form of analysis, but require large sample sizes and do not directly measure enzymatic activity. RT-PCR is compatible with small sample sizes, but measures levels mRNA, a proxy twice removed from ubiquitination. FRET-based reporters directly measure E3 ligase activity, but require genetic engineering approaches, requiring large initial numbers of cells and long development times.

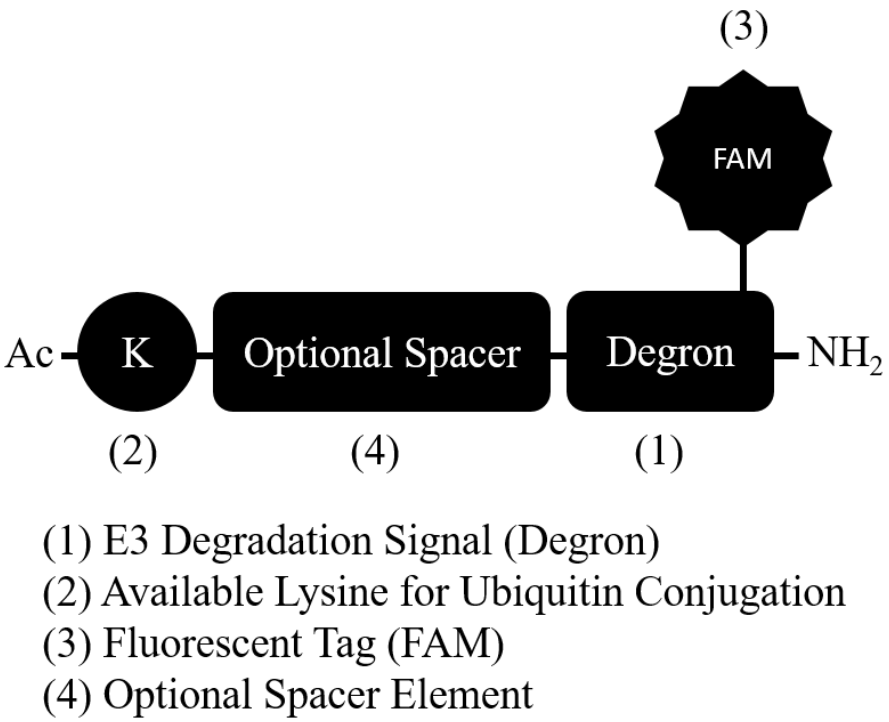

Figure 1.3 General design of peptide reporters. Reporters consist of three key elements: (1) an E3 binding recognition site (degron), (2) a proximal lysine residue for ubiquitin conjugation, and (3) a fluorescent tag for detection and quantification. Additionally (4), an optional spacer element, can be included to modulate reporter length for optimal posting of the proximal lysine.

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Chapter 2: Generating a Degron Reporter Library

2.1 Introduction

The ubiquitin proteasome system (UPS) is a key enzymatic pathway that plays a central role in cell homeostasis.[1, 2] The UPS is composed of a constellation of enzymes with diverse functions, which are collectively responsible for the degradation of misfolded and damaged proteins. This process is regulated through the post translation modification of proteins by ubiquitin (i.e., ubiquitination), which, in addition to its role in the degradation of proteins plays an important role in DNA repair, cell cycle regulation, and immune responses. The UPS is comprised of several enzyme families: Ubiquitin, E1 and E2 enzymes, and the E3 ligase. Ubiquitin is a small, 76 amino acid protein found throughout the cell and primarily functions to post translationally modify UPS-targeted proteins. E1 ubiquitin-activating enzymes chemically activate ubiquitin in a process consuming adenosine triphosphate (ATP) to chemically activate ubiquitin, which is then accepted by E2 ubiquitin-conjugating enzymes. Finally, E3 ligases interact with the E2-ubiqutin complex and facilitate the process of conjugating the activated ubiquitin to an available lysine residue on a targeted protein. Either the E3 ligases bring E2 enzymes and substrates together or they act as an intermediary for the activated ubiquitin to accomplish the conjugation. Additionally, E3 ligases recognize proteins through specific degradation signals, or degrons, on target protein substrates.[3] These substrates can be simple amino acid sequences, or more complex, requiring post translation modifications such as phosphorylation or oxidation. The E3 ligase is primarily responsible for the tightly controlled

regulation of the UPS; they are a highly diverse class of enzymes comprised of over 600 proteins in humans.

Following an initial ubiquitination event, the conjugated ubiquitin protein can accommodate further ubiquitin conjugations via one of its seven lysine residues. The number and subsequent position of ubiquitin molecules determine the signaling properties of the attached ubiquitin moieties.[4] For instance, ubiquitin chains (i.e., poly-ubiquitin) formed at the 48th position lysine residue target proteins to be sent to the 26S proteasome for degradation.[5] The 26S proteasome is a large protein complex that recognizes poly-ubiquitin, then unfolds and threads a targeted protein into its catalytic core, where it is subsequently degraded into small peptide fragments. Acting in opposition to E3 ligases are deubiquitinating enzymes (DUBs), which selectively cleave the conjugated ubiquitin from a protein, potentially rescuing it from degradation and allowing ubiquitin to be removed from a protein prior to degradation.

Dysregulation of the E3 ligases in particular has been observed in a number of diseases, including several cancers.[6-9] Drugs that target elements of the UPS, such as the proteasome inhibitor Bortezomib and the second generation Carfilzomib, have found clinical success treating multiple myeloma, demonstrating that components of the UPS are excellent candidates for targeted therapeutics.[7, 10] Dysregulation of the E3 ligases in particular has been observed in a number of diseases, including several cancers.[6-9] While there is intense interest in the research community to investigate components of the UPS, current analytical tools used to probe the enzymatic activity of these components leave much to be desired.[1]

Several technologies including Western blots, qPCR (quantitative polymerase chain reaction), FRET (Förster resonance energy transfer) sensors, and proteomic approaches such as SILAC (stable isotope labeling with amino acids in cell culture) are used widely in the literature;

however, each have drawbacks that make them problematic for investigations into primary cell samples: They either probe enzymatic activity indirectly, require genetic engineering, or require large numbers of cells and thus only provide bulk analyses.[11-19] Chemical cytometry overcomes these limitations by using peptide-based reporters as substrates for enzymes of interest, making it an attractive alternative analytical strategy.[20] This strategy revolves around the use of peptide based reporters that act as substrates for enzymes of interest. These reporters can be introduced into cells or cell lysates, where they are recognized by select enzymes and undergo post-translational modifications, (e.g., phosphorylation).[21-24] The reporters can be conjugated to a fluorescent tag such as fluorescein, allowing highly sensitive laser induced fluorescence (LIF) detection to be used. This, when paired with the extremely small sample size requirements, exceptional separation efficiency, and peak capacity of capillary electrophoresis (CE), can detect enzymatic activity in samples as small as single cells. Through well-established solid phase peptide synthesis (SPPS), peptides can be rapidly synthesized and prototyped, by passing genetic engineering requirements, and peptides measure rates of post-translational modification, allowing for direct measures of enzymatic activity.

Given the importance of E3 ligases in regulating and facilitating protein ubiquitination, their penultimate position in the UPS, and their observed dysregulation in several diseases, they present an excellent target for new peptide reporter technologies. Peptide reporters require three key components in order to investigate E3 ligase ubiquitination kinetics: (1) a degron for E3 ligase recognition, (2) an available lysine residue for ubiquitination, and (3) a fluorescent tag for detection (Figure 2.1A). We also speculate that a fourth element, a spacer, might be required to prevent steric hindrance between elements of the reporter. This work describes the development of a small library of nine reporters that contain elements from both natural and artificial sources,

to determine an optimal substrate for measuring ubiquitination.[1, 25] Particular interest was paid to the formation of poly-ubiquitin chains versus multiple mono-ubiquitination events. An additional library of iterations of the top performing initial reporters were synthesized to investigate the effect of the spacer element and determine the position of the most rapidly ubiquitinated lysine residue. Additionally, a computational model was developed to quantitate and describe the ubiquitination process.

2.1.1 Initial Selection of Reporter Library and Reporter Design

Initially, nine degrons were selected by Dr. Adam Melvin and Dr. Kaiulani Houston for their potential use as an E3 ligase reporter.[26-35] The origin and sequence of each degron used in the initial reporter library are listed in Table 2.1, along with the E3 ligases known to recognize them. These selected peptides were the result of an extensive literature search, and come from several representative classes of degrons, including degrons that require phosphorylation prior to recognition by E3 ligases (TAZ, IFNAR1, SRC3, and Cyclin D1), and degrons that are oxidation dependent (HIF-1α). Degrons were also pulled from a number of different sources including: tumor suppressors (p53), proteins that are key to cell homeostasis (iNOS, β-Catenin), and artificial sources (Bonger).[26-36]

All degrons were next incorporated into a standard design for peptide reporters. Each peptide reporter consisted of a degron, an N-terminal lysine, and a C-terminal fluorescent tag. Because the degrons pulled from the literature had highly variable sequence lengths that ranged from as few as four amino acids (Bonger) to as many as 25 (p53), spacer elements were incorporated to standardize the size of the initial reporters and minimize steric effects between reporter elements. Reporters containing small degron sequences were extended to approximately 25 amino acids using protein sequences from the NCBI database. In the event that the degron

sequence did not contain a lysine proximal to the degron, as was the case for iNOS and SRC3, we incorporated polyethylene glycol (PEG) into the reporter to provide functional distance between the degron and lysine residue. During the initial round of synthesis, a triple lysine C-terminus was designed into each peptide to provide net positive charge and thus promote peptide solubility in aqueous solution, and also to minimize steric interactions between the fluorescein tag and amide resin during synthesis.

2.2 Experimental Design

2.2.1 Peptide Reporter Synthesis

All reporters were synthesized using SPPS, either manually or with an automated synthesizer (Creosalus TetrasUI Peptide Synthesizer, Louisville, KY). Synthesis was carried by Dr. Adam Melvin, Dr. Kaiulani Houston, and Gregery Woss, with the Waters laboratory providing expertise and guidance. Peptide synthesis was performed on CLEARamide resin beads (Peptides International Louisville, KY), using Fmoc-[N]-protected amino acids purchased from Advanced Chem Tech (Louisville, Kentucky). The Fmoc-[N]-protected unnatural amino acids Fmoc-NH-(PEG)-COOH, Fmoc-Ser[PO(OBzl)-OH]-OH, Fmoc-Thr[PO(OBzl)-OH]-OH, and Fmoc-Lys(ivDde)-OH were purchased from EMD Biosciences (Billerica, MA). 5,6-carboxyfluorescein acquired from Sigma (Saint Louis, MO) was used to fluorescently tag peptides.

Fmoc deprotection was carried out by gently agitating beads in a DMF (dimethylformamide) solution containing 2% piperidine and 2% DBU (1,8-diazobicyclie[5.4.0]undec-7-ene). Deprotection was carried out twice over the course of 15 minutes. For peptides containing aspartate residues, an alternative deprotection solution containing 20% piperidine and 0.1 M HOBt (hydroxybenzotriazole) in DMF was used. Amino acid activation was carried out in a solution of NMP (N-methyl-2-pyrrolidone) containing four equivalents of HBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate), four equivalents of HOBt,

and five equivalents of DIPEA (N,N-diisopropylethylamine). Amino acid coupling was carried out twice for 30-60 minutes each. To conserve regents, unnatural amino acids were coupled in a single reaction over the course of four hours using five equivalents of the unnatural peptide and four equivalents of HBTU, HOBt, and DIPEA.

Following the addition of the final amino acid, peptides had their N-terminus acetylated. This was accomplished by reacting the peptide with 5% acetic anhydride and 6% 2,6-lutidine in DMF for 35 minutes. Next, the ivDde protecting group was removed to allow for conjugation of the fluorescent tag. This was done by mixing the peptide with a solution of 3% anhydrous hydrazine in DMF for three minutes three times. A Kaiser test confirmed the ivDde group removal. To conjugate the peptide and fluorescent tag, four equivalents of 5,6-carboxyfluorescein and four equivalents of HOBt and PyBOP (benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate) were mixed in DMF overnight in the dark. Finally, peptide was cleaved from the resin using a mixture of 95% TFA (trifluoroacetic acid), 2.5% TIPS (triisopropylsilyl) and 2.5% water for 3 hours. Excess TFA was evaporated under a steady flow of nitrogen, and cleavage products were precipitated into ice cold ether. This mix was extracted into water, lyophilized, and stored at -4°C until purification.

Following synthesis, all peptides were purified using reverse phase HPLC on an Atlantis C-18 semi-preparative column (Waters, Morrisville, NC). Purification was carried out by either Dr. Melvin or Gregery Woss. Peptides were first purified using a 60-minute gradient from 95% acetonitrile, 5% H_2O and 0.1% TFA to 95% H_2O, 5% acetonitrile, and 0.1% TFA. Peptide masses were verified using MALDI (matrix-assisted laser desorption/ionization) mass spectrometry. Following verification of peptide mass, the peptides were lyophilized and then purified a second time using the same gradient as above but extended to a 100-minute duration.

After purification, peptides were suspended in 50 mM phosphate buffer (pH 8.0) and quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA) at the 5,6-carboxyfluorescein tag absorbance wavelength, 492 nm. Peptides were aliquoted and stored at -20 °C.

2.2.2 Generation of HeLa S100 Lysates and Cell Cultures

To ensure a consistent and potent source of UPS components, HeLa S100 lysates were generated and frozen for use throughout the peptide library development process. HeLa S100 lysates were generated by either Dr. Adam Melvin or Gregery Woss. HeLa S3 cells (ATCC Manassas, VA) were initially grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% v/v bovine calf serum (HyClone, GE Healthcare Logan UT) and maintained in a 37 °C, 5% CO_2 environment. Following initial thaw, cells were grown in T-75 culture flasks until they reached 70% confluence. Following this, cells were rinsed with PBS and treated with 2 mL trypsin-EDTA for five minutes or until detached, at which point they were transferred to a centrifuge tube and spun at 800g for 10 minutes. Media was discarded and cells were resuspended in fresh media and expanded into additional flasks. This process continued until eight flasks reached 70% confluence. Cells were then pooled together, the media was discarded, and the cells were resuspended in suspension growth media (Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 5% v/v bovine calf serum) to a density of 2 x 10^5 cells/mL in a 250 mL spinner flask. Cells were monitored closely and fresh media was added daily to maintain a cell density of 2 x 10^5 and 5 x 10^5 cells/mL. Once media volume exceeded 250 mL, cells were spun down at 800g for 10 min and resuspended in fresh media in a 1 L spinner flask at a density of 2 x 10^5 cells/mL. This process was repeated until the suspension

volume reached 1 L, at which point the above process was repeated and the cells transferred to a 3 L spinner flask.

Cells were allowed to grow with fresh media being added periodically until the suspension reached a volume of 3 L and a density of 4-6 x 10^5 cells/mL. The media was removed and cells were washed in ice cold PBS. Cells were spun at 1200g for 10 minutes at 4 °C. The resulting pellet volume was recorded as packed cell volume (PCV). PBS supernatant was removed from the cells and replaced with 5 x PCV hypotonic buffer (10 mM HEPES, pH 8.0, 10 mM KCl, 1.5 mM $MgCl_2$, and 1 mM DTT), which caused the cells to swell over 10 minutes at 4 °C. Cells were spun at 1800g for 10 minutes at 4 °C and the hypotonic buffer was removed. Cells were resuspended in 2 x PCV hypotonic buffer and lysed using approximately 10 strokes of a Dounce glass homogenizer. The resulting lysate was centrifuged for 10 minutes at 1200g, at which point the resulting supernatant was collected and supplemented with 0.11 x PCV of buffer B (0.3 M HEPES, pH 8.0, 1.4 M KCl,and 30 mM $MgCl_2$). The resulting lysates were placed in an ultra-centrifuge and spun at 100,000g for 60 minutes at 4 °C. The cytosolic supernatant was collected and dialyzed twice in buffer D (20 mM HEPES, pH 8.0, 100 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, 1 mM DTT, and 20% v/v glycerol) at 4 °C, first overnight and then again for four hours. The reduced lysate was centrifuged one final time at 33,000g for 20 minutes at 4 °C to remove membrane proteins. The cytosolic lysates were quantified using a Nanodrop 2000, aliquoted, and stored at -80 °C.

For studies using other cell lines, fresh cell lysates were prepared shortly before each experiment. HeLa, HL-60, THP-1, and U937 cells were all acquired from the Lineberger Tissue Culture Facility at the University of North Carolina at Chapel Hill. HeLa cells were grown in DMEM with 10% v/v fetal bovine serum (FBS, Atlanta Biologics, Flowery Branch, GA). THP-1

cells were grown in RPMI 1640 supplemented with 10% v/v FBS and 0.05 mM 2-mercaptoethanol. U937 cells were cultured in RPMI 1640 with 10% v/v FBS. HL-60 cells were grown in RPMI 1640 with 10% v/v FBS and 1% v/v penicillin-streptomycin.

2.2.3 In Vitro Ubiquitination Assay

Peptide performance was measured using an *in vitro* ubiquitination assay using HeLa S100 lysates as the source of UPS components. *In vitro* ubiquitination assays were carried out by Dr. Adam Melvin or Gregery Woss. The reaction was carried out in 20 μL of a reaction buffer consisting of 10 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, and 2 mM DTT. This buffer was supplemented with 1 x ATP energy regenerating solution (ATP-ERS, Boston Biochem, Cambridge, MA), 100 mM of the proteasome inhibitor MG-132 (EMD Chemicals Billerica, MA), 20 mg/mL ubiquitin aldehyde (Boston Biochem, Cambridge, MA), 400 mg/mL ubiquitin or methylated ubiquitin (Enzo Life Sciences Farmingdale, NY), and 750 ng of inhibitors cOmplete ULTRA and PhosSTOP (Roche). HeLa S100 (2 mg/mL) lysates were thawed and immediately added to this reaction mixture prior to the experiment. The reaction was initiated with the addition of 750 ng of peptide, and the sample was incubated at 37 °C for a set period of time. The reaction was halted with the addition of 40 μL tricine sample buffer (Bio-Rad, Hercules, California) followed by boiling for 5 minutes.

2.2.4 Ubiquitin Pull Down Assay

In order to validate ubiquitination of peptide reporters, pull down experiments using agarose beads coated in ubiquitin antibodies were employed. Pull down experiments were carried out by either Dr. Melvin or Gregery Woss. The reaction buffer described in the previous section was prepared using 5x the original reaction volume (100 μL) while keeping the reagent concentrations constant. Pulldown reagents were prepared immediately prior to use. For each

sample, 20 µL of Control-Agarose bead slurry (LifeSensors Malvern, Pennsylvania) and 25 µL Agarose-TUBES1 slurry (Tandem Ubiquitin Binding Entity, LifeSensors Malvern, Pennsylvania) were thawed on ice and suspended in 1 mL of ice cold TBS-T (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% v/v Tween-20) and centrifuged at 1800g for 5 minutes at 4 °C. The TBS-T was removed from the bead pellet and discarded, then the beads were suspended in 1 mL of fresh TBS-T. This process was repeated for a total of 5 washes, after which beads were suspended in 100 µL of fresh TBS-T.

Following incubation of the reaction buffer, 300 µL of TBS-T was added to halt the reaction. Then, agarose control beads were added. The sample was then transferred to a tube rotator and incubated for 1 hour at 4 °C. Samples were centrifuged at 1800g for 5 min to pellet the control beads and the sample supernatant was transferred to a fresh centrifuge tube. Agarose-TUBES1 beads were added to the sample, and the sample was returned to the tube rotator and incubated overnight at 4 °C. Samples were centrifuged at 1800g, the supernatant discarded, and the beads suspended in 1 mL of TBS-T. This process was repeated five times to rinse away non-specifically adsorbed analytes. Following the final wash, beads were suspended in 50 µL tricine sample buffer and heated at 90 °C for five minutes to elute any analytes bound to the bead. Samples were centrifuged for 5 min at 13000g, and the resulting supernatant collected. Samples were either immediately analyzed or kept at -20 °C until analysis.

2.2.5 Ubiquitination Assays Carried out in Cell Lysates

Ubiquitination assays in cell lysates were carried out using the same protocol as the *in vitro* ubiquitination pull downs, with the major exception being the substitution of freshly prepared cell lysates for HeLa S100 lysates, with a final lysate protein concentration of 2 mg/mL. Ubiquitination assays in cell lysates were performed by either Dr. Melvin or Gregery

Woss. Lysates were prepared from tissue cultured cell lines immediately prior to the assay. Once cells had reached the appropriate confluence or cell density, cells were centrifuged at 800g for 2 minutes, and washed twice in PBS. Cells were pelleted and re-suspended in approximately 1 x cell pellet volume using MPER lysis buffer (Mammalian Protein Extraction Reagent, Fisher, Hampton, NH). This was vortexed at 900 RPM for 10 minutes at 25 °C, then centrifuged at 4 °C for 15 minutes at 14,000 x g. The resulting supernatant was collected and its protein concentration was quantified using an M5 spectrophotometer (Molecular Devices, Sunnyvale, California). The sample was stored on ice until use.

2.2.5 SDS-PAGE Analysis

Samples were separated using SDS-PAGE and analyzed using fluorescence imaging. SDS-PAGE analysis was carried out by either Dr. Melvin or Gregery Woss. Samples were loaded into precast 16.5% Mini PROTEAN Tris-Tricine (Bio-Rad Hercules, CA) using 20 μL of sample per well. The gel was subjected to a 120 V differential until tricine buffer bands reached the bottom of the gel. Benchmark fluorescent ladder (ThermoFisher, Waltham, MA) was included in all gels as a standard to estimate molecular weight of analytes. Gels were visualized using a Typhoon Imager (GE Healthcare Life Sciences, Pittsburgh, PA) at the highest sensitivity possible without saturating the detector. Gels were quantified using ImageJ software (US National Institute of Health), and measured sample band intensity relative to the unreacted parent peptide band intensity.

2.2.6 Data Analysis

Dr. Adam Melvin was primarily responsible for the development of computational models. Data was analyzed using Origin 7.0 (OriginLab), equations were solved in MATLAB

using ODE15s. Additional information about the mathematical model used can be found in reference 25.

2.3 Results and Discussion

An initial library of nine reporters was initially synthesized using SPPS, then purified by two rounds of reverse phase HPLC. The molecular weight of the reporter was verified following purification using MALDI-TOF, after which it was reconstituted into 50 mM phosphate buffer (pH 8.0). Reporter concentrations were quantified using absorbance measurements. The peptides were stored at -20 °C until use.

While developing and evaluating the peptide library, it was key to have a potent and consistent source of UPS components. Cell lysates provided an excellent source of these components, as they contain a multitude of E1, E2, and E3 enzymes, enabling ubiquitination of peptides even with unknown E3 ligases. Due to cell heterogeneity, it was vital to prepare a large batch of lysates to perform separate ubiquitination experiments under reproducible conditions. To this end, HeLa S100 cytosolic lysates were selected as a source of UPS components. These lysates contain a litany of concentrated cytosolic enzymes, and could be prepared in large batches and frozen until use.

For ubiquitination experiments, HeLa S100 lysates were mixed with buffering reagents and supplemented with $MgCl_2$, ATP, and inhibitors for peptidases, phosphatases, and the proteasome. Excess ubiquitin was also added to the reaction buffer, either in the form of wild type ubiquitin or methylated ubiquitin (MeUb). MeUb was chosen to probe mono-ubiquitination event kinetics because each of its lysine residues is blocked by methyl group; thus, MeUb is incapable of forming poly-ubiquitin chains.

Each of the nine initial reporters were evaluated in a time course assay and analyzed using SDS-PAGE (examples shown in Figure 2.2). Early work was done by Dr. Adam Melvin and Dr Kaiulani Houston, with Gregery Woss joining the project after initial synthesis and evaluation of several reporters. The fluorescent tag allowed for easy and sensitive detection of analytes without the need for antibodies. All nine initial reporters demonstrated an increase in ubiquitination over time, as expected; however, formation of ubiquitinated products differed between reporters, with some reporters producing more intense ubiquitination bands than others. Analysis also showed the formation of a low molecular weight band over time, attributed to the degradation of the parent peptide over time, which was expected even with the addition of peptidase inhibitor.

Of particular note was that the incorporation of MeUb into the assay did not always result in the formation of a single ubiquitination band (Figure 2.2D). This result strongly indicated that either multiple mono-ubiquitination events occurred at several of the reporter's lysine residues (multi-mono-ubiquitination), or that endogenous ubiquitin from the HeLa S100 was incorporated, contributing to a poly-ubiquitin chain. The latter was considered unlikely, as MeUb was calculated to exceed endogenous ubiquitin by a factor of 100 or more.[37]

In order to further validate that the ubiquitinated peptide accounted for the observed high molecular weight bands, an antibody-based pull down assay was carried out. Following the previously described ubiquitination reaction, samples were incubated with agarose beads coated in ubiquitin-specific antibodies. This allowed for the collection of only analytes containing an ubiquitin moiety, providing excellent supporting evidence that the observed bands were ubiquitinated reporter. As an additional benefit, the antibody pulldown also allowed for the concentration of ubiquitinated analytes (Figure 2.3).

2.3.1 Initial Comparison of Degron Ubiquitination Rates

All nine developed peptide reporters demonstrated time-dependent ubiquitination. In order to compare performance between reporters, the intensity of each band was normalized to the band intensity of an unmodified peptide that was not subjected to the ubiquitination assay. Within the nine peptide reporters surveyed, there was a high degree of variability in the formation rate of high molecular weight bands, demonstrating the importance of the degron component. Four of the initial reporters, Bonger, p53, and iNOS, and β-Catenin, showed particularly rapid ubiquitination kinetics, especially compared to the other five reporters (Table 2.2). Initial studies indicated that the reporter incorporating the degron from p53 was most rapidly ubiquitinated, and close inspection of the results revealed the presence of bands correlating to di-, tri-, and tetra-ubiquitination events (~21, ~30, and 39 kDa) in addition to mono-ubiquitination events. The origin of these bands were likely multi-mono-ubiquitination events, as the ratio of added MeUb was calculated to be in large excess of endogenous wild type ubiquitin (>100 times).[37] While this result was initially unexpected, it can be explained by the presence of multiple lysine residues on each reporter, either in the original degron sequence or as part of the triple lysine C-terminus added to aid in synthesis.

2.3.2 Effects of Degron Size and Lysine Position on Ubiquitin Rate

The next step in the reporter library development was to further refine the top reporters by investigating the effects of both the reporter size and the position of the most rapidly ubiquitinated lysine residue. Initially, all reporters were standardized in length (~25 amino acids); however, it was unclear if the additional residues were required or if they affected ubiquitination rates. For Bonger, iNOS, and β-Catenin peptides, a smaller minimal degron had been reported, whereas the minimal degron size for p53 was unknown.[26, 30, 35] Additionally, while the presence of multiple lysine residues could potentially enhance the overall

ubiquitination rate, multiple ubiquitination sites can confound kinetic analysis by convoluting poly- and multi-mono-ubiquitination events. To detect poly-ubiquitination chains is desirable not only because it could deconvolute kinetic measurements, but also because poly-ubiquitin chains serve as the recognition element for the 19S regulatory particle of the 26S proteasome. To investigate these effects, four reporter sub-libraries were synthesized using the top four preforming peptides (p53, Bonger, iNOS, and β-Catenin).

Initial work was done using β-Catenin, which performed well in early trials. The β-Catenin reporter initially incorporated a phosphorylated serine into the degron component, but during the design of the expanded library, this residue was substituted with phosphomimetic glutamic acid, which prevents dephosphorylation during a ubiquitination reaction. The substitution did not appear to have an effect on the ubiquitination rate (Figure 2.4D). Continued work proceeded to examine the effect of peptide length on ubiquitination rate, as work by Wu *et al.* had previously established the importance of the spacing between lysine residues and β-Catenin's minimal degron element.[35] A shortened β-Catenin was synthesized using the minimal degron element DEGIHEG; however, this version of the reporter exhibited markedly decreased ubiquitination kinetics (Figure 2.4D, lane 3). Given this finding, it is highly likely that the spacer amino acids play an important structural role in the ubiquitination of β-Catenin.

Following peptide length studies, we investigated the position of the most rapidly ubiquitinated lysine residue. Five additional variants of the reporter were synthesized, three full length versions and two shortened ones, with all but a single lysine residue substituted for either valine on the N-terminus or arginine on the C-terminus. The results demonstrate a clear and pronounced preference for a lysine residue on the C-terminal side of the full length peptide (Figure 2.4D lanes 4-8). Moving the lysine residue a single position away from the C-terminus

slightly decreased ubiquitination, but not as much as having an N-terminal lysine. Both shortened reporters had comparable and significantly reduced ubiquitin kinetics.

Similar to β-Catenin, development of the Bonger reporter focused on the importance of the proximal distance between the portable degron (RRRG) and the ubiquitination site. Three variants of the reporter were synthesized, beginning with a compact, nine amino acid variant (KRRRGK(FAM)KKK), as well as two variants incorporating PEG monomers: (K(PEG)RRRGK(FAM)KKK) and (K(PEG)$_2$ RRRGK(FAM)KKK). The triple lysine C-terminus was left in place to investigate degron size, but it also had an effect on poly-ubiquitination versus multi-mono-ubiquitination. The modified reporters were compared to the initial 'full sized' reporter (KTRGVEEVAEGVVLLRRRGNK(FAM)KKK) used in the initial screening process using a pull-down assay incorporating MeUb (Figure 2.4A, lanes 1-3). These results demonstrated that the Bonger based reporter only required the compact degron for ubiquitination, and that removing the spacing element did not negatively impact the ubiquitination rate. Additionally, we determined that the length of the reporter did not appear to play a role in the formation of multi-mono-ubiquitination.

Next, the preferential ubiquitination site was isolated. This was done by synthesizing variants of the reporter using either a triple arginine or triple glycine C-terminus. Both of these variants demonstrated marked decreases in ubiquitin band intensity, suggesting the preferential ubiquitination site was likely on the C-terminus (Figure 2.4A, lanes 4,5). This finding corresponded well with reports in the literature about the Bonger peptide. A new variant was synthesized that incorporated a single lysine residue on the C-terminus (VRRRGK(FAM)GGK); valine was chosen to replace lysine on the N-terminus in an effort to increase stability based on the N-end rule.[38] This new variant produced increased ubiquitin band intensity relative to that of

peptide reporters with only an N-terminal lysine, suggesting that the C-terminus lysine was preferentially ubiquitinated (Figure 2.4A lane 6).

A similar sub-library was generated for iNOS based around its portable degron (DINN). Similar to β-Catenin, removing the amino acid spacing elements significantly decreased the rate of ubiquitination (Figure 2.4B, lanes 1,2). Integrating a PEG spacer element did not recover the ubiquitination rate, suggesting that the amino acid spacer element may play an important role in enhancing ubiquitination rates, perhaps through an interaction with an E3 ligase. Further experiments demonstrated that the replacement of the triple lysine C-terminus with a triple arginine sequence did not further reduce the rate of ubiquitination, suggesting that the N-terminus contained the preferential ubiquitination site (Figure 2.4B, lane 3).

Refining the reporter containing the p53 degron was less straightforward because its portable degron was unknown. Because of this uncertainty, the site of the preferentially ubiquitinated lysine was first investigated. Variants of the p53 reporter where available lysine residues only presented on the N- or C-termini yielded similar results, which, paired with the presence of di-ubiquitin, suggested that a lysine residue in the degron sequence might be contributing to the rate of ubiquitination (Figure 2.4C, lanes 1,2). To test this hypothesis, a second set of peptides were synthesized with a single available lysine residue, either on the N-terminus or in the middle of the peptide. Both variants produced similar amounts of ubiquitination, with the N-terminal lysine variant demonstrating slightly faster kinetics. These results suggested that having a free lysine residue available in the reporter was sufficient for ubiquitination (Figure 2.4C, lanes 3,4). Once it was determined that no single free lysine was preferentially ubiquitinated, work was done to identify whether a degron sequence could be truncated without compromising the reporter ubiquitination rate. This was done by synthesizing three reporters (N-terminal, a middle

segment, and a C-terminal) using a shortened sequence selected at random (~9-10 amino acids). While all three reporters demonstrated ubiquitination, the peptide incorporating the middle segment performed best. These results suggest the previously reported portable degron for p53 might be further reduced and refined (Figure 2.4C, lanes 5-8).

In order to investigate whether reporter optimization had an impact on poly-ubiquitination, a final set of experiments was carried out with the optimized substrates for β-Catenin, Bonger, iNOS, and p53, using wild type recombinant ubiquitin (Figure 2.5). Each peptide reporter contained either a one (Bonger, iNOS, β-Catenin) or two (p53) free lysine residues, and upon analysis demonstrated the characteristic poly-ubiquitin ladder formation indicative of tetra-ubiquitin plus chain lengths.

2.3.3 Performance of Reporters in Different Cell Lines and *In Vitro* Ubiquitination Assay

The initial peptide library screen identified and optimized four top-performing peptide reporters incorporating degrons from Bonger, p53, iNOS, and β-Catenin. The next step in reporter development was to validate their performance outside of HeLa S100 lysates (i.e., in freshly prepared cell lysates), and to examine how they were affected by variable E3 ligase compositions and concentrations. HeLa S100 lysates used in the reporter development presented a potent and reproducible source of UPS associated enzymes, but only allowed investigation into a single, artificial cellular environment containing a fixed concentration of only cytosolic enzymes. Additionally, it was unknown what the effect of reporter optimization had on E3 ligase specificity. In order to investigate these effects, four cell lines were selected to investigate peptide performance: HeLa (adenocarcinoma), U937 (histiocytic lymphoma), THP-1 (acute monocytic leukemia), and HL-60 (acute promyelocytic leukemia). These assays were carried out in a similar fashion to those used in the HeLa S100 studies, with the substitution of freshly

prepared lysates and the addition of recombinant wild-type ubiquitin. While the precise concentration and composition of UPS components of each cell line was unknown, it was assumed that there would be variability between cell lines. This hypothesis appears to be supported by the results: HeLa lysates produced markedly similar results to those of the HeLa S100 lysates, as did U937 lysates. However, THP-1 and HL-60 lysates produced substantially diminished rates of ubiquitination, potentially indicating variability in UPS-associated enzymes between cells of different disease states (Figure 2.6A).

In order to investigate the specificity of the optimized p53 based reporter, experiments were carried out using a cell-free *in vitro* assay. The p53 reporter was incubated with recombinant E1, E2, and Hdm2 enzymes in the presence of ATP` and ubiquitin. When all components were present, the reporter was ubiquitinated; however, excluding either E1 or E2 enzymes prevented the reaction from proceeding. In contrast, while the absence of Hdm2 did affect the ubiquitination rate, it did not prevent ubiquitination. This indicated that Hdm2 was no longer essential for ubiquitination and suggested that the reporter was no longer specific to Hdm2 (Figure 2.6B, lanes 1-4). Follow-up studies using the Hdm2 inhibitors HLI-373 and NSC-66811 provided additional evidence supporting this idea, as neither drug prevented reporter ubiquitination. It should be noted that a high dose of NSC-66811 (1 μM) did reduce the ubiquitination rate, though it is unclear whether this is an effect of Hdm2 activity loss or a non-specific effect of the inhibitor. (Figure 2.6B, lanes 5-8)

2.3.4 Computational Modeling of Ubiquitination Rates

Kinetic analysis was computationally modeled by Dr. Adam Melvin using the generated peptide library; however, it warrants discussion as it informed further development of the reporter library. The goals of the kinetic model were to describe the ubiquitination process in a

stepwise function, ultimately determining the kinetics of ubiquitin conjugation to the peptide reporter. This model relied on first order kinetics to account for the addition of ubiquitin (both poly- and multi-mono events) as well as the degradation of ubiquitinated products. The model uses a Markov Chain Monte Carlo algorithm to fit the data collected from pulldown experiments using both wild-type and methylated ubiquitin (Figure 2.7). Based on these results, approximate rate constants for all nine reporters in the initial library screen were obtained (Table 2.2).

2.4 Conclusions

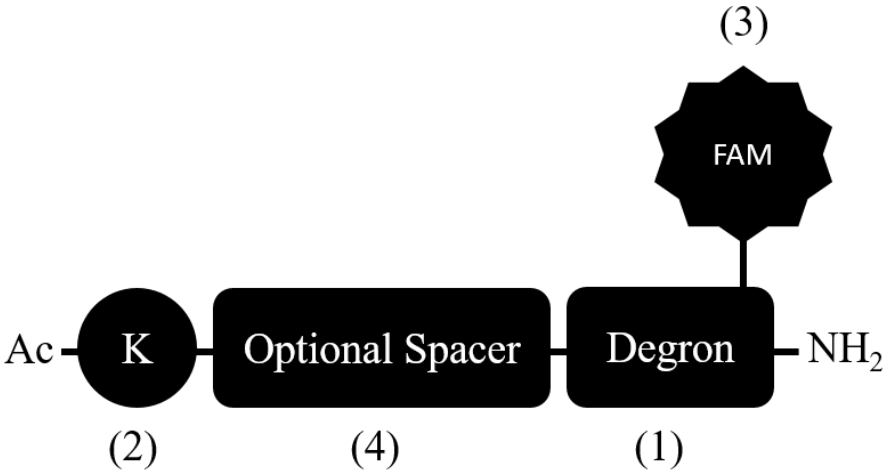
A library of nine novel peptide reporters that incorporated degrons from several sources were synthesized and analyzed using an *in vitro* cell-based assay. This work ultimately identified four candidate reporters for future development based on ubiquitination rates versus peptidase-mediated degradation. With growing interest in the UPS and the role it plays in disease, these reporters serve as the groundwork for a new technology to fill a current gap in analytical tools capable of measuring E3 ligase activity. Current techniques for investigating UPS activity often require large populations of cells or genetic modifications, restrictions which often exclude the potential for measuring samples obtained directly from a patient.
Peptide reporters offer a means to overcome these shortcomings, especially when paired with chemical cytometry techniques such as CE-LIF. In addition, peptide reporters boast several distinct advantages over current state of the art technologies. Peptide reporters act as substrates for E3 ligases and thus directly measure enzymatic activity, which allows them to account for upstream effects in the UPS. They are rapidly synthesized using SPPS, allowing rapid prototyping and refinement, as demonstrated by the construction of this library. While not explored in this work, they are also capable of being loaded into cells in a variety of ways, including: pinocytosis, incorporation of a TAT sequence, or the addition of a myristoyl tag.[39]

These reporters were initially developed in HeLa S100 lysates, which provided a reproducible source of E1, E2, and E3 enzymes. To quantify the kinetic parameters of the reporters, lysates were enriched with either wild-type or methylated ubiquitin. To further validate the reporters, additional pull down experiments were performed allowing for analysis of only analytes with an ubiquitin moiety. The kinetic parameters of the initial reporters were derived from time course assays, from which the best performers were selected for optimization. While kinetic studies have been done for some E3 ligases and specific degrons, there have been few comprehensive studies that examine multiple degrons under the same conditions.[40-43] This work directly compared degron performances from several sources in both cytosolic lysates and freshly prepared lysates. While the rates calculated for degrons with available data were lower than their predicated literature values, these values were obtained from artificial conditions, such as optimized *in vitro* recombinant protein assays or from using concentrated nuclear lysates.[41, 44]

One important characteristic of peptide reporters is their specificity, as there are several reports of promiscuity in kinase reporters throughout the literature; however, none have explored E3 ligases.[45-47] Studies using p53 suggested that Hdm2 is no longer required for ubiquitination, and that the p53 reporters may have significantly reduced specificity. This may prove to be an advantageous quality, as it could facilitate the development of a more global UPS reporter.

In conclusion, the body of work described in this chapter details the initial development of a peptide-based E3 ligase reporter that should be compatible with CE-LIF. The work identified four key degrons that were optimized and further refined to enable optomized kinetic analysis of ubiquitination rates.

2.5 Figures and Tables



(1) E3 Degradation Signal (Degron)
(2) Available Lysine for Ubiquitin Conjugation
(3) Fluorescent Tag (FAM)
(4) Optional Spacer Element

1.1

Figure 2.1 General design of library peptide reporters. Reporters consist of three key elements: (1) an E3 binding recognition site (i.e., degron), (2) a proximal lysine residue for ubiquitin conjugation, and (3) a fluorescent tag for detection and quantification. Additionally, (4) an optional spacer element was included to standardize reporter length.

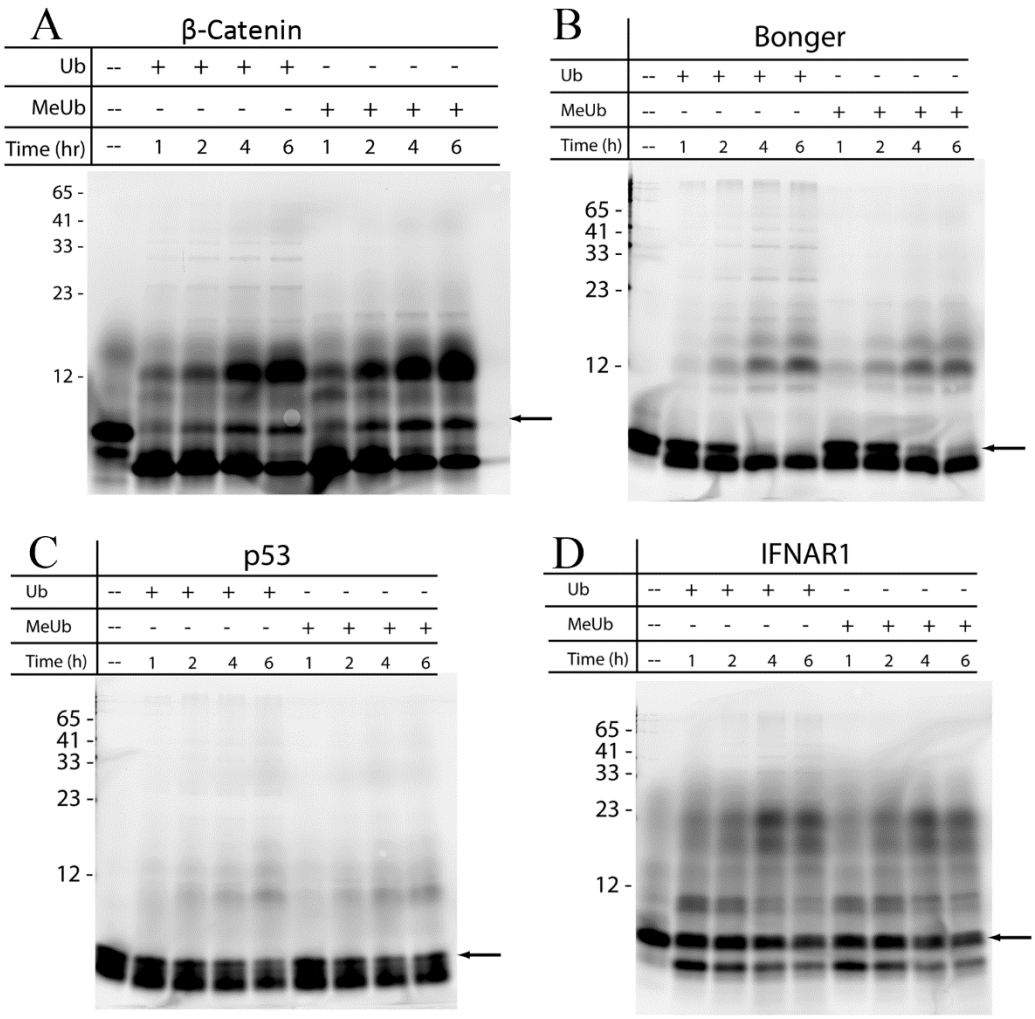


Figure 2.2 Preliminary validation of degron-based substrates. Four example results of *in vitro* ubiquitination assays supplemented with either wild-type or methylated ubiquitin (MeUb). As expected, the degree of ubiquitination increases for (A) β-Catenin, (B) Bonger, (C) p53, and (D) IFNAR1 peptide reporters over time, and at different rates, demonstrating the importance of the degron element. Unreacted peptide was run in the left-most lane to standardize results and to identify unreacted peptide, as indicated by the arrows.

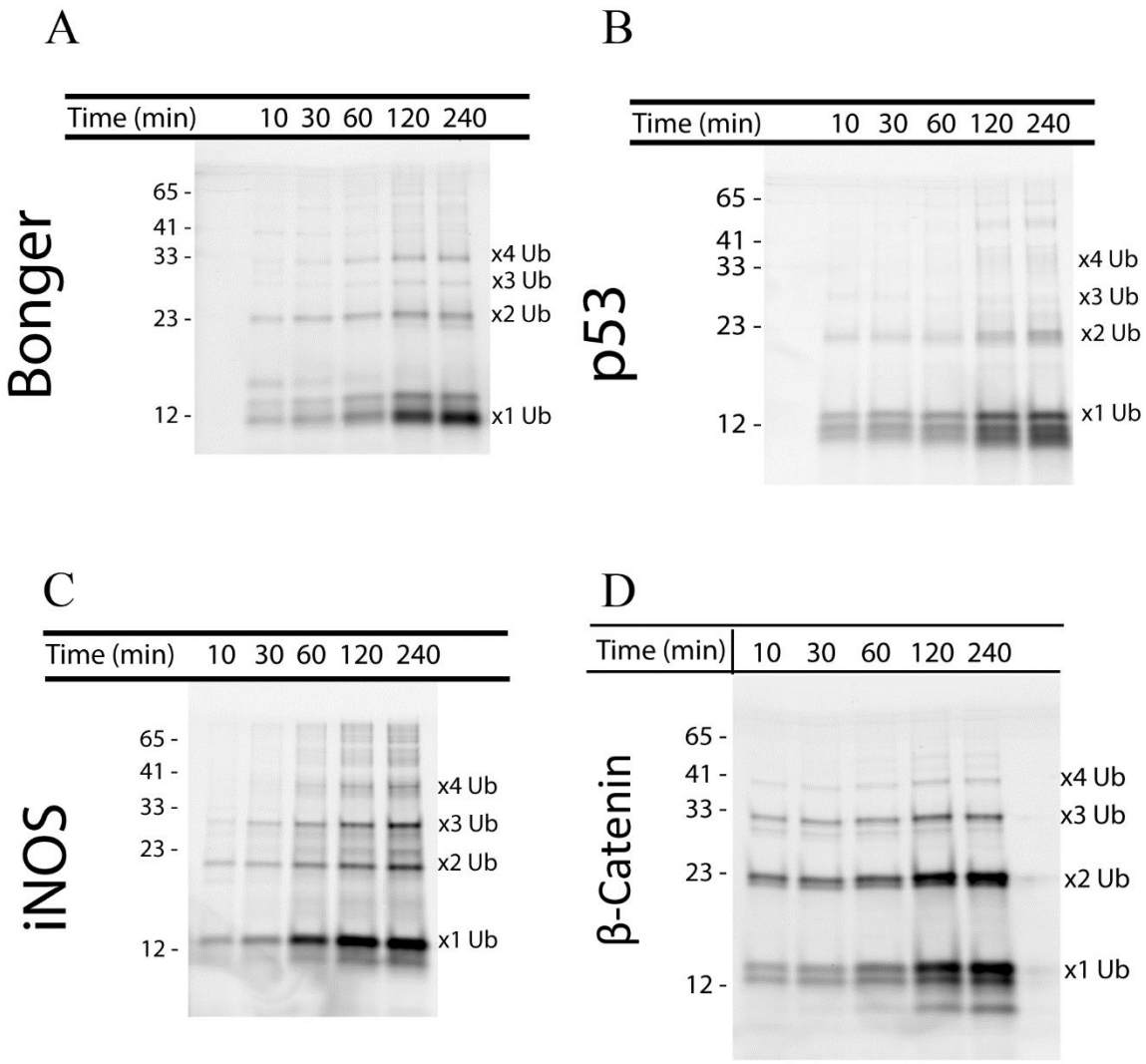


Figure 2.3 Ubiquitination assay pull down results. The results of ubiquitin pull down assays using wild type ubiquitin. The four best performing reporters are shown, which incorporated degrons from: (A) β-Catenin, (B) Bonger, (C) p53, and (D) iNOS. Assays were carried out in triplicate and used to calculate the kinetic parameters in Table 2.

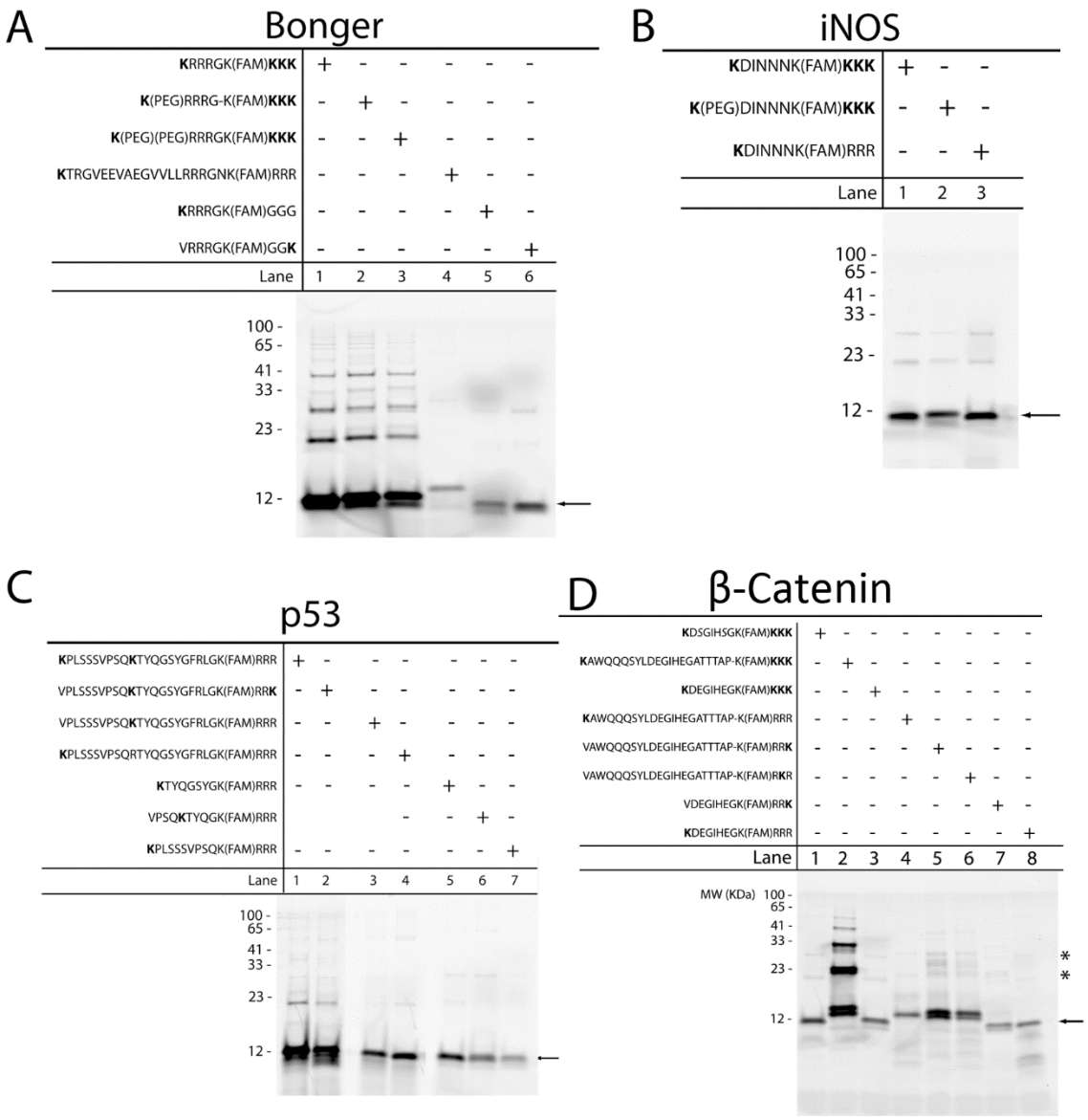


Figure 2.4 Pulldown results of the expanded libraries. The expanded peptide library for (A) Bonger, (B) iNOS, (C) p53, and (D) β-Catenin. *In vitro* ubiquitination assays supplemented with ubiquitin were carried out followed by an ubiquitin pulldown. Each expanded library was used to identify the optimal reporter length and lysine position. All samples were incubated in the *in vitro* ubiquitination assay for 2 hours.

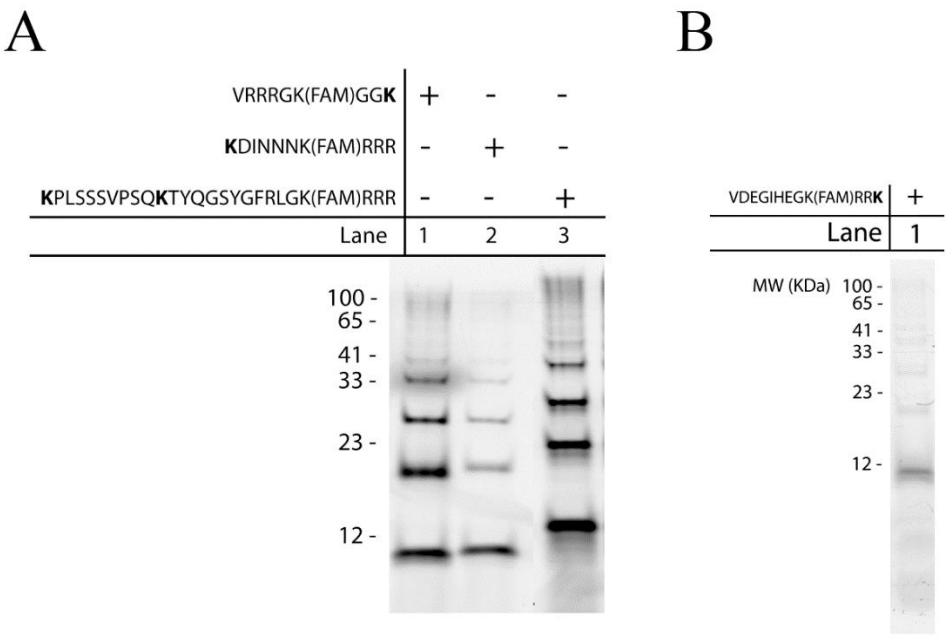


Figure 2.5 Poly-ubiquitination of optimized reporters. Optimized reporters for Bonger (A, lane 1), iNOS (A, lane 2), p53 (A, lane 3), and β-Catenine (B, lane 1) were subjected to an *in vitro* ubiqutination assay supplimented with wild-type ubiqutin for 2 hours, followed by a ubiquitin pull down. Poly-ubiqutination was observed in all cases, to varying degrees.

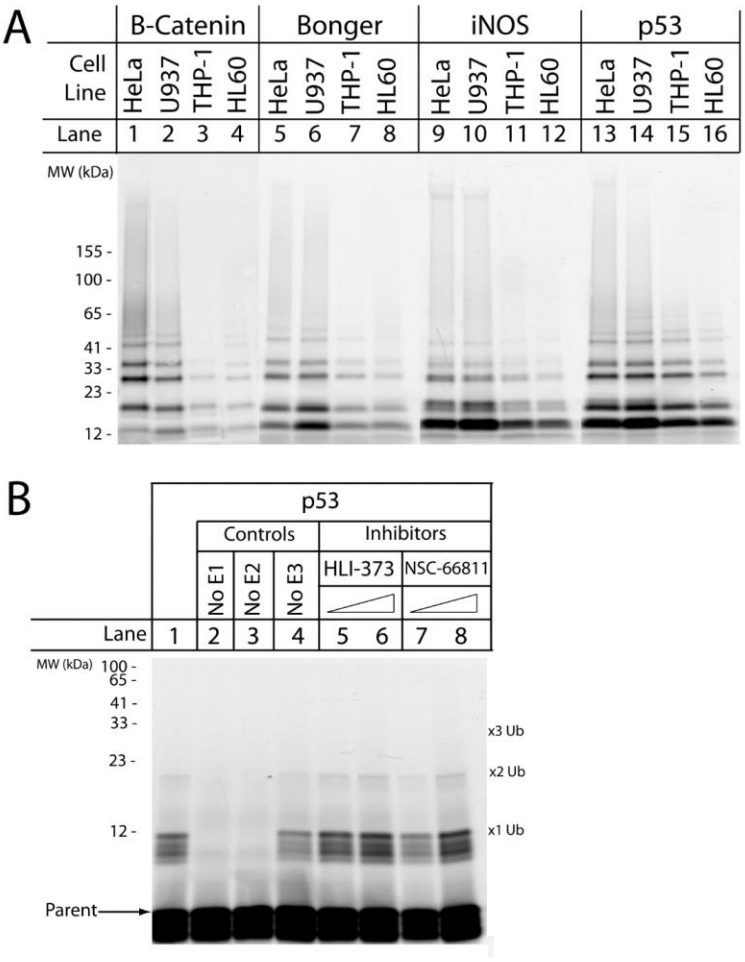


Figure 2.6 Optimized reporters ubiquitinated in cell lysates. (A) Top performing peptides were incubated for 2 hours in lysates from four different cell lines (HeLa, U937, THP-1, and HL60), supplemented with wild-type ubiquitin, then subjected to a ubiquitin pulldown. (B) A cell-free *in vitro* ubiquitination assay was carried out using the optimized p53-based reporter. Lane 1 contains all assay components. Lanes 2–4 are control experiments with key enzymatic components removed. The assay was probed using two commercial Mdm2 inhibitors: HLI-373 (1,10 mM, lane 5–6 respectively) and NSC-66811 (1,10 mM, lanes 7–8, respectively). All reactions were incubated for 2 hours at 30 °C prior to halting the reaction with sample buffer. Arrow indicates unmodified substrate.

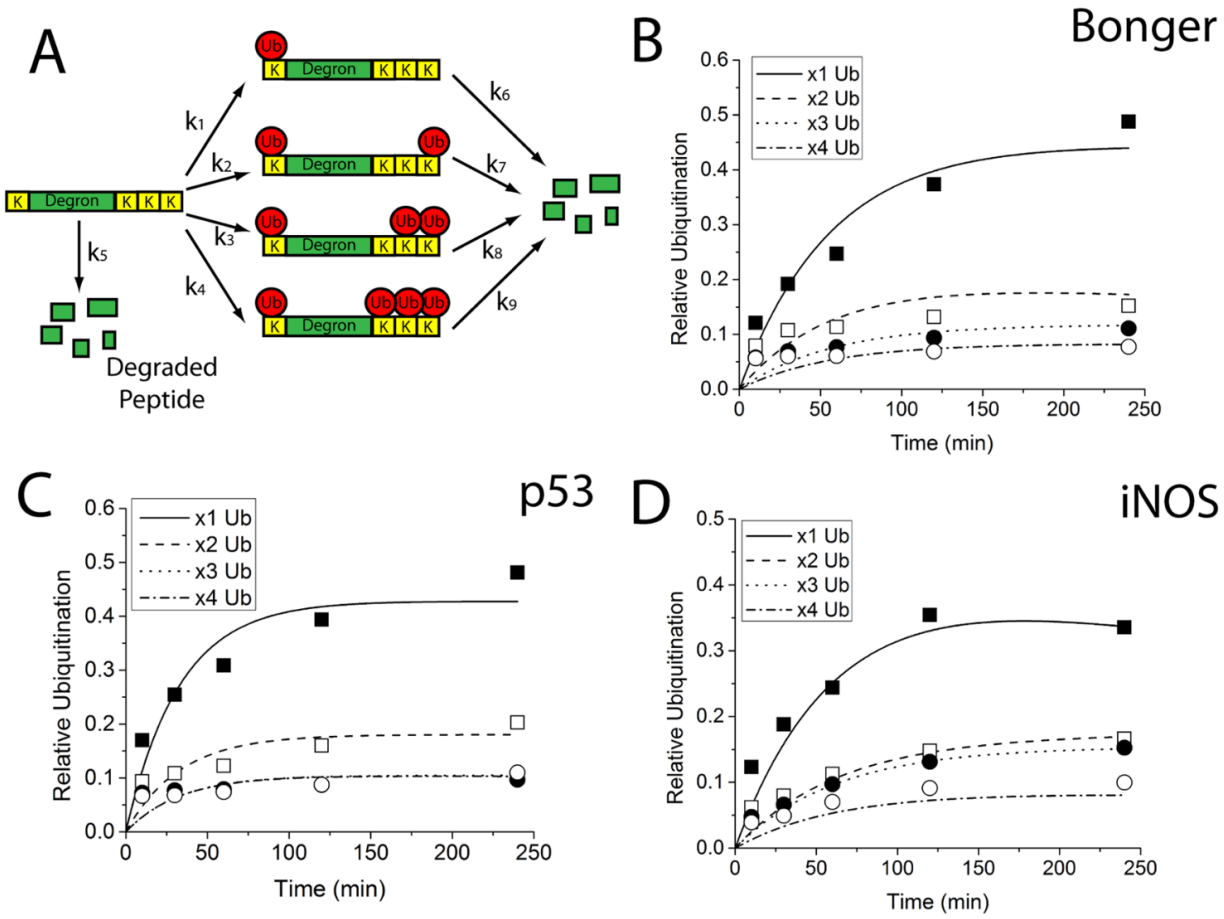


Figure 2.7 Kinetic analysis of reporter ubiquitination. (A) Proposed model for multi-mono-ubiquitination of the degron-based substrates. Rate constants correspond to Equations S1–S6 representing substrate ubiquitination and peptidase dependent degradation (see reference 25). Computational fits (lines) for the three degron based substrates: Bonger (B), p53 (C), and iNOS (D), based on experimental observations (symbols).

Table 2.1 Reporter library and degron origins. Reporters were named according to either the protein from which they derived, or the researcher who first described them. Underlined regions indicate the minimal degron (if known). Bold amino acids represented phosphorylated residues. The N-terminus of all peptides were acetylated and the C-terminus of all peptides were amidated.

Parent Protein	E3 Ligase	Peptide Sequence	Reference
Bonger	Unknown	KTRGVEEVAEGVVLLRRRGNK(FAM)KKK	26
TAZ	$SCF^{\beta TrCP}$	KPFLNGGPYHSREQ**S**TD**S**GLGLGSYK(FAM)KKK	27
HIF-1α	VHL	ASADLDLEALAPYIPADDDFQLRK(FAM)KKK	28
iNOS	Unknown	K-(PEG)-KEEKDINNNVKKTK(FAM)KKK	30
SRC3	SPOP	K-(PEG)-DVQKADV**SS**TGQGIDSK(FAM)KKK	31
Cyclin D1	SCF^{FBX4}	KAAEEEEVSLAS**T**PTDVRDVDIK(FAM)KKK	32
IFNAR1	$SCF^{\beta TrCP}$	KKYSSQTSQD**S**GNYSNK(FAM)KKK	29
p53	Mdm2	KPLSSSVPSQKTYQGSYGFRLGK(FAM)KKK	34
β-Catenin	$SCF^{\beta TrCP}$	KAWQQQSYLD**S**GIH**S**GATTTAPK(FAM)KKK	35

Table 2.2 Reporter rate constants determined by kinetic analysis. Rates k_1-k_4 represent ubiqutination, while k_5-k_9 represent peptidase-dependent degradation as determined by a computational model (see Figure 2.7 and reference 25).

(1/min)*10^3	Bonger	p53	iNOS	IFNAR1	TAZ	Cyclin D1	SRC3	HIF-1α	β-Catenin
k_1	8.1	13.1	6.8	7.2	6.1	3.4	2.8	2.6	6.2
k_2	3.6	5.5	2.7	5.2	7.2	5.9	1.5	3	6.4
k_3	2.1	3.2	2.5	2.1	4.7	4.1	1.2	1.9	2.6
k_4	1.5	3.2	1.4	1.2	2.2	2.1	1.0	1.0	1.4
k_5	4.0	8.7	4.2	7.5	2.3	7.2	8.5	7.0	4.0
k_6	0.01	0.01	1.0	0.01	1.5	0.01	0.01	0.1	0.1
k_7	1.0	0.01	0.01	0.01	0.1	0.01	0.01	0.1	0.1
k_8	0.01	0.01	0.01	0.01	0.5	0.01	0.01	0.4	1.0
k_9	0.01	0.33	0.3	0.1	0.1	1.8	0.33	0.8	3.0
k_1/k_5	2.03	1.51	1.62	0.96	2.65	0.47	0.33	0.37	1.55
cSSD	2.988	3.444	2.431	2.802	4.495	4.184	1.897	1.689	2.203

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Chapter 3: Exploration of Expanded p53 Degron Library

3.1 Introduction

The ubiquitin proteasome system (UPS) is the primary enzymatic pathway for the degradation of misfolded or damaged proteins in cells.[1, 2] At the heart of this process is the conjugation of the small protein ubiquitin to proteins, a process known as ubiquitination, which targets proteins to the proteasome for degradation. Ubiquitination is a tightly regulated process that principally involves a cascade of three enzymes (in order of increasing levels of specificity): E1 activating, E2 conjugating, and E3 ligases.[3] E3 ligases are the final arbitrators of the ubiquitination process that recognize the specific degradation signals, or degrons, on target proteins and facilitate their ubiquitination. The importance of E3 ligases is highlighted in their abundance and diversity, with hundreds of known E3 ligases in humans.[1, 4]

Once E3 ligases recognize a degron on a target protein, they transfer activated ubiquitin to a proximal lysine residue on the protein, forming an isopeptide bond between the ubiquitin C-terminal carboxylate and the lysine ε-amino group. This process can be repeated such that additional ubiquitin proteins form a poly-ubiquitin chain off of a single lysine residue. The ultimate fate of the protein depends on which of the ubiquitin molecule's several lysine residues the chain originates. For example, chains formed on the 48th lysine residue are targeted to the proteasome for degradation, while chains formed on the 63rd lysine play a role in signal transduction for DNA repair.[4] Acting in direct opposition to E3 ligases are deubiquitinating enzymes (DUBs), which selectively cleave the isopeptide bond between a protein and ubiquitin and thereby rescues the protein and allows ubiquitin to be reused in future ubiquitination events.

Because the UPS plays a key role in precisely controlling cellular protein levels, a vital cellular function, it has become a topic of intense interest as a target for molecularly targeted therapeutics. Proteasome inhibitors such as Bortezomib and Carfilzomib revolutionized the treatment of multiple myeloma and kicked off a rush to develop new inhibitors for different components of the UPS, including E3 ligases, DUBs, and the proteasome.[2, 5] However, with the push to develop these new therapeutics comes the need to develop tools capable of precisely quantifying the activity of UPS components. The current gold standards, such as Western blots or ELISA (enzyme-linked immunosorbent assay), require relatively large sample sizes and can only analyze bulk cell lysate samples. This makes discovery of subpopulations of cells impossible, as they only report a bulk population average.[6]

Chemical cytometry is capable of overcoming these limitations; coupling fluorescently labeled peptide reporters with capillary electrophoresis (CE-LIF) has enabled precise quantification of enzymatic activity in samples as small as single cells.[7] Peptide reporters have been used in previous work to investigate kinase, phosphatase, and peptidase activity in single cancer cells.[8-10] Additionally, peptide reporters boast several distinct advantages: they can be rapidly synthesized and prototyped, do not require genetic engineering techniques, and are compatible with patient samples. One of the most significant drawbacks to peptide reporters is their susceptibility to peptidases in cells, which rapidly degrade reporters, limit reporter lifetime, and convolute kinetic analyses. We have developed a strategy to overcome this drawback using a β-hairpin 'protectide' to produce a new generation of robust reporters that are resistant to peptidases.[11, 12] In order to develop these next generation peptide reporters for E3 ligase activity, compact degron components must be identified and incorporated into the reporter design. Studies investigating several degron signals have demonstrated that their integration into native

proteins results in dramatically increased proteasome mediated degradation.[13, 14] Though several degron sequences have been reported in the literature, the ability of the sequences to function once incorporated into peptide reporters appears to greatly depend on peptide length or posttranslational modifications such as phosphorylation.[6, 15] The development of future E3 ligase peptide reporters would benefit greatly from a library of compact, potent degrons.

The goal of this work was to build on previously reported work; specifically, to identify a shortened degron sequence taken from the tumor suppressor protein, p53. Work done by Gu et. al. had previously established a 26 amino acid degron element that was key to E3 ligase MDM2 (mouse double minute 2 homolog) recognition of p53.[16] Follow-up work used this sequence as a starting point to develop an E3 ligase peptide reporter that incorporated the p53 degron.[17] However, the long peptide length proved to be problematic, as the reporter was rapidly degraded by peptidases in cell lysates. A small library was synthesized to investigate the preferred ubiquitination site of the 26-amino acid sequence and made rough attempts to further shorten the original sequence while preserving the rates at which the reporter was ubiquitinated. These early attempts suggested that the original reporter degron could be further refined, potentially producing a compact degron with improved kinetics. This p53 degron therefore was an excellent candidate to be paired with a protectide; however, a shortened sequence first needed to be identified in order to facilitate the protectide strategy. In this work, a library of nine reporters was synthesized and evaluated in an *in vitro* reaction using recombinant enzymes, then evaluated using SDS-PAGE. More precise quantification was performed using reverse phase high performance liquid chromatography (HPLC) in order to calculate reporter ubiquitination kinetics. Finally, a compact 5 amino acid degron component was tested against a panel of

inhibitors to investigate the HDM2 (human double minute 2 homolog) ligase specificity of the reporter.

3.2 Experimental Design

3.2.1 Synthesis of p53 library

All peptides were synthesized using SPPS manually or using an automated peptide synthesizer (Creosalus TetrasUI Peptide Synthesizer, Louisville, KY). Synthesis was carried out by Dr. Adam Melvin, Gregery Woss, or Lukas Dumberger, with Lukas carrying out the majority of the synthesis. Synthesis was done on CLEARamide resin (Peptides Internationals) using Fmoc-[N]-protected amino acids (Advanced Chem, Louisville, KY). Removal of Fmoc was done by gentle agitation in a solution of 2% DBU (1,8-diazobicyclie[5.4.0]undec-7-ene) and 2% piperidine suspended in DMF (dimethylformamide) for 15 min twice. Amino acids were activated using 4 equivalents of HBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) and 4 equivalents of HOBt (hydroxybenzotriazole) in DMF and NMP (N-methylpyrrolidone) in the presence of 5 equivalents of DIPEA (N,N-diisopropylethylamine). For all natural amino acids, the coupling reaction was carried out twice for 30 minutes to 1 hour. In the case of Fmoc-Lys(ivDde)-OH, a single reaction was carried out using standard coupling agents over four hours to reduce reagent cost. Following synthesis of the peptide, the N-terminus was acetylated using 5% acetic anhydride and 6% 2,6-lutidine in DMF for 35 minutes.

In order to conjugate a fluorescent tag to the peptide, ivDde was removed by gentle agitation in a solution of 3% hydrazine monohydrate in DMF for three minutes three times; removal of the ivDde protecting group was confirmed using the Kaiser test. Next, the peptide was gently agitated overnight in the dark while in the presence of 4 equivalents of 6-carboxyfluorescien (Chem Impex, Wood Dale, IL), 4 of equivalents HOBt, 4 equivalents of PyBOP (benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate), and 8 equivalents of DIPEA in DMF.

The peptide was then cleaved from the resin using TFA (trifluoroacetic acid), TIPS (triisopropylsilane), and H_2O in a ratio of 95:2.5:2.5 for 3.5 hours, after which remaining TFA was evaporated under nitrogen. The peptide products were then precipitated into ice cold ether, extracted into water, then lyophilized.

Peptides were purified twice using reversed phase HPLC (Atlantis C-18 semi-preparative column), first using a 60-minute gradient of 95%/5% acetonitrile/water + 0.1% TFA to 5%/95% acetonitrile/water + 0.1% TFA, and then again using a 100-minute gradient. Purification was carried out either by Dr. Adam Melvin, Gregery Woss, or Lukas Dumberger, with Lukas performing the majority of the purification. The peptide's molecular weight was validated using ESI or MALDI mass spectrometry. Peptides were then suspended in 50 mM phosphate buffer (pH 8.0) and quantified using a Nanodrop 2000 spectrophotometer.

3.2.2 Tissue Culture

HeLa S100 lysates were generated to provide components of the UPS that could be used reproducibly and consistently throughout the development process. HeLa S100s were generated by Dr. Adam Melvin and Gregery Woss. HeLa S3 cells (ATCC, Manassas, VA) were grown in Dulbecco's modified eagle medium (DMEM) with 10% v/v bovine calf serum (HyClone, GE Healthcare Logan UT) and maintained in a 37 °C, 5% CO_2 environment. Cells were initially grown in T-75 culture flasks until 70% confluent. Cells were then rinsed with phosphate-buffered saline (PBS) and treated with 2 mL trypsin-EDTA for five minutes or until detached, at which point they were then spun down at 800-1200g for 10 min, resuspended in fresh media, and expanded into additional flasks. Once 8 flasks had reached 70% confluence, cells were treated with trypsin-EDTA as described above and re-suspended in suspension growth media (RPMI 1640 supplemented with 5% v/v bovine calf serum) to a density of 2 x 105 cells/mL in a 250 mL

spinner flask. Cells were then allowed to grow with fresh media added to prevent exceeding a cell density of 5 x 105 cells/mL. After 2-3 days of growth, cells were spun down at 800-1200g for 10 min and resuspended in fresh media in a 1 L spinner flask. This process was repeated until the suspension volume reached 1 L, at which point the above process was repeated, the cells were transferred to a 3 L spinner flask to grow with fresh media added until the suspension reached a volume of 3 L and a density of 4-6 x 10^5 cells/mL, and the cells were harvested. Cells were spun out of solution as described above, then washed with ice cold PBS and pelleted at 1200g for 10 minutes. The cell pellet volume was recorded as packed cell volume (PCV). 5xPCV hypotonic buffer (10 mM HEPES pH 8.0, 10 mM KCl, 1.5 mM MgCl2, and 1 mM DTT) was then used to swell the cells over the course of 10 minutes at 4 °C, at which point cells were centrifuged at 18006g for 10 min. This pellet was then resuspended in 2xPCV in hypotonic buffer and then lysed with approximately 10 strokes in a Dounce glass homogenizer. The resulting lysed cells were then centrifuged for 10 min at 1200g.

Following centrifugation, the resultant supernatant was collected as and supplemented with 0.11xPCV of buffer B (0.3 M HEPES pH 8.0, 1.4 M KCl, and 30 mM $MgCl_2$). This solution was then centrifuged at 100,0006g for 60 minutes at 4 °C. The resulting supernatant was then collected and dialyzed twice, first overnight and again for 4 hours, in buffer D (20 mM HEPES pH 8.0, 100 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, 1 mM DTT, and 20% v/v glycerol) at 4 °C. The lysate was then centrifuged one final time at 33,0006g for 20 min at 4 °C. The resulting cytosolic lysates were then quantified using a Nanodrop 2000 (Thermo Scientific), aliquoted, and stored at -80 °C.

3.2.3 In vitro p53 ubiquitination assay using recombinant enzymes

Evaluation of p53 ubiquitination was done using an *in vitro* ubiquitination assay using recombinant human enzymes. This reaction was initially adapted from cell lysate-based ubiquitination reactions by Dr, Adam Melvin based on recommendations by the company Boston Biochem. The reaction was then optimized by Gregery Woss. The reactions were carried out by Lukas Dumberger and Gregery Woss, with Lukas carrying out the majority of them. The reaction buffer was composed of 10 mM Tris-HCl (pH 7.6), 2 mM $MgCl_2$, 2 mM DTT, 300 μM ubiquitin (Boston Biochem, Cambridge, MA), 1X ATP-Energy Regenerating Solution (ERS) (Boston Biochem, Cambridge, MA), 1 μM Ube1 (E1, Boston Biochem, Cambridge, MA), 10 μM UbcH5b (E2, Boston Biochem, Cambridge, MA), and 1 μM HDM2 (E3, Boston Biochem, Cambridge, MA). In order to determine kinetic parameters, a range of peptide reporter concentrations was used (1 μM, 5 μM, 10 μM, 20 μM, and 30 μM). Reactions were carried out at 30 °C for 2 h and halted either by freezing the sample prior to analysis via HPLC or by the addition of 40 μL Tricine Sample Buffer (BioRad, Hercules, CA) and heating at 90 °C for 5 min. For analysis by gel electrophoresis, samples were loaded into precast 16.5% Mini-PROTEAN Tris Tricine SDS-PAGE gels (Bio-Rad, Hercules, CA). Gels were visualized with a Typhoon Imager (GE Healthcare Life Sciences, Pittsburgh, PA). Quantification of gels was done using ImageJ software (US National Institute of Health). Kinetic analysis was performed by comparing sample band intensity to unreacted parent peptide band intensity.

3.2.4 HPLC analysis

Analysis of reporter ubiquitination was carried out using an Agilent 100 HPLC system with a fluorescence detector and a Phenomenex Jupiter 300 C-18 column. Following optimization of separation conditions by Gregery Woss, Lukas Dumberger carried out the large majority of HPLC runs. Samples prepared in the reaction outlined above were stored at -20°C

until immediately prior to analysis, at which point they were quickly thawed to room temperature, spiked with an internal standard (10 μM fluorescein), and injected into the column. Separations occurred under a 15-minutemin gradient of 99.9% acetonitrile + 0.1% TFA to 99.9% water + 0.1% TFA. As an internal control, unreacted parent peptide was analyzed prior to each round of analysis to ensure consistent column performance and validate peak migration times. Experiments were carried out in triplicate at all concentrations with the exception of the negative control peptide, which was only analyzed in triplicate at the 10 μM in order to conserve reagents.

3.2.5 Inhibitor panel of p53 library

Peptides were evaluated for ubiquitination activity in HeLa S100 lysates. Reactions using cell lysates were carried out by Gregery Woss. A 100 μL reaction buffer was prepared containing 10 mM Tris-HCl pH. 7.6, 5 mM $MgCl_2$, 2 mM DTT, 20 μg per mL ubiquitin aldehyde (Boston Biochem, Cambridge, MA), 400 μg per mL ubiquitin (Boston Biochem, Cambridge, MA), 1X ATP ERS, 100 μM MG-132 (EMD Chemicals, Billerica, Massachusetts), 1 μL PhosSTOP (Roche, Basel, Switzerland), 1 μL cOmplete ULTRA (Roche, Basel, Switzerland) and HeLa S100 cytosolic lysates diluted to a concentration of 2 mg/mL. For experiments utilizing inhibitors, the reaction buffers were allowed to pre-incubate for 60 min following the addition of inhibitor but prior to the addition of the peptide reporter. The inhibitor compounds were all obtained from LifeSensors (Malvern, PA), and were suspended in fixed volume of DMSO. Inhibitors were used at 3x their listed IC50 value, with the following concentrations: 30 μM serdementan, 0.27 μM nutlin-3, 150 μM SKPin C1, 300 μM SMER3, 90 μM thalidomide, 30 μM PYR-41, or a DMSO vehicle control. Following addition of 10 μM peptide, samples were incubated for 2 hours at 37 °C.

To validate and pre-concentrate products containing an ubiquitin moiety, we utilized agarose beads coated with antibodies specific for ubiquitin (Agarose TUBES1, LifeSensors, Malvern, PA). Once the ubiquitination reaction had been allowed for the desired time, it was halted by diluting with 280 μL TBS-T buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, and 0.1% v/v Tween-20). 20 μL Control-Agarose beads (LifeSensors, Malvern, PA) were then added to the sample to control for non-specific binding and the sample was placed on a tube rotator and agitated for 1 hour at 4 °C. Control beads were then pelleted by centrifuging the sample at 1800g for 5 minutes. The supernatant was collected and added to a fresh centrifuge tube containing 100 μL of the Agarose-TUBES1 in TBS-T buffer. Samples were kept on a tube rotator over night at 4 °C then spun down at 1800g for 5 minutes. The supernatant was then removed and the beads washed in 1 mL ice cold TBS-T five times, re-centrifuging at 1800g for 5 minutesmin. Following the final centrifugation, the beads were suspended in 50 μL of tricine sample buffer and heated for 5 minutesmin at 90 °C. Samples were then stored at -20 °C until being loaded into gel for analysis.

3.2.6 Data Analysis

Data was analyzed by Dr. Adam Melvin, Gregery Woss, and Lukas Dumberger, with Lukas performing analyses for the majority of HPLC runs. Pseudo-Michaelis-Menten kinetics analysis was spearheaded by Dr. Adam Melvin. Data was analyzed using Origin 7.0 (OriginLab) to calculate the following parameters: the area of the ubiquitinated product peak, A_{ub}; the area of the unreacted peptide, A_p; the width of each peak ($W_{\frac{1}{2},p}, W_{\frac{1}{2}ub}$); and the migration time of each peak (t_p, t_{ub}). The additional parameter, column length, (L) was obtained directly form the column used. These variables were used to calculate the following parameters: The fraction of

the peptide ubiquitinated (f_{ub}), average peak width ($W_{\frac{1}{2},Avg}$), resolution (R), theoretical plates (N) and theoretical plate height (H). The equations used to calculate these metrics are as follows:

$$f_{ub} = \left(\frac{A_{ub}}{A_{ub} + A_p}\right)$$

$$W_{\frac{1}{2},Avg} = \frac{W_{\frac{1}{2},p} + W_{\frac{1}{2}ub}}{2}$$

$$R = \frac{0.589\left(t_p - t_{ub}\right)}{W_{\frac{1}{2}Avg}}$$

$$N = \frac{5.55t^2}{W_{\frac{1}{2}Avg^2}}$$

$$H = \frac{L}{N}$$

3.3 Results and Discussion

3.3.1 Refinement of the p53 Degron Sequence

Work done in the previous chapter detailed the original investigation of the 26 amino acid p53 degron identified by Gu et. al.[16] A peptide reporter incorporating amino acids 92-112, an available lysine residue, and a fluorescent tag [KPLSSSVPSQKTYQGSYGFRLGK(FAM)KKK], were ubiquitinated in HeLa S100 cell lysates.[15] The N-terminal lysine residue was not originally part of the degron sequence, but was previously incorporated into early studies and found to be a preferential ubiquitination site. Initial work also demonstrated that peptide reporters were capable of having multiple ubiquitination events occur at each lysine residue. In order to simplify kinetic analysis, this reporter was redesigned to substitute the three C-terminal lysine residues with three arginine

residues. Eight additional peptides were derived from this starting point (Figure 3.1A). All peptides in this library were between 8-14 amino acids in length, each with a single ubiquitination site. It is important to note that the lysine residue near the peptide C-terminus is conjugated to fluorescein and thus is not available for ubiquitination. Peptides 2, 4, 7 and 8 had N-terminal lysine residues, whereas peptides 3, 5, and 6 contained an internal lysine residue. The lysine residue of peptide 2 was replaced with a valine residue in the negative control peptide 9, as valine is incapable of being ubiquitinated.

Peptides were assayed using recombinant human enzymes to allow for analysis in the absence of competing cellular reactions, such as degradation. Peptides were incubated with Ube1 (an E1 activating enzyme), UbcH5b (an E2 conjugating enzyme) and HDM2 (an E3 ligase) along with ATP and buffering reagents to recapitulate cellular conditions. Samples were separated using SDS-PAGE electrophoresis and analyzed using a fluorescent imager. The presence of a fluorescent tag allowed for imaging without the need for antibodies, a major advantage of the reporter design. Peptides 1-8 were all ubiquitinated, but to variable degrees, as seen in Figure 3.1B. As expected, peptide 9 was not ubiquitinated, confirming that a lysine residue was required for reporter ubiquitination. Quantifying ubiquitination bands revealed that peptides 2, 7, and 8 all performed well, exceeding the ubiquitination rate of peptide 1. This is especially notable given that peptide 1 contained two potential ubiquitination sites, suggesting that peptide length plays a critical role in the ubiquitination kinetics of these reporters. While peptides 7 and 8 appeared to be the most rapidly ubiquitinated, poor signal to noise ratio made precise quantification of these bands difficult.

Control experiments were carried out using peptide 1 to validate whether observed bands were due to ubiquitination of peptides (Figure 3.1C). As expected, ATP, ubiquitin, Ube1, and

UbcH5b were are required for ubiquitination. Omitting HDM2 did not prevent reporter ubiquitination, but did result in a depressed ubiquitination rate. This result was not entirely unexpected; previous examples of similar behavior have been reported in the literature. For example, UbcH5 has been observed to directly ubiquitinate Otub1, while other work suggested HMD2 primarily serves as a 'landing pad' for Ubch5b and p53.[18, 19] Given the shortened nature of the peptides, and the abundance of UbcH5b-activated ubiquitin complexes, it is possible that a significant amount of reporter is directly interacting with the E2 enzyme, though ubiquitination is carried out more efficiently in the presence of HDM2.

3.3.2 Separation of Peptide Reporter Ubiquitinated Reporter by HPLC

A more precise analytical method than SDS-PAGE was necessary to precisely quantify the reporter ubiquitination rates. Analytical HPLC has a well-established background as a means for measuring posttranslational modifications such as phosphorylation, deubiquitination, and ubiquitination.[20-22] HPLC enables reproducible and precise quantification using relatively small sample sizes, and can accommodate controls, such as internal standards. In order to evaluate the ubiquitination kinetics of the peptide reporter library, HPLC analysis methodology was developed.

Peptide 7 was used to optimize the method of HPLC analysis based on its high performance in the initial trials. Peptide 7 was subjected to the *in vitro* ubiquitination assay detailed in the previous section, then analyzed using a 15-minutemin gradient elution, which produced three distinct peaks (Figure 3.2A). Parent peptide peak, labeled peak 2, was identified by spiking the reaction mix with unreacted peptide. The identity of peak 3 was confirmed to be ubiquitinated peptide through a combination of mass spectrometry and experimental controls (Figure 3.2C). In addition to these expected peaks, an unexpected peak appeared in all experiments (peak 1).

Control experiments and mass spectrometry were unable to identify the peak, but given its consistent area and appearance it is believed to be a contaminant in the ubiquitination assay mixture. The peak area was relatively small compared to other peaks, so it was omitted for data analysis. The separation of peptide and ubiquitinated peptide had a resolution of 3.37 ±0.33, with the parent peak having a theoretical plate number of 14157 ± 6360 and a theoretical plate height of 0.126 ± 0.13. This separation laid the groundwork for the analysis of the peptide library by HPLC.

3.3.3 Kinetic Analysis of the p53 Library by HPLC Quantification

In order to determine the rate of ubiquitination for the peptide library, peptides were analyzed across a range of concentrations, from 1 μM up to 30 μM. Separation of the parent peptide (peak 2) and ubiquitinated peptide (peak 3) was observed for all peptides at all concentrations (Figures 3.3A, 3.3C 3.3E, 3.4A, 3.4C, 3.4E, 3.5A, and 3.5C). The area of the unreacted peptide (A_p) and ubiquitinated reporter (A_{ub}) were used to calculate the fraction of ubiquitinated reporter, which was then used to calculate the ubiquitinated reporter concentration. This was used to calculate the rate of ubiquitinated peptide formation, which was plotted against the initial concentration of reporter (Figures 3.3B, 3.3D, 3.3F, 3.4B, 3.4D, 3.4F, 3.5B, and 3.5D). These plots demonstrated strong linear correlations, as demonstrated by their calculated correlation coefficients (R^2) presented in Table 3.1. This linear approximation between reaction velocity and substrate concentration suggested the reaction could be described using pseudo-Michalis-Menten kinetics at very low substrate concentrations ($K_m >> C$). With this in mind, an aggregate v_{max}/K_m was calculated to describe the reaction rates of the p53 peptide library (Table 3.1).

In line with the results of the SDS-PAGE analysis, peptide 7 appeared to have the most rapid ubiquitination kinetics ($v_{max}/K_m = 2.55 \times 10^{-4}$ min^{-1}). The disparity between peptides highlights the impact that reporter length and lysine position play in ubiquitination kinetics. These results also suggest that the amino acid sequence GSYG (missing from peptides 3-6 and peptide 8) is important to ubiquitination. While peptides 1 and 2 also contained this GSYG sequence, their relatively lower ubiquitination rate suggests that large sizes play a role in determining their ubiquitination rate. These observations are also in line with earlier work done with the p53 peptide, in which a reporter containing the GSYG amino acid sequence performed better than p53 reporters lacking it.[15] Taken together, this work suggests that peptide 7 is a potent compact degron that is ubiquitinated by UbcH5b and HDM2.

3.3.4 Ubiquitination of the p53 Based Reporter in HeLa S100 Lysates

The strong performance of peptide 7 suggested that it would be a good candidate for integration into a more robust peptide reporter. However, it was it was key to validate the peptide's performance in cell lysates prior to this step. Though the recombinant ubiquitination assay was advantageous for reporter development and allowed us to minimize competing reactions, it was not clear that it provided an accurate recapitulation of the cellular environment. To investigate this, peptide 7 was evaluated in HeLa S100 cytosolic lysates. This assay was similar to the recombinant enzyme assay with a key difference being the substitution of HeLa S100 lysates for the recombinant E1, E2, and E3 enzymes. In order to verify ubiquitination and concentrate any ubiquitinated products, agarose beads coated with antibodies specific for ubiquitin (TUBES) were employed. Additionally, we investigated the effect that six commercially available UPS inhibitors had on the ubiquitination of peptide 7 in HeLa S100 lysates to see if the inhibitor of any enzyme class decreased ubiquitination rate. The inhibitors,

nutlin-3, serdementan, SKPin C1, SMER3, and thalidomide were all E3 ligase inhibitors while PYR-41 was an E1 ligase (Figure 3.5).[23-27] Lysates were treated with 3x the IC_{50} value of each inhibitor 60 minutesmin before peptide was added.

As demonstrated by Figure 3.6, lane 1, peptide 7 was readily ubiquitinated in HeLa S100 lysates, forming mono-ubiquitination bands as well as bands characteristic of di-, tri-, and tetra-ubiquitination. The E3 ligase inhibitors nutlin-3, serdementan, and thalidomide had no apparent effect on ubiquitination rate (Figure 3.6, lanes 2, 3, and 6). SKPin C1, which inhibits the E3 ligase SCF-Skp2, appeared to produce a slight decrease in poly-ubiquitination of the peptide but did not appear to effect mono-ubiquitination (Figure 3.5, lane 4). SMER-3, which inhibits the E3 ligase SCF-MET30, had a more pronounced effect, apparently inhibiting both mono- and poly-ubiquitination (Figure 3.6, lane 5). These results suggest that the shortened degron incorporated into peptide 7 might serve as a substrate for the SCF family of enzymes. These enzymes consist of three large subunits: Skp1 (the bridging protein), cullin (the major structural scaffold), and RBX1 (the RING binding domain responsible of the binding of the activated E2-ubiqutin complex). The specificity of the SCF family of enzymes stems from the F-box contain proteins (such as Skp2 or β-TrCP), which recognize target proteins. It is possible that peptide 7 is recognized and ubiquitinated by the SCF complex; the minimal degron sequence in peptide 7, GSYG, shares some similarities with degron sequences of proteins ubiquitinated by SCF E3 ligases, including β-catenin (DSGIHSG), IFNAR1 (DSGNYS), and TAZ (HSREQSTDSG).[15] Interestingly, these sequences typically require a phosphorylate serine, unlike the GSYS degron.

While the shortened substrate apparently loses its specificity for HDM2, it also gains a broader applicability, potentially acting as a more global indicator of ubiquitination rates. This may be due to its very compact size, as the protein structure may play a large role in limiting

proteins interaction with E3 ligases. These results also demonstrate that this minimal degron sequence was ubiquitinated in cell lysates, making it a good potential tool for next generation peptide reporter development.

3.4 Conclusions

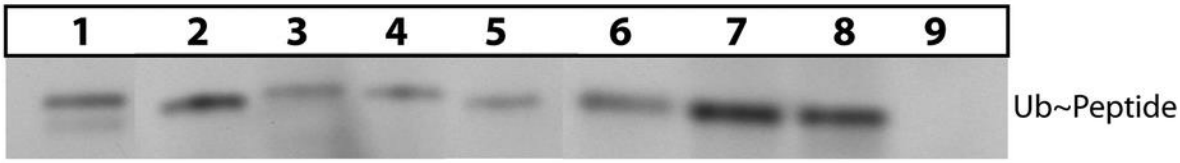
This work sought to expand upon earlier work and isolate a compact degron sequence from a previously established p53-based E3 ligase peptide reporter that retained or enhanced its ubiquitination kinetics. A library of nine peptides was synthesized and their ubiquitination kinetics in an *in vitro* ubiquitination assay were quantified. The top performing peptide, KGSYGK(FAM)RRR, was then validated in cell lysates and demonstrated to be ubiquitinated. Experiments with inhibitors suggest that the new compact degron (GSYG) might act as a substrate for the SCF family of E3 ligases. This shortened degron should be a useful tool in the development of future E3 ligase reporters, due to both its small size and broad applicability. When paired with new strategies, such as β-hairpins to increase peptide robustness and CE-LIF analysis, this work lays the ground work for the next generation of E3 ligase reporters.

3.5 Figures and Tables

A)

Peptide	Sequence
1	**K**PLSSSVPSQ**K**TYQGSYGFRLGK(FAM)RRR
2	**K**TYQGSYGK(FAM)RRR
3	VPSQ**K**TYQGK(FAM)RRR
4	**K**PLSSSVPSQK(FAM)RRR
5	SQ**K**TYK(FAM)RRR
6	VPSQ**K**K(FAM)RRR
7	**K**GSYGK(FAM)RRR
8	**K**TYQK(FAM)RRR
9	VTYQGSYGK(FAM)RRR

B)



C)

Ubiquitin	+	--	+	+	+	+
ATP-ERS	+	+	--	+	+	+
Ube1	+	+	+	--	+	+
UbcH5b	+	+	+	+	--	+
HDM2	+	+	+	+	+	--
Lane	1	2	3	4	5	6

Figure 3.1 *In vitro* ubiquitination of peptide substrates based on a p53 degron. (A) Library of the nine peptides reporters evaluated in this study. Bold amino acid residues represent potential ubiquitination site lysines. FAM represents 6-carboxyfluorescein. (B) SDS-PAGE results demonstrating *in vitro* ubiquitination the peptide library. Pictured are fluorescent bands corresponding to ~12 kDa, which is the expected weight of a mono-ubiquitinated peptide. (C) SDS-PAGE data showing control experiments using peptide 1 with the exclusion of a single

assay component (lanes 2–6) followed by SDS-PAGE. Pictured are fluorescent bands corresponding to ~12 kDa, which is the expected weight of a mono-ubiquitinated peptide.

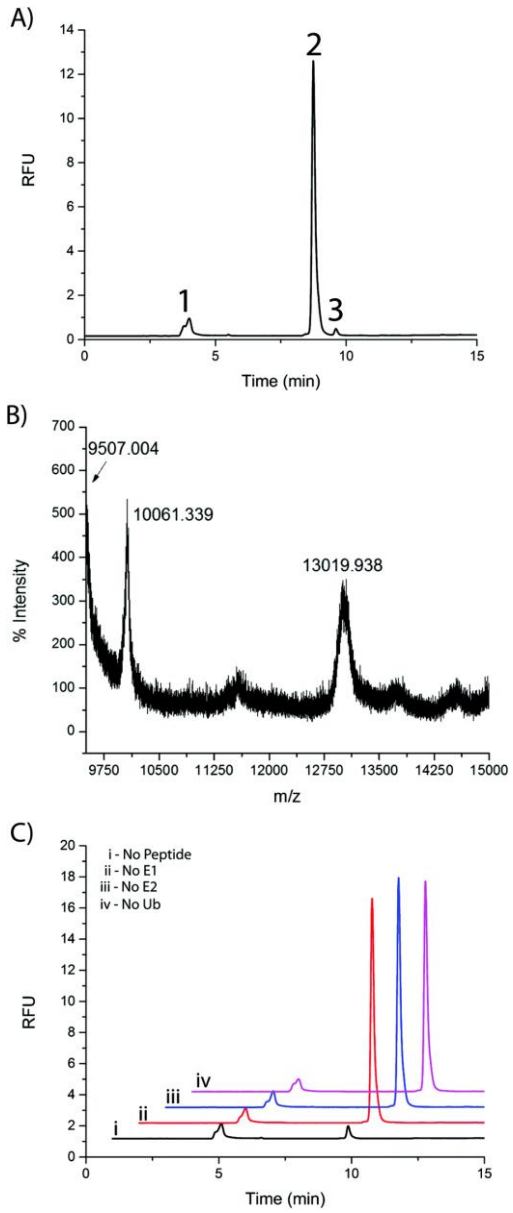


Figure 3.2 HPLC separation of parent peptide from ubiquitinated peptide. (A) Representative chromatogram showing the ubiquitination of peptide 7 (10 μM) and its separation using a C18-bonded silica analytical column. The parent peptide is labeled as peak 2, and the ubiquitinated reporter is labeled peak 3. Peak 1 is believed to be a contaminant. (B) Mass

spectrometry data (MALDI-TOF) of peak 3. (C) Control experiments demonstrating the absence of peak 3 with the exclusion of peptide (i), E1 enzyme (ii), E2 enzyme (iii), or ubiquitin (iv).

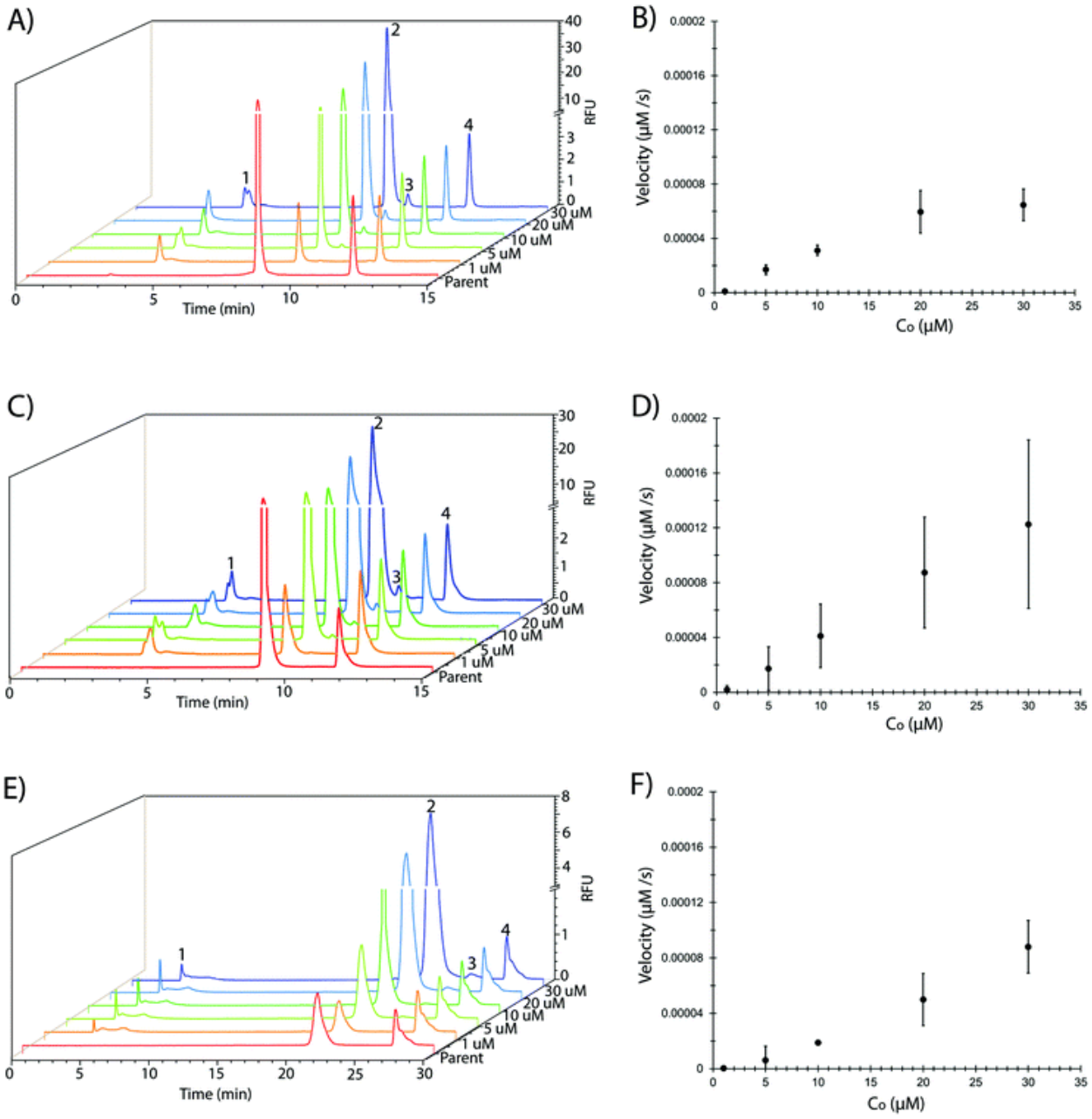


Figure 3.3 Quantification of top performing reporter ubiquitination kinetics. Presented chromatograms showing the concentration-dependent ubiquitination of peptides 2 (A), 7 (C), and 1 (E). Peak 1 represents unidentified, non-reactive contaminant, peak 2 represents parent peptide, peak 3 represents ubiquitinated reporter, and peak 4 an internal standard (FAM, 6-carboxyfluorescein). Initial concentration of peptides 2 (B), 7 (D) and 1 (F) were plotted against the velocity of peptide ubiquitination µM s^{-1}) (n = 3).

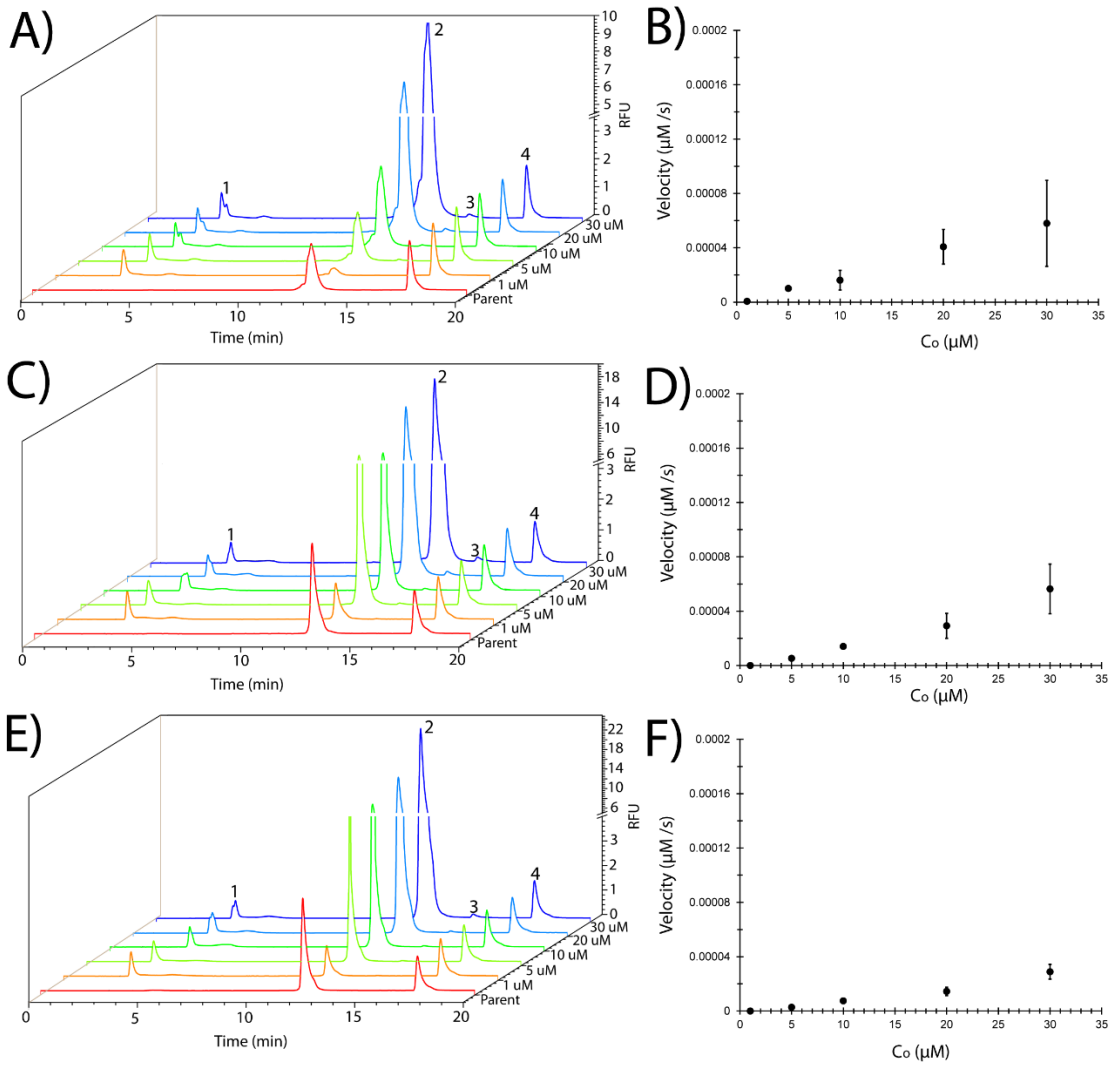


Figure 3.4 Quantification of reporter library ubiquitination kinetics (1/2). Presented chromatograms showing the concentration dependent ubiquitination of peptides 3 (A), 4 (C), and 5 (E). Peak 1 represents unidentified, non-reactive contaminant, peak 2 represents parent peptide, peak 3 represents ubiquitinated reporter, and peak 4 an internal standard (FAM, 6-carboxyfluorescein). Initial concentration of peptides 3 (B), 4 (D), and 5 (F) were plotted against the velocity of peptide ubiquitination (µM s^{-1}) (n = 3).

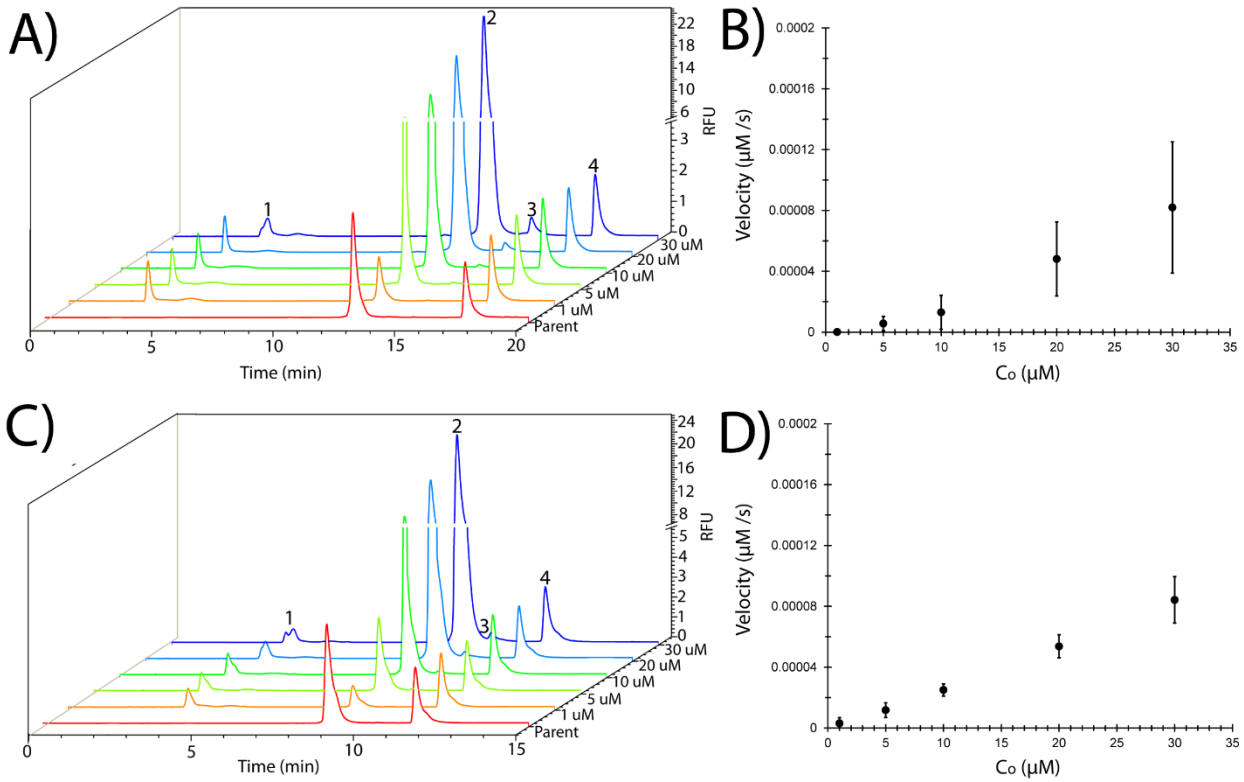


Figure 3.5 Quantification of reporter library ubiquitination kinetics (2/2). Presented chromatograms showing the concentration dependent ubiquitination of peptides 6 (A), and 8 (C). Peak 1 represents unidentified, non-reactive contaminant, peak 2 represents parent peptide, peak 3 represents ubiquitinated reporter, and peak 4 an internal standard (FAM, 6-carboxyfluorescein). Initial concentration of peptides 6 (B), and 8 (D) were plotted against the velocity of peptide ubiquitination (µM s^{-1}) (n = 3).

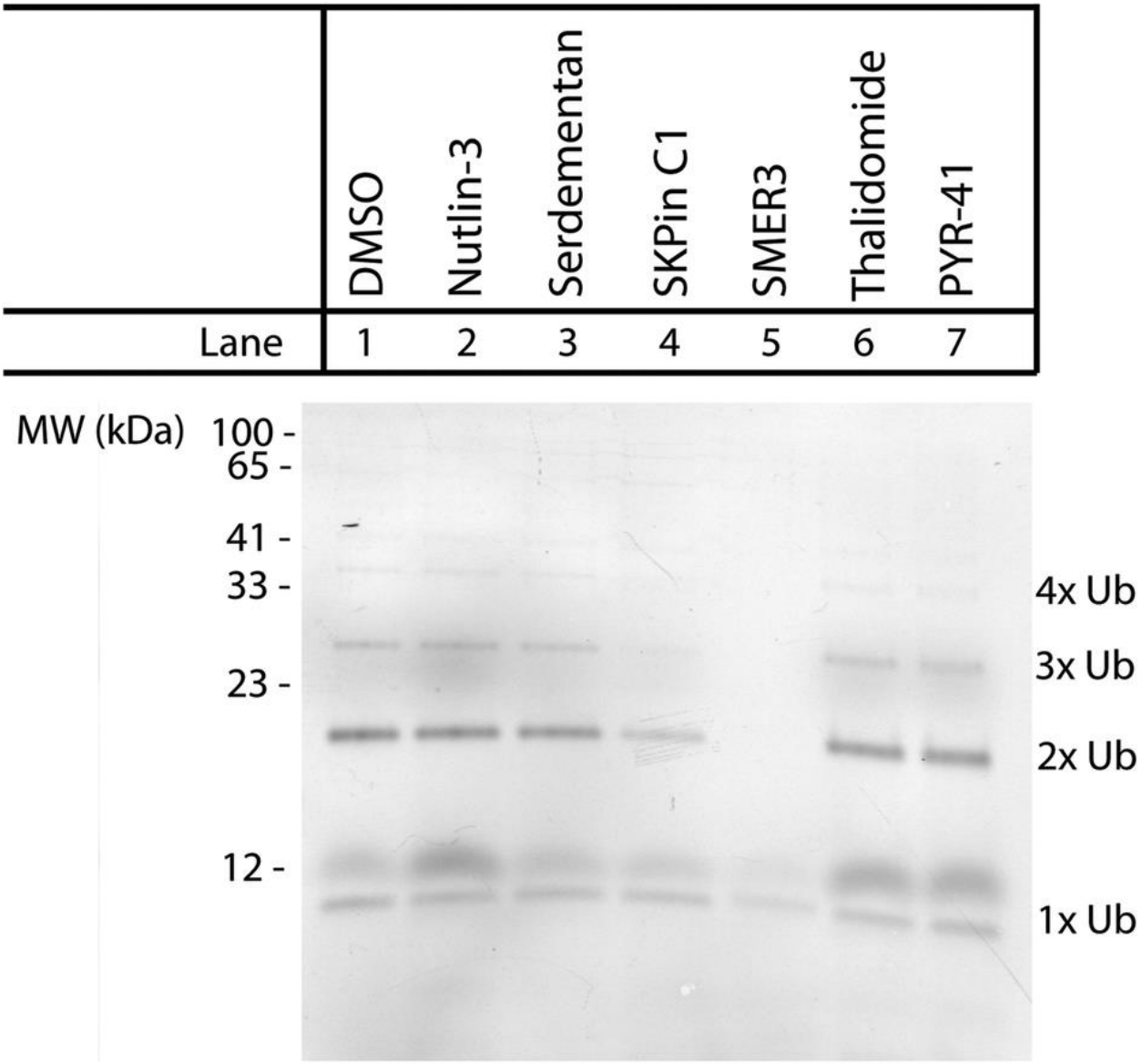


Figure 3.6 Ubiquitination of a p53-based peptide substrate in cell lysates. Peptide 7 (10 μM) was incubated in HeLa S100 cytosolic lysates for 2 h. For inhibitor studies E1 and E3 enzyme inhibitors were added 1 h before the reaction. Each inhibitor was added with the following concentrations: nutlin-3 (0.27 μM), serdementan (30 μM), SKPin C1 (150 μM), SMER3 (300 μM), thalidomide (90 μM), PYR-41 (30 μM), or a DMSO control.

Table 3.1 Ubiquitinated kinetic constants for p53-based peptide library. Calculated kinetic parameters for each peptide along with their coefficient of determination (n=3).

Peptide	v_{max}/K_m $(min^{-1})\times 1000$	R^2
1	0.184 ± 0.028	0.987
2	0.134 ± 0.022	0.932
3	0.120 ± 0.064	0.994
4	0.114 ± 0.029	0.981
5	0.058 ± 0.009	0.976
6	0.175 ± 0.084	0.979
7	0.255 ± 0.117	0.997
8	0.169 ± 0.024	0.998

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Chapter 4: Characterization of OWRWR

4.1 Introduction

The controlled synthesis, post translational modification, and degradation of proteins are crucial to cell homeostasis, migration, and proliferation.[1] The ubiquitin proteasome system (UPS) is a collection of enzymes that play a key role in this process, acting to modify proteins by conjugating them to the small protein, ubiquitin (i.e., ubiquitination).[2] This post translational modification can evoke a litany of downstream effects, one of the most dramatic being the unfolding and degradation of ubiquitinated proteins. Ubiquitination typically involves a cascade of increasingly specific enzymes, each beginning with the 76-amino acid-long ubiquitin protein. Adenosine triphosphate (ATP) drives ubiquitin activation via E1 activating enzymes, which form a thioester bond with ubiquitin. This complex is transferred to an E2 conjugating enzyme, which then interacts with an E3 ubiquitin ligase to further facilitate the transfer of ubiquitin to a targeted protein. A ubiquitination event completes once the E3 ligase conjugates the C-terminal carboxylate of ubiquitin to an ε-amino group of a proximal lysine, which can occur either directly or indirectly.[3]

Following the initial ubiquitination event, additional ubiquitin proteins can conjugate to the original lysine-conjugated ubiquitin, creating a poly-ubiquitin chain. The ultimate effect of ubiquitination depends on the length of these chains, as well as at which lysine residue the chains form. For example, chains formed from the 46th lysine residue are recognized by the regulatory elements of the enzyme ultimately responsible for protein degradation: the proteasome. Recent work demonstrated that the ubiquitin chain length that is required for degradation depended

partially on the protein size, with a single ubiquitin being sufficient for proteins smaller than 150 amino acids.[4] Proteins smaller than 20-30 amino acids appear to not be degraded by the proteasome regardless of ubiquitin chain length, and may be too small for the proteasome to unfold and thread them into its catalytic core properly. The UPS system is stringently regulated in large part by E3 ligases, which recognize degradation signals (degrons) encoded within proteins. Being both plentiful and diverse, with >600 varieties known to exist in humans, E3 ligases are capable of sensing degrons that range from simple amino acid sequences to complex signals that require additional post translation modifications.[5]

Given the central role the UPS plays in cell homeostasis; it is unsurprising that it has become a focal point in drug discovery for the treatment of a broad span of diseases ranging from cancer to neurodegenerative diseases.[6] The success of drugs targeting the proteasome, and the number of potentially targetable proteasome components have prompted hopes that UPS inhibitors may one day be comparable to kinase/phosphatase inhibitors.[6, 7] The rapid increase in interest in the UPS has opened up a need to develop new and more powerful technologies to measure the activity of its components, in particular the E3 ligases.

The two gold standards for measuring E3 ligase activity are Western blotting and ELISA (enzyme-linked immunosorbent assay); however, both come with significant drawbacks, such as being unable to identify sub-populations of cells, making them unsuited for analyzing patient samples.[2, 8] Chemical cytometry overcomes these limitations, and can analyze individual cells. Notably, coupling the use of fluorescently labeled peptide reporters with capillary electrophoresis with laser induced fluorescence detection (CE-LIF) permits the monitoring of enzymes responsible for post translation modifications, such as kinases and phosphatases.[9-11] Peptide reporters themselves boast several advantages: they can be synthesized rapidly,

optimized through iterative processes, and do not require complex techniques such as genetic modifications in order to be incorporated into cells.[12] Early work developing peptide reporters established a library of degrons that, when incorporated into a peptide reporter, resulted in ubiquitination in cell lysates.[8] One substantial drawback to peptide reporters is that these substrates are rapidly degraded by intracellular peptidases.[12] In the past, this degradation has been diminished through the incorporation of unnatural amino acids, but this process must be carefully tailored to individual reporters.[12] In addition to unnatural amino acids, secondary structures have been shown to increase peptide lifetimes in the presence of intracellular peptidase.[13] Working in close collaboration with the Waters lab, who provided their experience and expertise, Professors Allbritton and Waters hypothesized that by appending a well-folded β-hairpin to the N-terminus, the unstructured degron element of our reporters would be protected, and the β-hairpin would thus serve as a 'protectide'.[14]

4.2 Experimental Design

4.2.1 Peptide Synthesis

All peptides were synthesized using solid phase peptide synthesis (SPPS) manually or using a TetrasUI automated peptide synthesizer (Creosalus, Louisville, KY). Peptides were synthesized by Dr. Adam Melvin, Dr. Kaiulani Houston, Dr. Effrat Fayer or Gregery Woss. Peptides were formed on CLEARamide resin (Peptides International, Louisville, KY) beads. Synthesis was done using Fmoc-[N]-protected amino acids (Advanced Chem, Louisville, KY). Removal of Fmoc group was done mixing resin in in a solution of DMF (dimethylformamide) containing 2% DBU (1,8-diazobicyclie[5.4.0]undec-7-ene) and 2% piperidine over 15 minutes two times. Amino groups were activated using 4 equivalents of HBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate), 4 equivalents of HOBt (hydroxybenzotriazole), and 5 equivalents of DIPEA (N,N-diisopropylethylamine) suspended in

DMF and NMP (N-methylpyrrolidone). The coupling reaction was carried out twice for 30 minutes to 1 hour for all natural amino acids. Fmoc-Lys(ivDde)-OH was coupled in a single reaction using standard coupling agents over a 4-hour period in order to reduce reagent cost. Following the addition of all amino acids, the peptide's N-terminus was acetylated through a reaction with 5% acetic anhydride and 6% 2,6-lutidine in DMF for 35 minutes.

The ivDde protecting group was next removed with gentle mixing in a solution of 3% hydrazine monohydrate in DMF for three minutes three times, and was confirmed using the Kaiser test. The peptide was fluorescently tagged by reacting overnight in 4 equivalents of 6-carboxyfluorescien (Chemi Impex, Wood Dale, IL), 4 of equivalents HOBt, 4 equivalents of PyBOP (benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate), and 8 equivalents of DIPEA in DMF. The peptide was then cleaved from the resin using a mixture of TFA (trifluoroacetic acid), TIPS (triisopropylsilane), and H_2O in a ratio of 95:2.5:2.5 over 3.5 hours, after which the remaining TFA was evaporated under nitrogen. 50 mL of ice cold ether was then used to precipitate peptide products, which were then extracted into water. These products were then frozen and the sample lyophilized.

The peptides were next purified using reverse phase HPLC (Atlantis C-18 semi-preparative column). Two solvent systems were used: solvent A (95% acetonitrile, 5% water, 0.1% TFA) and solvent B (5% acetonitrile, 95% water, 0.1% TFA). Initially, the peptide was purified over a 60-minute gradient from solvent A to solvent B, at which point product peaks were identified using ESI (electrospray ionization) or MALDI (matrix-assisted laser desorption/ionization) mass spectrometry. Following initial purification, peptides were subjected to a second round of purification over a 100-minute gradient to minimize the possibility of closely migrating impurities. Peptides were then lyophilized and re-suspended in 50 mM phosphate buffer (pH

8.0) or DMSO and quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). Peptides were stored at -20 °C until use; peptides suspended in DMSO were mixed with diluted 100 fold with 50 mM phosphate buffer (pH 8.0) prior to use.

4.2.2 Cell culture and S100 Lysate generation

Cell lysate experiments were carried out by Dr. Adam Melvin, Dr. Kaiulani Houston, or Gregery Woss. OPM-2 cells (obtained Donald McDonnell, Duke University) were maintained in a 37 °C, 5% CO_2 environment using RPMI 1640 media (Fisher, Hampton, NH) supplemented with 12% fetal bovine serum (HyClone, GE Healthcare Logan UT), 21.8 mM glucose, 8.6 mM HEPES (pH 7.4) and 1.0 mM sodium pyruvate.

HeLa S3 cells (ATCC, Manassas, VA) were used to produce HeLa S100 lysates. Cells were grown in Dulbecco's modified eagle medium (DMEM) with 10% v/v bovine calf serum (HyClone) and maintained in a 37 °C, 5% CO_2 environment. Cells were grown in T-75 culture flasks until 70% confluent. Cells were rinsed with PBS and treated with 2 mL trypsin-EDTA for five minutes at which point they were detached using gentle agitation and centrifuged at 800g for 2 minutes, resuspended in fresh media and expanded into additional flasks. Once 8 flasks had reached 70% confluence cells, cells were detached and re-suspended in a 250 mL spinner flask using suspension growth media (RPMI 1640 supplemented with 5% v/v bovine calf serum) with an initial density of 2 x 10^5 cells/mL. Cells were allowed to grow with fresh media added periodically to prevent cells from exceeding a cell density of 5 x 10^5 cells/mL. After 2-3 days of growth, cells were spun down at 800g for 10 minutes and resuspended in a 1 L spinner flask using fresh media. Fresh media was added periodically to maintain a cell density between 2 x 10^5 cells/mL to 5 x 10^5 cells/mL until the total volume reached 1 L. The process was then repeated and cells were transferred to a 3 L spinner flask. Cells were harvested once media

reached 3 L and cells researched a density of 4 x 10^5 cells/mL to 6 x 10^5 cells/mL. Cells were centrifuged out of solution, then washed with ice cold PBS. They were then pelleted at 1200g for 10 minutes. The volume of this cell pellet was recorded as packed cell volume (PCV). Cells were suspended in 5 x PCV of hypotonic buffer (10 mM HEPES pH 8.0, 10 mM KCl, 1.5 mM $MgCl_2$, and 1 mM DTT) and allowed to sit for 10 minutes at 4 °C, then centrifuged at 18006g for 10 minutes. This pellet was then resuspended in 2xPCV in hypotonic buffer and then lysed using approximately 10 strokes in a Dounce glass homogenizer. The resulting lysate was centrifuged for 10 minutes at 1200g. The resulting supernatant was collected as and supplemented with 0.11 x PCV using a buffer comprised of (0.3 M HEPES pH 8.0, 1.4 M KCl, and 30 mM $MgCl_2$). This mix was then centrifuged at 100,000g for 60 minutes at 4 °C. The supernatant was collected and then dialyzed at 4 °C in 1 L of 20 mM HEPES pH 8.0, 100 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, 1 mM DTT, and 20% v/v glycerol for 8 hours and then again for 4 hours. The concentrated lysate was then centrifuged a final time at 33,000g for 20 minutes at 4 °C. Lysates were then quantified using a Nanodrop 2000 (Thermo Scientific), aliquoted, and stored at -80 °C until use.

4.2.3 Ubiquitin pull down assay

Peptides were evaluated for ubiquitination activity in HeLa S100 lysates and validated using an antibody based pull down reaction. Pull down reactions were carried out by Dr. Adam Melvin or Gregery Woss. A 100 µL reaction buffer was prepared composed of 10 mM Tris-HCl pH. 7.6, 5 mM $MgCl_2$, 2 mM DTT, 20 µg/mL ubiquitin aldehyde (Boston Biochem), 400 µg/mL ubiquitin or methylated ubiquitin (Boston Biochem, Cambridge, MA), 1X ATP ERS (Boston Biochem, Cambridge, MA), 100 µM MG-132 (EMD Chemicals, Billerica, Massachusetts), 1 µL PhosSTOP (Roche, Basel, Switzerland), 1 µL cOmplete ULTRA (Roche, Basel, Switzerland)

and HeLa S100 cytosolic lysates diluted to a concentration of 2 mg/mL. For experiments utilizing inhibitors, the reaction buffers containing cell lysates were allowed to pre-incubate for 60 minutes prior to addition of the peptide reporter. The inhibitor compounds were all obtained from LifeSensors (Malvern, PA) and were suspended in fixed volume of DMSO. Inhibitors were used at three times their listed IC50 value, with the following concentrations: 30 μM serdementan, 0.27 μM nutlin-3, 150 μM SKPin C1, 300 μM SMER3, 90 μM thalidomide, 30 μM PYR-41, or a DMSO vehicle control. The reaction was started with the addition of 4.2 μg peptide reporter and incubated for 2 hours at 37 °C.

In order to validate and pre-concentrate products containing an ubiquitin moiety, agarose beads coated with antibodies specific for ubiquitin (Agarose TUBES1, LifeSensors) were utilized. Once the ubiquitination reaction had been allowed for the desired time it was halted by diluting with 280 μL TBS-T buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, and 0.1% v/v Tween-20). 20 μL Control-Agarose beads (LifeSensors, Malvern, PA) were then added to the sample to control for non-specific binding and the sample was placed on a tube rotator and agitated for 1 hour at 4 °C. Control beads were then pelleted by centrifuging the sample at 1800g for 5 minutes, and the supernatant was collected and added to a fresh centrifuge tube containing 100 μL of the Agarose-TUBES1 in TBS-T buffer. Samples were kept on a tube rotator over night at 4 °C then spun down at 1800g for 5 minutes. The supernatant was then removed and the beads washed in 1 mL ice cold TBS-T five times, re-centrifuging at 1800g for 5 minutes. Following the final centrifugation, the beads were suspended in 50 μL of tricine sample buffer and heated for 5 minutes at 90 °C. Samples were then stored at -20 °C until being loaded into gel for analysis.

Peptides were evaluated for ubiquitination activity in HeLa S100 lysates. A 100 μL reaction buffer was prepared containing 10 mM Tris-HCl pH. 7.6, 5 mM $MgCl_2$, 2 mM DTT, 20 μg per mL ubiquitin aldehyde (Boston Biochem), 400 μg per mL ubiquitin (Boston Biochem), 1X ATP ERS (Boston Biochem), 100 μM MG-132 (EMD Chemicals), 1 μL PhosSTOP (Roche), 1 μL ULTRA (Roche) and HeLa S100 cytosolic lysates diluted to a concentration of 2 mg/mL. For experiments utilizing inhibitors, the reaction buffers were allowed to pre-incubated for 60 minutes following the addition of inhibitor but prior to the addition of peptide reporter. The inhibitor compounds were all obtained from LifeSensors and suspended in fixed volume of DMSO. Inhibitors were used at three times their listed IC50 value, with the following concentrations: 30 μM serdementan, 0.27 μM nutlin-3, 150 μM SKPin C1, 300 μM SMER3, 90 μM thalidomide, 30 μM PYR-41, or a DMSO vehicle control. Following addition of peptide, samples were incubated for 2 hours at 37 °C.

Ubiquitinated reporter was isolated using a ubiquitin pull-down assay. Reporter was concentrated and the cell lysate debris removed to readily detect and quantify the reporter on SDS-PAGE gels with the aid of a fluorescein tag. The ubiquitin pull down assay was carried out at the indicated times at 37 °C in a total reaction volume of 100 μL containing assay buffer (10 mM Tris-HCl pH 7.6 and 5 mM $MgCl_2$) with 2 mM DTT, 20 μg/mL ubiquitin aldehyde (Boston Biochem), 100 μM MG-132 (EMD Chemicals), 400 μg/mL ubiquitin, methylated ubiquitin, or no Lys ubiquitin (Boston Biochem), 1X ATP energy regenerating solution (ATP-ERS, Boston Biochem), 4.2 μg of indicated peptide substrate, and 2 mg/mL HeLa S100 cytosolic lysates as the source of E1, E2, and E3 enzymes. At the end of the indicated times, samples were incubated with Control-Agarose beads (LifeSensors), diluted in TBS-T buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, and 0.1% v/v Tween-20), for 60 minutes on a tube rotator at 4 °C. Samples were

subsequently centrifuged at 1800g for 5 minutes to pellet and remove control beads. The supernatant was transferred to a solution of Agarose-TUBEs (LifeSensors, Malvern, PA) diluted in TBS-T and incubated overnight on a tube rotator at 4 °C. Ubiquitin-bound beads were washed 5X with 1X TBS-T and then the samples were eluted off of the bead with 2X tricine sample buffer, heated for 5 minutes (>90 °C), and then isolated by centrifugation for 5 minutes at 13000g. Samples were loaded onto SDS-PAGE gels (precast 16.5% Mini PROTEAN Tris-Tricine, Bio-Rad) using 1X tris-tricine running buffer and visualized with a Typhoon Imager (GE Healthcare Life Sciences). Gels were quantified using ImageJ (US National Institute of Health) by comparing sample intensity to unreacted parent peptide intensity, which is not depicted in the gels.

Pull down assays performed in the presence of chemical inhibitors were completed as described above, except that the reaction mixture contained 10 μM peptide and was supplemented with 1 μL of cOmplete ULTRA and 1 μL of PhosSTOP. The HeLa S100 lysates were pre-incubated for 60 minutes with the following single inhibitor compounds (all obtained from LifeSensors) at the following concentrations prior to adding the remaining reaction mixture components (each are three times the listed IC50 value for each compound): 30 μM serdementan, 0.27 μM nutlin-3, 150 μM SKPin C1, 300 μM SMER3, 90 μM thalidomide, or 30 μM PYR-41. The reaction mixture was incubated at 37 °C for 2 hours and then subjected to ubiquitin-conjugated protein isolation as described above. Inhibitor studies were initially started by Dr. Kaiulani Houston, then re-optimized and carried out by Gregery Woss. Samples were separated using SDS-PAGE and visualized with a Typhoon imager.

4.2.4 Circular dichroism

Peptide concentrations were determined based on absorbance of FAM at 492 nm. CD spectroscopy data was collected by Dr. Kaiulani Houston using an Applied Photophysics Chiroscan Circular Dichroism Spectrophotometer. Spectra were generated at 25 °C with a wavelength scan (260-185 nm) using 0.5 s scanning in a 0.1 cm cell. All peptides were at a final concentration of 40 μM in 10mM sodium phosphate buffer (pH 8.02).

4.2.5 Peptidase degradation reactions

Initial degradation studies were done by Dr, Kaiulani Houston using commercially available peptidases with analysis by HPLC; however, following several setbacks, peptide degradation studies were carried out in lysates prepared from the OPM2 cell line by Gregery Woss. Briefly, 1 x 10^6 cells/mL OPM2 cells were harvested, washed twice in PBP (137 mM NaCl, 10 mM Na_2HPO_4, 27 mM KCl, and 1.75 mM KH_2PO_4 at pH 7.4) and pelleted at 800g. The cell pellet was re-suspended in an approximately equivalent volume of mammalian protein extraction reagent (MPER, ThermoFisher, Wilmington, DE), then vortexed for 10 minutes at room temperature. Following this, the mixture was centrifuged at 14,000g for 15 minutes at 4 °C and the supernatant transferred to a fresh centrifuge tube and stored on ice until use. Total protein concentration was determined using fluorescamine as previously described[12]. To investigate peptide stability, 10 μM peptide was incubated with lysates diluted to a total protein concentration of 2 mg/mL in an assay buffer (10 mM Tris-HCL, pH 7.6) at 37 °C in the dark. Aliquots of the reaction mixture were removed at set intervals, at which point further peptidase activity was quenched by heating the aliquots at 90 °C for 5 minutes followed by immediately freezing in liquid nitrogen and then storage at -20 °C until analysis by CE. The 0 minute time point measurements were made using lysates that were heat killed prior to peptide incubation. Samples were analyzed using a Beckman Coulter ProteomeLab™ PA800 automated CE system

(Brea, CA) (CE-LIF, 488 nm) as previously described.[12] Prior to CE analysis, samples were thawed to room temperature and diluted 1:100 in separation buffer (125 mM Sodium Tetraborate, 3% v/v Tween-20, pH 8.5). The capillary was washed with 1 M NaOH for 2 minutes, dH_2O for 2 minutes, and separation buffer for 2 minutes. Separation was carried out in a fused-silica capillary (30 μm inner capillary, 360 μm outer capillary, Polymicro Technologies, Pheonix, AZ) with a total length of 30 cm and an effective length of 20 cm. Samples were injected into the capillary by applying 0.5 psi pressure for 5 seconds, and separation was initiated by applying a negative voltage of 6.5 kV. Percent intact peptide remaining was calculated by the corrected area under the parent peptide peak divided by the total corrected peak area (i.e., the area under all peaks of the electropherogram) for each time point. The identity of the parent peptide peak was confirmed using the parent peptide alone and verified with the t=0 minute electropherogram. The data was analyzed using commercial software (32 Karat, version 8.0, Beckman Coulter). All degradation reactions and analysis were performed in triplicate.

4.3 Results and Discussion

4.3.1 Ubiquitination of protectide based reporters

Previous work demonstrated that peptide reporters could be ubiquitinated in cell lysates.[2, 8, 15] The basic structure of these reporters consisted of a short degron sequence, an available lysine reside to which ubiquitin could conjugate, and a fluorescent tag for detection and quantification. Early studies had demonstrated that an artificial degron developed by Bonger *et. al.* had rapid ubiquitination kinetics in cytosolic lysates.[8, 16] Unfortunately, the peptide was also rapidly degraded by intracellular peptidases, mitigating its usefulness as an E3 ligase reporter. It was hypothesized that a more robust reporter could be produced by incorporating a large, well-folded β-hairpin, or 'protectide', onto the N-terminus of the peptide. Working in close collaboration with the Waters lab, and in particular Dr. Kaiulani Houston who did much of the initial work on

this project, Dr. Houston designed a protectide-degron conjugate, which was a modified version of a protectide that was previously characterized, OWOWO, which improved peptide stability in the presence of peptidases (Figure 4.1A).[13, 14] A reporter consisting of an N-terminal OWOWO protectide and the compact Bonger degron (RRRG) joined by a PEG (polyethylene glycol) spacing element was synthesized using SPPS (OWOWO-RRRG, Figure 4.1A). This peptide was then tested using an *in vitro* ubiquitination reaction with HeLa S100 cytosolic lysates supplemented with methylated ubiquitin (MeUb). These analytes were confirmed as ubiquitinated products using agarose beads coated in ubiquitin-specific antibodies (TUBEs). As expected, the peptide was ubiquitinated in a time-dependent manner (Figure 4.1B, lanes 1,2); surprisingly, the results also indicated multiple ubiquitination products despite using MeUb, which is incapable of forming poly-ubiquitin chains. This result suggested that multiple mono-ubiquitination events occurred, and because OWOWO-RRRG contained a single lysine residue, we suspected that the ornithine residues within the OWOWO protectide itself were being ubiquitinated.

To test this hypothesis, OWOWO was subjected to the same ubiquitination assay as OWOWOW-RRRG using both MeUb and wild-type ubiquitin (Figure 4.1B, lanes 3-6). Interestingly, OWOWO was ubiquitinated in a time-dependent fashion in both cases, suggesting not only were the ornithine residues capable of being ubiquitinated, but that OWRWR itself was acting as a degron. Though initially unexpected, the ornithine ubiquitination is not entirely surprising given the similarity of lysine and ornithine structures. The results of initial experiments with MeUb were indicative multi-mono-ubiquitination events, a phenomenon previously observed in peptides with multiple potential ubiquitination sites. To further confirm this, control experiments with OWOWO were carried out using an assay supplemented with

mutated lysine (No K Ub) without lysine residues (K→R), as well as lysates not supplemented with ubiquitin at all, in order to confirm that bands were not the result of endogenous ubiquitin (Figure 4.2). These results indicated that endogenous ubiquitin may play a small role in the formation of multi-ubiquitination bands even when the lysate is supplemented with MeUb or No K Ub.

In order to further characterize OWOWO as a degron, a sub-library of four new peptides was synthesized: OWRWR, RWOWR, RWROW, and RWRWR (Figure 4.1A). The majority of the work comparing these four peptides was done by Dr. Adam Melvin and Dr. Kaiulani Houston. The first three peptides were similar to OWOWO but with two of the ornithine residues replaced with arginine residues; arginine was selected because it possessed a positive charge but could not be ubiquitinated. By eliminating all but a single potential ubiquitination site on the reporter, the preferential ubiquitination site could be determined. RWRWR served as a negative control, with all ornithine residues (e.g., potential ubiquitination sites) replaced with arginine residues. These peptides were each tested in HeLa S100 lysates supplemented with either wild-type ubiquitin or No K Ub (Figure 4.1C). OWRWR exhibited the highest degree of ubiquitination, comparable to OWOWO. RWOWR and RWRWO both exhibited ubiquitination, but to a smaller extent. As expected, RWRWR exhibited no ubiquitination, further supporting that the ornithine residues in the OWOWO library were the sites of ubiquitin conjugation.

4.3.2 Kinetic Analysis of β-hairpin Ubiquitination

Initial studies using both OWOWO-RRRG and OWOWO indicated that the two peptides had comparable ubiquitination rates, suggesting that in addition to being highly degradation-resistant, OWOWO and OWRWR were potent degrons in their own right. To compare OWOWO and OWRWR ubiquitination kinetics to those of other peptides, the kinetic parameters of each

peptide were determined. Additionally, an unstructured variant of OWOWO (Scram-OWOW) was synthesized and evaluated to determine what role, if any, the β-hairpin structure played in the ubiquitination of these degrons. Kinetic data was obtained using a time course assay in HeLa S100 lysates supplemented with No K Ub (Figure 4.3A, 3C, and 4A). Multi-ubiquitination was observed with both peptides, likely the result of multi-mono-ubiquitination or poly-ubiquitination by endogenous ubiquitin. The intensity of each ubiquitination band was normalized to a standard of unreacted peptide and the fraction of the peptide ubiquitinated over time was calculated. These values were used to generate kinetic parameters for each reporter, using a stepwise ubiquitination kinetics model developed by Dr. Adam Melvin, similar to the one presented in Chapter 2 of this document.[8] This computational model used the observed ubiquitination rate to produce first order reaction kinetics based off of four kinetic rate constants: mono- (k_1), di- (k_2), tri- (k_3) ubiquitinated species, and the loss of peptide via either degradation or de-ubiquitination (k_4). (Figure 4.3B, 4.3D, 4.4B)

Interestingly, mono-ubiquitination was the dominant reaction product for both the OWOWO and OWRWR peptides (k_1 = 2.5 and 3.5, k_2 = 0.9 and 0.9, k_3= 0.8 and 0.9 respectively, Figure 4.3B, 4.3D) while Scram-OWOWO exhibited a higher degree of both di- and tri-ubiquitination (k_1 = 2.8 and 3.5, k_2 = 1.4, k_3= 1.4, Figure 4.4B). This is suspected to be due to a lack of secondary structure in the peptide, which likely increased the availability of the internal ornithine residues for multi-mono-ubiquitination. The kinetic model achieved a good fit for all three peptides tested, with low values for the cSSD and Pearson correlation coefficient values near 1 for most of the species tested (Figure 4.3F and 4.4C).

4.3.3 Characterization of the β-hairpin Degron Structure

Previously described and well-characterized β-hair peptides, WKWK and WRWR, served as the template for OWOWO and, subsequently, OWRWR.[17, 18] WKWK and WRWR structures were well-folded according to NMR (nuclear magnetic resonance spectroscopy) and CD (circular dichroism) characterization (>93% in both cases); however, it was unknown what effect the modifications made to OWOWO and OWRWR would have on these well-folded structures. To investigate these effects, CD experiments were carried out using OWOWO, OWRWR, and an unstructured, scrambled version of OWOWOs sequence: Scram-OWOWO.

These experiments were carried out by Dr. Kaiulani Houstan, but warrant discussion here. The CD spectrum of the OWOWO peptide displayed the expected characteristics for this class of β-hairpin peptides with a minimum near 205 nm (Figure 4.5). Additionally, a minimum near 215 nm and maximum near 225 nm were observed that derived from the exciton coupling of the cross-strand Trp residues' indole rings, providing further evidence of the correct fold and register.[19] These spectra are consistent with the CD spectrum of the related peptide, WKWK, and while the mean residue ellipticities of OWOWO and OWRWR differ, neither exhibited the peak at 195 nm characteristic of a random coil structure. Scram-OWOWO served as a negative control for these experiments, and differed significantly from OWOWO, with a minimum near 195 nm that did suggest a random coil structure.

4.3.4 Resistance of OWRWR to Intracellular Peptidases

Work by Cline *et al.* had previously demonstrated that β-hairpins increased resistance to peptidase-mediated degradation in cytosolic environments relative to unstructured peptides.[14] CD experiments suggested that OWRWR had a similar structure to a β-hairpin, so it was strongly expected that OWRWR would increase peptidase resistance comparably. To test this hypothesis, OWRWR, an unstructured version of OWRWR (Scram-OWRWR), and an

unstructured control peptide (III-67B) were subjected to freshly-prepared OPM2 lysates. Peptides were incubated at 37 °C at varying lengths of time, after which cellular reactions were halted by heating the sample at 90 °C for 5 minutes. Peptides were then analyzed using CE, which allowed for separation of degraded peptide fragments from un-degraded peptide.

OWRWR was highly robust in OPM2 lysates (Figure 4.6, squares), with 80% of the peptide intact after 180 minutes, corresponding to a half-life of 630 minutes. Scram-OWRWR (Figure 4.6, circles) quickly degraded with a half-life just 30 minutes. Finally, an additional control peptide, III-67B (FAM-GGAYAATKKKA), which had been previously characterized was used as an additional control, was found to have a half-life of only 4 minutes in OPM2 lysates (Figure 4.6, triangles).[12] These results highlight the importance of the well-folded β-hairpin structure in conferring resistance to intracellular peptidases.

4.3.5 Determination of OWRWR E3 Ligase Specificity

Experiments using cytosolic lysates demonstrated that OWRWR was ubiquitinated by cells, but because it was not derived from a known degron, it was unclear what components of the UPS were responsible for its ubiquitination. In order to investigate which classes of E3 ligases were potentially responsible for OWRWR ubiquitination, *in vitro* ubiquitination experiments were carried out in the presence of a panel of E3 ligase inhibitors as well as an E1 inhibitor. Cytosolic lysates were incubated for one hour with one of five E3 ligase inhibitors (serdemetan nutlin-3, SKPin C1, SMER3 [small molecule enhancer of rapamycin 3], and thalidomide), the E1 enzyme inhibitor PYR-41, or a DMSO control (Figure 4.7)[20-24]. Each inhibitor was added at a concentration three times that of its established IC50 value. The reaction buffer was also supplemented with wild-type ubiquitin, a DUB inhibitor (ubiquitin aldehyde) and a proteasome inhibitor (MG-132). OWRWR was allowed to incubate in this mixture for 2 hours at 37 °C.

The E3 ligase inhibitors nutlin-3, serdementan, and thalidomide (Figure 4.7, Lanes 2, 3, and 6, respectively) had quantifiable effect on the ubiquitination of the OWRWR peptide, suggesting that the enzymes they inhibit were not primarily responsible for OWRWR ubiquitination. SMER3 significantly reduced both the poly- and mono-ubiquitination of OWRWR (Figure 4.7, Lane 5). SKPin C1 (Figure 4.7, Lane 4) appeared to reduce poly-ubiquitination, but not mono-ubiquitination. SMER3 is a specific inhibitor of the E3 ligase SCF-MET30, whereas SKPin C1 inhibits the cullin-RING ubiquitin E3 ligase SCF-Skp2.[22, 23] Taken together, this suggests that OWRWR could act as a reporter for the SCF family of E3 ubiquitin ligases. This family of E3 ligases contains three core subunits and a variable F-box protein (e.g., Skp2 or Met30). This variable F-box protein enables these E3 family members to recognize and bind to specific target proteins. The results presented here suggest that the β-hairpin OWRWR was recognized and ubiquitinated by an SCF complex; however, it does not appear ubiquitination was constrained to a single F-box protein as a recognition metric.

SCF E3 ligase inhibitors have been known to target specific proteins in cells, however it is possible that they recognize a wide range of small degrons, such as OWRWR. Work in chapter 3 of this document found that SKPin C1 and SMER3 also impaired ubiquitination in a nine amino acid-long peptide reporter based on a MDM2 binding region on the tumor suppressor p53 (KGSYGK(FAM)RRR).[15] Given these observations, it is possible that the SCF family of E3 ligases may lack specificity when it comes to the ubiquitination of short peptide sequences, perhaps favoring polycationic peptides. Interestingly, The E1 ubiquitin-activating enzyme inhibitor PYR-41 had no quantifiable effect on the ubiquitination of OWRWR (Figure 4.7, Lane 7), suggesting that it may not target the E1 enzyme involved in OWRWR ubiquitination.

4.4 Conclusions

In this work, we present work characterizing OWRWR, a 12-residue β-hairpin peptide that acts an E3 ligase reporter, and demonstrated substantial robustness in the presence of intracellular peptidases. OWRWR was optimized from the protectide OWOWO, after it was determined that it functioned as a degron independently of an additional degron sequence. Following a screen of E3 ligase inhibitors, we found evidence to suggest that OWRWR is a potential substrate of the SCF family of E3 ligases, an important group of E3 ligases that has been linked to several diseases.[25] This protectide-based primary degron is thus a promising new tool as a reporter for detecting ubiquitination and potentially characterizing proteasome activity.

4.5 Figures and Tables

A)

Name	Sequence
OWOWO	Ac-**O**WV**O**VpGO(FAM)WI**O**Q-NH2
RWRWR	Ac-RWVRVpGO(FAM)WIRQ-NH2
OWRWR	Ac-**O**WVRVpGO(FAM)WIRQ-NH2
RWOWR	Ac-RWV**O**VpGO(FAM)WIRQ-NH2
RWRWO	Ac-RWVRVpGO(FAM)WI**O**Q-NH2
Scram-OWOWO	Ac-**O**WV**O**WIO(FAM)QV**O**pG-NH2
OWOWO-RRRG	Ac-**O**WV**O**VpGO(FAM)WI**O**Q-(PEG2)-RRRGGGG**K**-NH2
III-67B	FAM-GGAYAATKKKKA-NH2

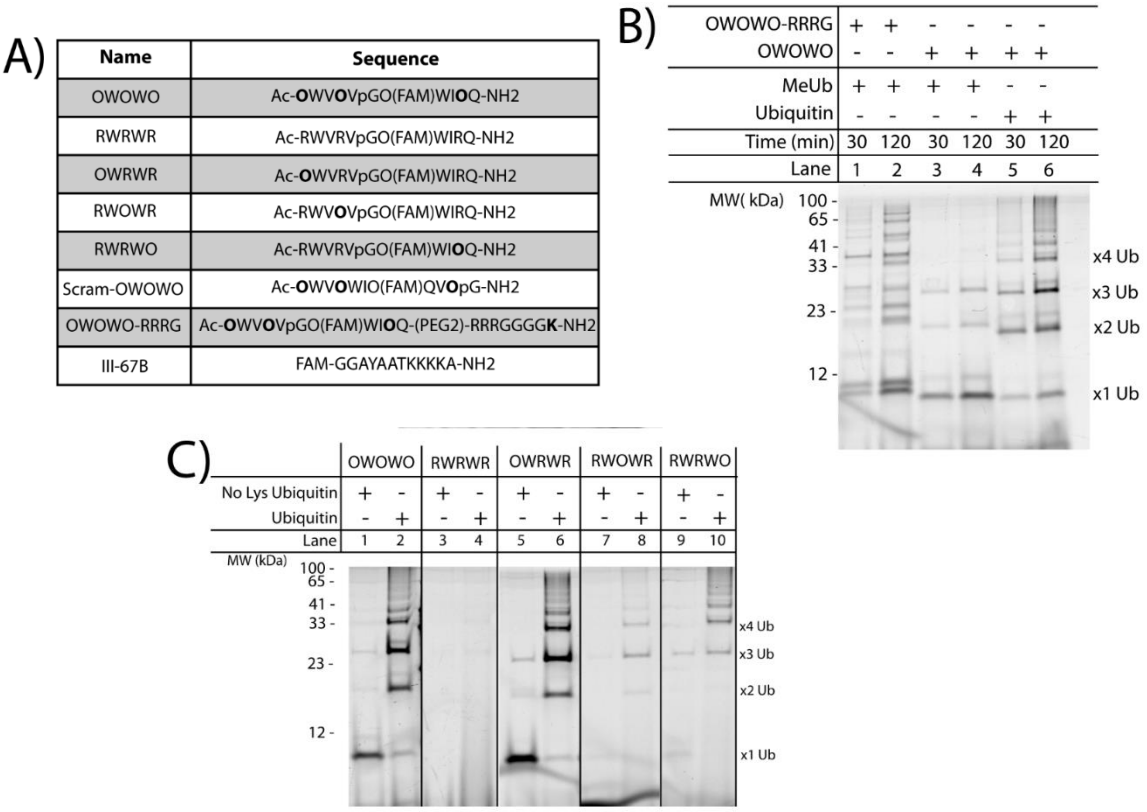


Figure 4.1 Ubiquitination of β-hairpin peptides in cell lysates. (A) Names and sequences of peptides studied. Bold amino acids (O = ornithine) correspond to potential ubiquitination sites. Peptides are acetylated at the N-terminus and amidated at the C-terminus. FAM denotes 6-carboxyfluorescein. (B) Ubiquitination of β-hairpin conjugated to a known degron (OWOWO-RRRG, Lane 1-2) compared to β-hairpin alone (OWOWO, Lane 3-6) using either ubiquitin or methylated ubiquitin (MeUb). Ubiquitin-conjugated peptides were purified using a ubiquitin pull down, separated using SDS-PAGE, and visualized using the fluorescein tag. Relative protein sizes (kDa) are compared to values obtained from a fluorescent protein marker (left of the gel). (C) The optimal ubiquitination site, ornithine, was identified using ubiquitin or no lysine ubiquitin. All reactions were incubated at 37 °C for 2 hours.

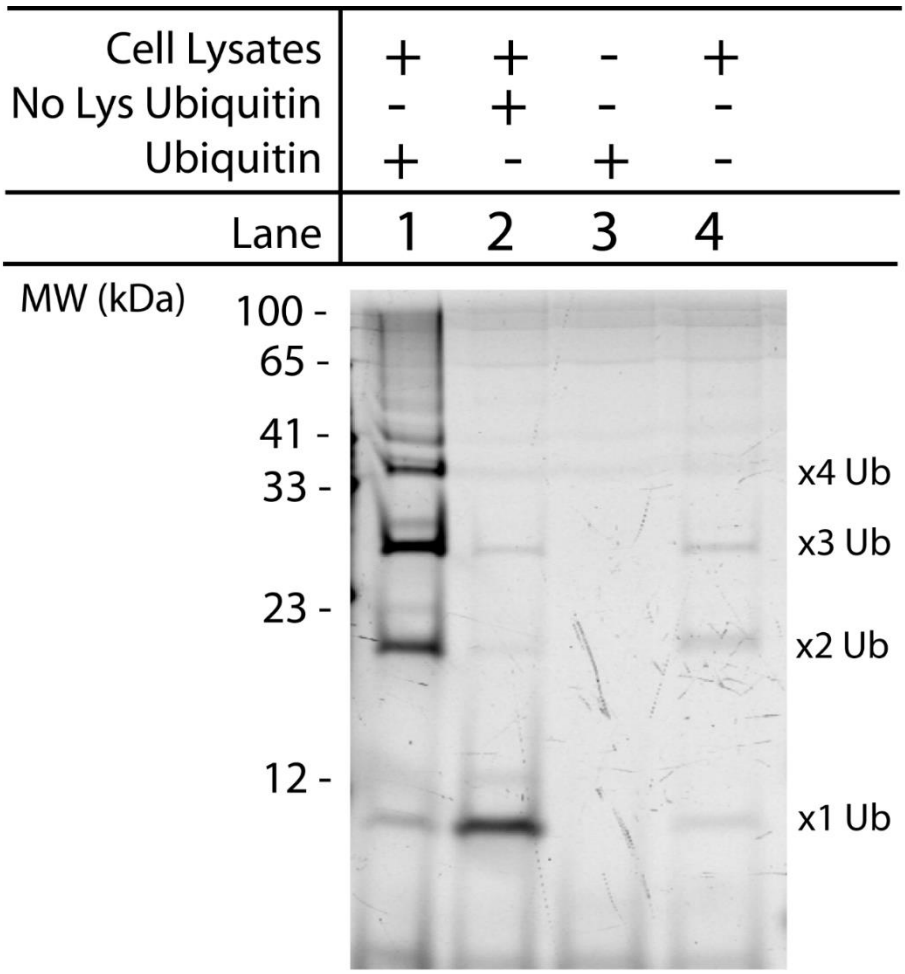
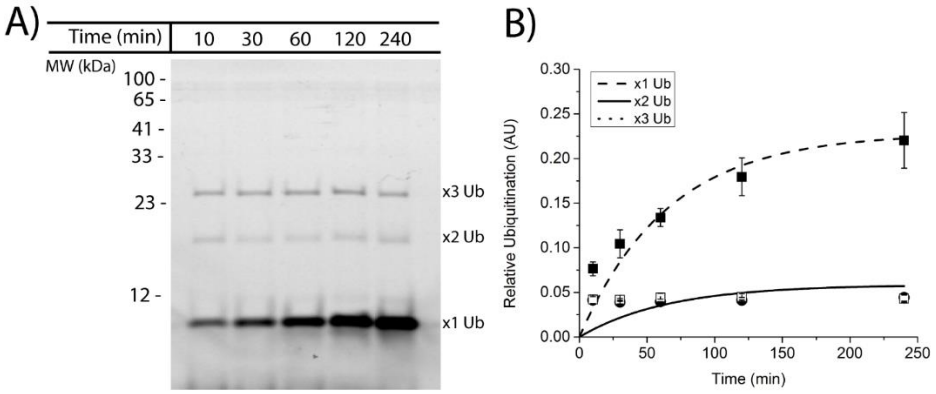


Figure 4.2 Ubiquitination of OWOWO in cell lysates. OWOWO demonstrates both multi-mono-ubiquitination and poly-ubiquitination. Ubiquitin-conjugated peptides were purified using a ubiquitin pull down, separated using SDS-PAGE, and visualized using the fluorescein tag. Relative protein sizes (kDa) are compared to values obtained from a fluorescent protein marker (left).

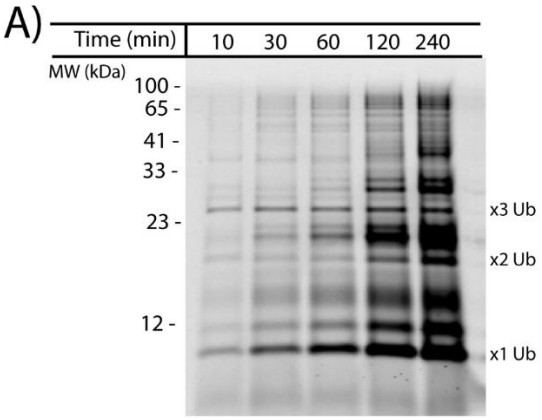


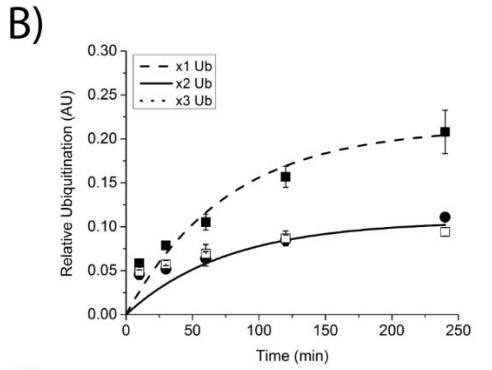
E)

	k_1	k_2	k_3	k_4	cSSD
OWOWO	2.5	0.9	0.8	7.5	2.767
OWRWR	3.5	0.9	0.9	10	2.858

	r_{xy}		
	Mono-Ub	Di-Ub	Tri-Ub
OWOWO	0.994	0.940	0.998
OWRWR	0.985	0.572	0.645

Figure 4.3 Time-dependent ubiquitination of OWRWR (A) and OWOWO (C) peptides. Experimental data points for mono-(closed squares), di-(closed circles), and tri-(open circles) ubiquitinated species for OWRWR (B) and OWOWO (D). The Y axis depicts the relative ubiquitination (C_i/C_o). The lines indicate the best fit of the data to the kinetic model. (E) Summary of the kinetic rate constants as determined by the kinetic model. The rate constants correspond to mono- (k_1), di-(k_2), or tri-(k_3) ubiquitinated species along with a term (k_4) for peptide unable to participate in the reaction. cSSD is the cumulative sum of the squared difference minimized in the model to achieve the desired fit. Pearson correlation coefficients (r_{xy}) describe goodness-of-fit between the model and experimental data for mono-, di-, and tri-ubiquitinated species.





C)

	k_1	k_2	k_3	k_4	cSSD
Scram-OWOWO	2.8	1.4	1.4	7.5	2.517

	r_{xy}		
	Mono-Ub	Di-Ub	Tri-Ub
Scram-OWOWO	0.977	0.963	0.996

Figure 4.4 Time-dependent ubiquitination of Scram-OWOWO (A). (B) Experimental data points for mono-(closed squares), di-(closed circles), and tri-(open circles) ubiquitinated species. The Y axis depicts the relative ubiquitination (C_i/C_o). The lines indicate the best fit of the data to the kinetic model. (C) Summary of kinetic rate constants as determined by the kinetic model. The rate constants correspond to mono- (k_1), di-(k_2), or tri-(k_3) ubiquitinated species along with a term (k_4) for peptide unable to participate in the reaction. cSSD is the cumulative sum of the squared difference minimized in the model to achieve the desired fit. Pearson correlation coefficients (r_{xy}) describe goodness-of-fit between the model and experimental data for mono-, di-, and tri-ubiquitinated species.

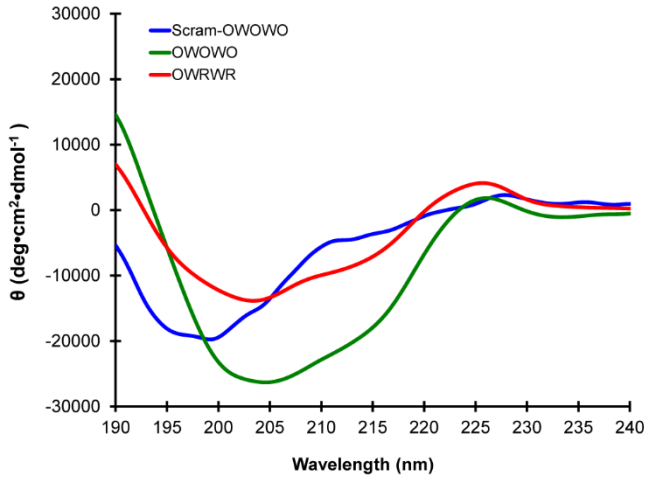


Figure 4.5 Circular dichroism spectra of a β-hairpin degron. Experiments were performed using 40 μM peptide in 10 mM sodium phosphate buffer, pH 8 at 25 °C. Scram-OWOWO (blue) was determined to be a random coil structure while OWOWO (green) and OWRWR (red) peptides exhibited spectra associated with a well-folded, β-sheet conformation.

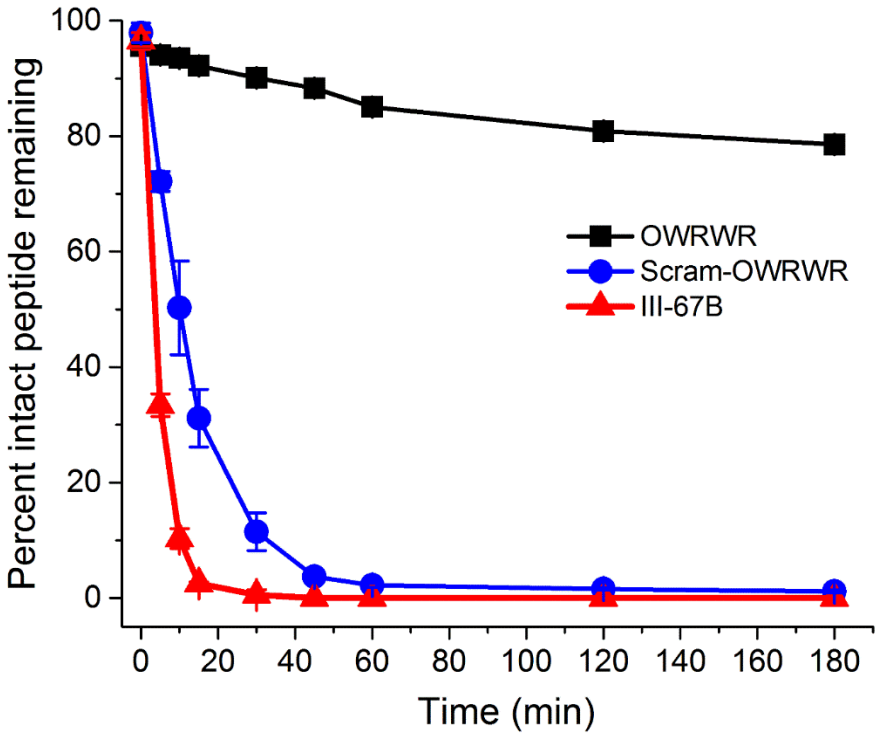


Figure 4.65 Capillary electrophoresis analysis of peptide degradation in OPM2 lysates. Stability of structured and unstructured peptides was evaluated by incubation with 2 mg/mL OPM2 lysates at 37 °C. Samples were removed from the enzymatic mixture at the indicated time points and separated using CE to identify and quantify the area of the peak corresponding to the parent peptide. Percent of intact peptide was calculated by dividing the area of the peak corresponding to the parent peptide by the total amount of fluorescence. The structured, β-hairpin degron OWRWR (black squares, $t_{1/2}$=626.2 minutes) was substantially more resistant to degradation than its unstructured counterpart Scram-OWRWR (blue circles, $t_{1/2}$=27.9 minutes) as evidenced by having a substantially greater amount of intact peptide and significantly higher half-life. An unstructured, rapidly degraded peptide (III-67B) was used as a positive control to demonstrate the activity of the OPM2 lysates (red triangles, $t_{1/2}$=3.96 minutes). The 0 minute time point used heat-killed lysates and served as the baseline for intact peptide. n=3.

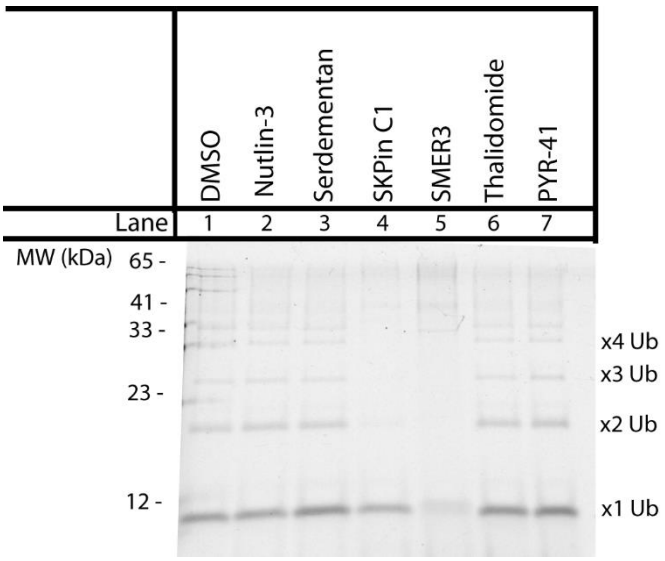


Figure 4.7 Selective inhibition of OWRWR ubiquitination in cell lysates. OWRWR peptide (10 μM) was incubated in HeLa S100 lysates for 2 hours at 37 °C in the presence of commercially-available E1 and E3 enzyme inhibitors. OWRWR (10 μM) was incubated in HeLa S100 cytosolic lysates for 2 hours. For inhibitor studies, E1 and E3 enzyme inhibitors were added 1 hour before the reaction. Each inhibitor was added with the following concentrations: nutlin-3 (0.27 μM), serdementan (30 μM), SKPin C1 (150 μM), SMER3 (300 μM), thalidomide (90 μM), PYR-41 (30 μM), or a DMSO control.

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Chapter 5: Adapting OWRWR to Analysis by Capillary Electrophoresis

5.1 Introduction

Ubiquitination, the conjugation of ubiquitin to proteins by components in the ubiquitin proteasome system (UPS), is a versatile post-translational modification (PTM), governing both proteasome-mediated degradation as well as key signaling pathways responsible for cell homeostasis.[1-3] Ubiquitination is principally carried out by E3 ligases, a diverse class of enzymes with at least 600 known in humans, organized into several families.[4] E3 ligases serve two major functions: (1) recognize target proteins for ubiquitination, and (2) facilitate the conjugation of ubiquitin to that protein, either directly or by bringing the protein and other elements of the UPS into close proximity with each other.[5]

Dysregulation of E3 ligases, as well as other UPS components, is suspected to play key roles in several human diseases including cancers and neurodegenerative disorders; this has prompted intense interest in developing therapeutics targeting E3 ligases as well as other components of the UPS.[6] The most prominent of these drugs are the blockbuster proteasome inhibitors Bortezomib and Carfilzomib; however, a slew of new drugs targeting numerous different components of the UPS are currently in clinical trials.[7-10] E3 ligases in particular are thought to be a fertile ground for such therapeutics, with drugs targeting E3 ligases including HDM2, cereblon, p97, and Parkin currently in clinical trials or approved for treatment.[7]

Research into E3 ligases is made difficult due to a lack of tools for directly assessing enzymatic activity. The self-regulatory nature of the UPS can confound proxy measurements for activity such as mRNA analysis, and Western blots, the gold standard, are limited by both their sample size requirements and the lack of specific antibodies.[1] Techniques such as FRET (Förster

resonance energy transfer) sensors, can directly measure E3 ligases activity and have been used to efficiently screen large numbers of compounds for E3 ligase inhibition, but these techniques require genetic engineering approaches as well as large sample sizes.[11] This leaves a gap in existing technologies for techniques compatible with small sample sizes, such as primary patient samples. Peptide-based reporters represent a new technique to fill this niche, but have only been analyzed using large scale techniques such as SDS-PAGE.[12]

This chapter reports on the development of a capillary electrophoresis (CE)-based assay using a previously described degradation resistant peptide reporter, OWRWR, that is ubiquitinated in cell lysates. This was done by identifying the electrophoretic separation conditions required for sub micro-molar analysis of ubiquitin by CE, and then optimizing the separation between ubiquitin and OWRWR. Following the identification of separation conditions, analysis of OWRWR was validated using both a recombinant enzyme ubiquitination assay as well as a cell-based ubiquitination assay. This work lays the foundation for further CE analysis optimization towards the analysis of very small numbers of cells.

5.2 Experimental Design

5.2.1 CE analysis

Samples were analyzed using a Beckman Coulter ProteomeLab™ PA800 automated CE system (Brea, CA) (CE-LIF, 488 nm) as previously described.[13] CE analysis was carried out by Gregery Woss, with buffers prepared with the assistance of Joshua Mu. Prior to CE analysis, samples were thawed to room temperature and diluted in separation buffer. The capillary was washed with 1 M NaOH for 5 minutes, dH_2O for 5 minutes, and separation buffer for 5 minutes by applying 20 psi to the capillary inlet. Separation was carried out in a fused-silica capillary (30 μm inner capillary, 360 μm outer capillary, Polymicro Technologies, Pheonix, AZ) with a total length of 30 cm and an effective length of 20 cm. Samples were injected into the capillary by

applying 0.5 psi to the capillary inlet for 5 seconds, and separation was initiated by applying a negative voltage to the outlet as determined by an Ohm's plot for each buffer. Analytes were quantified by calculating by the corrected area under the parent peptide peak divided by the total corrected peak area (i.e., the area under all peaks of the electropherogram) for each time point. The identity of each peak was confirmed using analytes alone or by spiking samples with standards. The data was analyzed using commercial software (32 Karat, version 8.0, Beckman Coulter). All optimization analyses were performed in triplicate.

5.2.3 Tissue Culture

HeLa S100 lysates were generated to provide components of the UPS that could be used reproducibly and consistently throughout the development process. Tissue culture was carried out by Gregery Woss. HeLa S3 cells (ATCC, Manassas, VA) were grown in Dulbecco's modified eagle medium (DMEM) with 10% v/v bovine calf serum (HyClone, GE Healthcare Logan, UT) and maintained in a 37 °C, 5% CO_2 environment. Cells were initially grown in T-75 culture flasks until 70% confluent. Cells were then rinsed with phosphate-buffered saline (PBS) and treated with 2 mL trypsin-EDTA for 5 minutes or until detached, at which point they were spun down at 800-1200g for 10 minutes, resuspended in fresh media, and expanded into additional flasks. Once 8 flasks had reached 70% confluence, cells were treated with trypsin-EDTA as described above and re-suspended in suspension growth media (RPMI 1640 supplemented with 5% v/v bovine calf serum) to a density of 2×10^5 cells/mL in a 250 mL spinner flask. Cells were allowed to grow with fresh media added to prevent exceeding a cell density of 5×10^5 cells/mL. After 2-3 days of growth, cells were spun down at 800-1200g for 10 minutes and resuspended in fresh media in a 1 L spinner flask. This process was repeated until the suspension volume reached 1 L, at which point the above process was repeated, and the cells

were transferred to a 3 L spinner flask to grow with fresh media added until the suspension reached a volume of 3 L and a density of 4-6 x 10^5 cells/mL, when the cells were harvested. Cells were spun out of solution as described above, then washed with ice cold PBS and pelleted at 1200g for 10 minutes. The cell pellet volume was recorded as packed cell volume (PCV). 5X PCV hypotonic buffer (10 mM HEPES pH 8.0, 10 mM KCl, 1.5 mM $MgCl_2$, and 1 mM DTT) was used to swell the cells over the course of 10 minutes at 4 °C, at which point cells were centrifuged at 1800g for 10 minutes. This pellet was resuspended in 2X PCV in hypotonic buffer and lysed with approximately 10 strokes in a Dounce glass homogenizer. The resulting lysed cells were centrifuged for 10 minutes at 1200g.

Following centrifugation, the resulting supernatant was collected and supplemented with 0.11X PCV of buffer B (0.3 M HEPES pH 8.0, 1.4 M KCl, and 30 mM $MgCl_2$). This solution was centrifuged at 100,000g for 60 minutes at 4 °C. The supernatant was collected and dialyzed twice, first overnight and again for 4 hours, in buffer D (20 mM HEPES pH 8.0, 100 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, 1 mM DTT, and 20% v/v glycerol) at 4 °C. The lysate was centrifuged one final time at 33,000g for 20 minutes at 4 °C. The cytosolic lysates were quantified using a Nanodrop 2000 (Thermo Scientific), aliquoted, and stored at -80 °C.

5.2.4 In vitro OWRWR ubiquitination assay using recombinant enzymes

OWRWR-Ubiquitin analytes were prepared using an *in vitro* ubiquitination assay using recombinant human enzymes. These reactions were carried out by Gregery Woss. The reaction buffer was composed of 10 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 2 mM DTT, 800 μg/mL ubiquitin (Boston Biochem, Cambridge, MA), 1X ATP (Sigma-Aldrich, Saint Louis, MO), 1.5 μM Ube1 (E1, Boston Biochem, Cambridge, MA), and 15 μM UbcH5b (E2, Boston Biochem, Cambridge, MA). Reactions were carried out at 37 °C for 4 hours, then all reagents other than

peptide were replenished. This replenishment was carried out again at 8 hours, and at 12 hours the reaction was halted by heating at 90 °C for 5 minutes. For analysis by gel electrophoresis, samples were mixed 2:1 with Tricine Sample Buffer (Bio-Rad, Hercules, CA) and loaded into precast 16.5% Mini-PROTEAN Tris Tricine SDS-PAGE gels (Bio-Rad, Hercules, CA). Gels were visualized with a Typhoon Imager (GE Healthcare Life Sciences, Pittsburgh, PA). Quantification of gels was done using ImageJ software (US National Institute of Health).

5.2.5 Cell lysate ubiquitination assay/Ubiquitin pulldown assay

Peptides were evaluated for ubiquitination activity in HeLa S100 or K562 lysates. These reactions were carried out by Gregery Woss. A reaction buffer was prepared containing 10 mM Tris-HCl pH. 7.6, 5 mM $MgCl_2$, 2 mM DTT, 20 μg per mL ubiquitin aldehyde (Boston Biochem), 800 μg/mL ubiquitin (Boston Biochem), 1X ATP (Sigma-Aldrich, Saint Louis, MO), and HeLa S100 cytosolic lysates or K562 lysates diluted to a set concentration. For some experiments, ubiquitinated reporter was confirmed using a ubiquitin pull-down assay. Following incubation, samples were incubated with Control-Agarose beads (LifeSensors), diluted in TBS-T buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, and 0.1% v/v Tween-20), for 60 minutes on a tube rotator at 4 °C. Samples were subsequently centrifuged at 1800g for 5 minutes to pellet and remove control beads. The supernatant was transferred to a solution of Agarose-TUBEs (LifeSensors, Malvern, PA), diluted in TBS-T, and incubated overnight on a tube rotator at 4 °C. Ubiquitin-bound beads were washed 5X with 1X TBS-T and the samples were eluted off the bead by incubating with either 2X tricine sample buffer, or 1X 1 mg/ml ubiquitin and heated for 5 minutes at 90 °C followed by centrifugation for 5 minutes at 13000 X g.

5.2.6 Data Analysis

Data was analyzed using commercial software (32 Karat, version 8.0, Beckman Coulter) to calculate the following parameters: the width of each peak ($W_{\frac{1}{2,}OWRWR}, W_{\frac{1}{2}OWRWR-Ub}$), and the migration time of each peak ($t_{OWRWR}, t_{OWRWR-Ub}$). Data was analyzed by Gregery Woss. These variables were used to calculate resolution (R) and the number of theoretical plates (N) using the following equations:

$$W_{\frac{1}{2,}Avg} = \frac{W_{\frac{1}{2,}OWRWR} + W_{\frac{1}{2}OWRWR-Ub}}{2}$$

$$R = \frac{0.589(t_{OWRWR} - t_{OWRWR-Ub})}{W_{\frac{1}{2}Avg}}$$

$$N = \frac{5.55t^2}{W_{\frac{1}{2}Avg^2}}$$

5.3 Results and Discussion

Capillary electrophoresis has been successfully used to separate and quantitate samples with ubiquitin moieties in several reports throughout the literature.[14, 15] These separations have been carried out at relatively high concentrations of ubiquitin (> 1 μM), using either high concentrations of salt or detergent, or highly acidic buffers. However, in order to function as a diagnostic tool, the separation of OWRWR and Ub-OWRWR must be demonstrably fast, efficient, capable of detecting ubiquitin at nano-molar concentrations, and highly reproducible. Preliminary experiments were carried out using ubiquitin conjugated to a single FAM tag (FAM-Ub), which provided a readily available and inexpensive proxy for OWRWR-ubiquitin. Experiments using reported buffer compositions demonstrated a non-linear decrease in signal versus the concentration of FAM-Ub at sub-milli-molar concentrations, suggesting that the globular ubiquitin was potentially adsorbing to the walls of the capillary. When an initial buffer

screen using common buffers failed to identify conditions that recovered signal at sub-milli-molar FAM-Ub concentrations, an extensive buffer screen was carried out using combinations of buffers in conjunction with a diverse range of additives (Table 5.1). Interestingly, the addition of unlabeled ubiquitin to buffers recovered the FAM-Ub signal down to the micro-molar level, providing some evidence that ubiquitin was adhering to the walls of the capillary; however, this proved to be an unreliable and expensive additive. Several high salt buffers were able to produce micro-molar levels of sensitivity; however, a single buffer additive, polysorbate 20 (TWEEN 20, Sigma-Aldrich, Saint Louis, MO) was capable of recovering FAM-Ub signal into the nano-molar range. This additive was paired with a buffer salt that had previously performed well, sodium tetraborate, to produce a starting point for an optimization screen for the separation of OWRWR and FAM-Ub. The concentration of polysorbate 20 (Figure 5.1A), buffer salt concentration (Figure 5.1B), and buffer pH (Figure 5.1C), were iterated in order to optimize the resolution and theoretical plates for each analyte. The optimized separation in shown in Figure 5.1D (125 mM sodium tetraborate with 3% TWEEN 20 at pH 8.5), with the resolution between analytes being 14.5 and the theoretical plates being 25,000 and 31,500 for OWRWR and FAM-Ub, respectively. Following the identification of separation conditions using OWRWR and FAM-Ub, the next step was to validate the separation using OWRWR and ORWRW-Ubiquitin. While ORWRW-Ubiquitin is difficult to chemically synthesize, OWRWR is ubiquitinated in cell lysates. This can serve as a source of OWRWR-Ubiquitin; however, the ubiquitination reaction occurs at a similar rate to degradation reactions, potentially confounding analysis. Previous experiments in the last chapter demonstrated that OWRWR ubiquitination was diminished when lysates were treated with inhibitors of the SCF family of E3 ligases, behavior that had previously been observed in a minimal p53 based reporter.[16] This suggested that OWRWR could potentially be

ubiquitinated by the same recombinant enzymes, Ube1 (an E1 activating enzyme) and UbcH5b (and E3 conjugating enzyme). Control experiments (Figure 5.2A) and pulldown experiments (Figure 5.2B) demonstrated the ubiquitination of OWRWR in the presence of these enzymes, resulting in a relatively clean OWRWR-Ubiquitin standard. OWRWR and OWRWR-Ubiquitin were cleanly separated using the previously identified CE buffer, as demonstrated in Figure 5.3.

Following the confirmation that OWRWR and OWRWR-Ubiquitin could be separated by CE, further experiments were carried out to demonstrate that OWRWR-Ubiquitin formation could be observed in cell lysate experiments by CE. The results of these experiments show an OWRWR-Ubiquitin peak that migrates at the expected time, and can be confirmed by spiking experiments (Figures 5.4). An unidentified peak, mostly likely a degradation fragment of OWRWR, migrates very close to the OWRWR-Ubiquitin peak, making precise quantification of the peak difficult. Though not yet complete, this work marks the first time the ubiquitination of a peptide reporter has been observed by CE, laying the ground work for future separations optimizations.

5.4 Conclusions

CE separation conditions were identified for the analysis of the peptide reporter OWRWR. Buffer conditions were identified and optimized for the separation of OWRWR and FAM-Ub following an extensive buffer screen. Cell-free recombinant enzyme ubiquitination experiments using OWRWR produced OWRWR-Ubiquitin standards, which were used to confirm the separation of OWRWR and OWRWR-Ubiquitin. Finally, the ubiquitination of OWRWR in cell lysates was observed by CE, a first for peptide-based E3 ligase reporters.

5.5 Figures and Tables

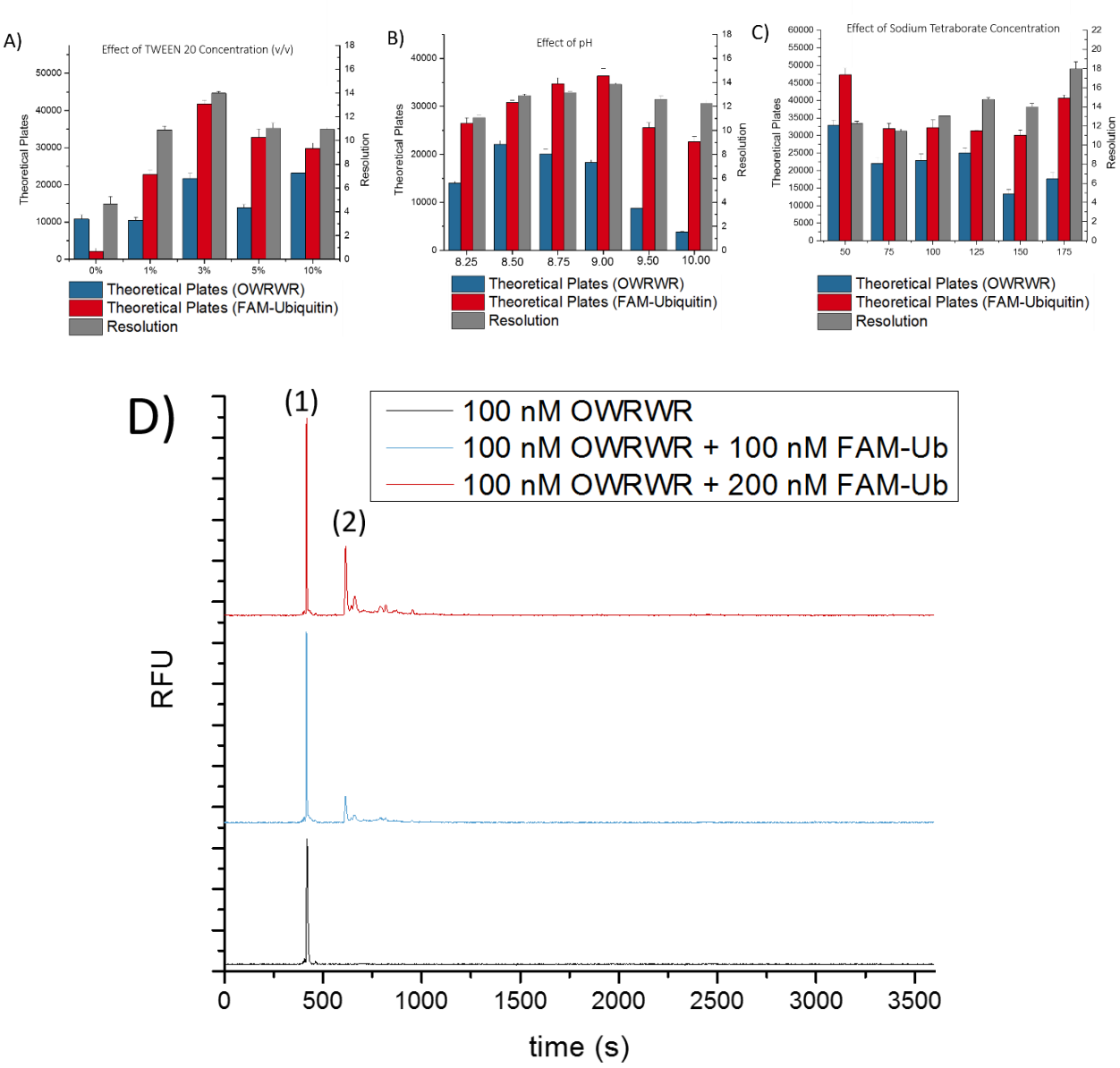

Figure 5.1 Optimization of OWRWR FAM-Ubiquitin Speraration. The effect of TWEEN 20 (A), sodium tetrabroate (B), and pH (C) on the separation of OWRWR and FAM-Ub; 125 mM sodium tetraborate with 3% TWEEN 20 at pH 8.5 was selected to maximize the number of theortical plates for each species while retaining a high level of resolution. (D) The separation of OWRWR (1) and FAM-Ub (2) using optimized buffer condtions.

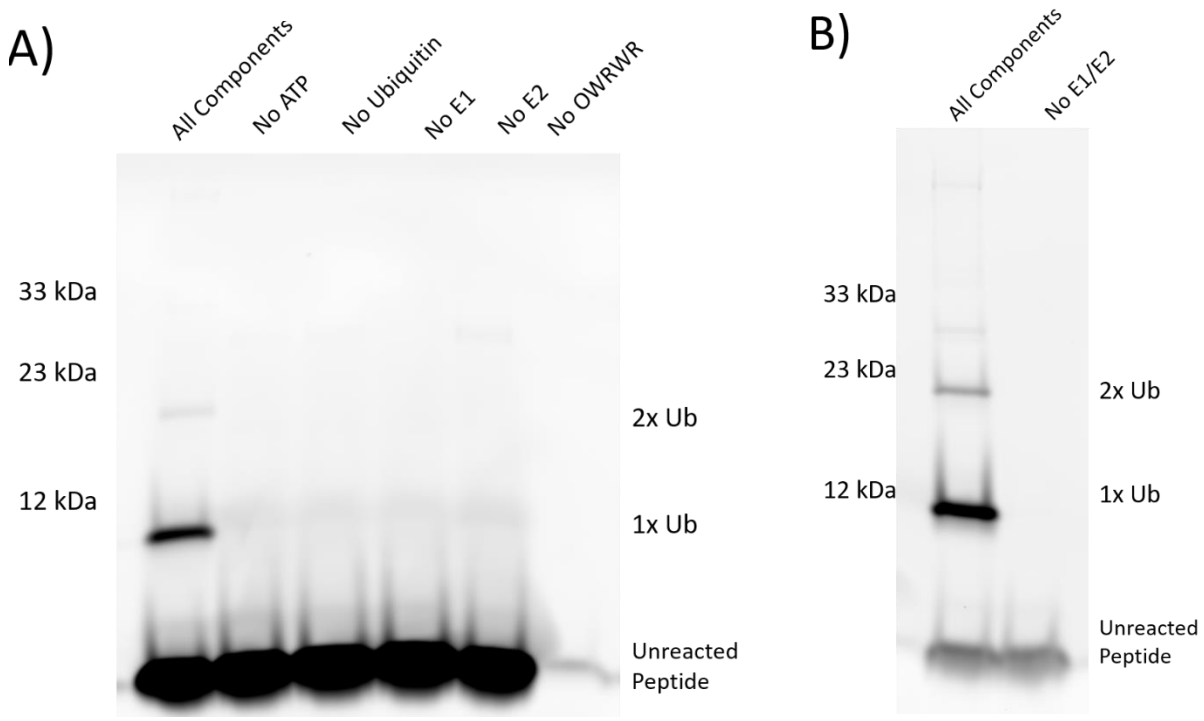

Figure 5.2 *In vitro* ubiquitination of OWRWR. A) OWRWR is ubiquitinated when incubated in the presence of Ube1 (E1) and UbcH5b (E2) ATP and ubiquitin. Ubiquitination can be seen at ~12 kDa and ~20 kDa. B) Ubiquitination bands were confirmed using a pull down assay.

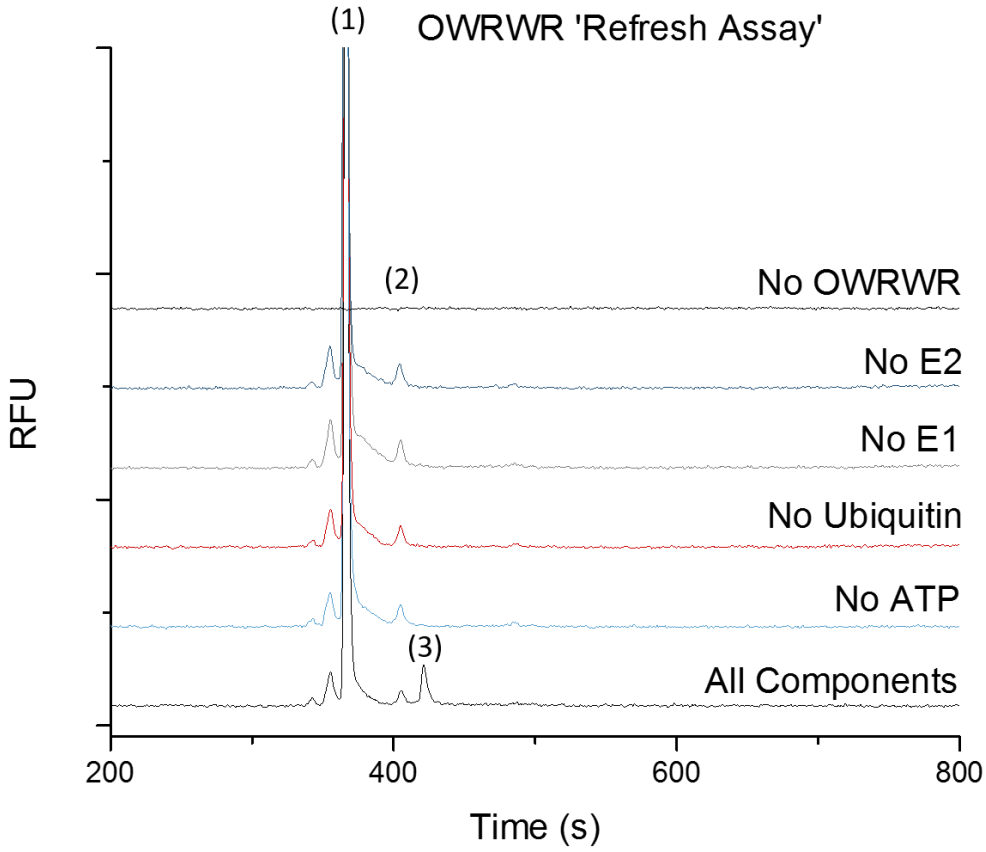

Figure 5.3 CE Analysis of OWRWR Ubiquitination. Electropherograms showing the formation of ubiquitinated products from an *in vitro* ubiquitination assay. Peaks (1) and (2) were confirmed as parent OWRWR and a peptide impurity, respectively, by standard spiking experiments. Peak (3) corresponds to ubiquitinated product. Data is zoomed in for clarity, peak (1) does not maximize the signal (not shown).

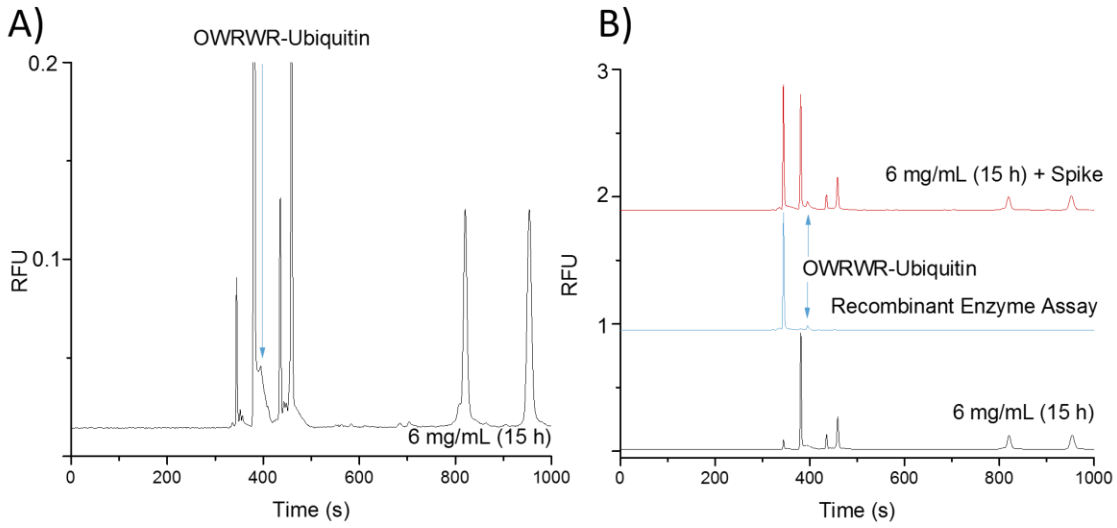

Figure 5.4 CE Analysis of OWRWR Ubiquitination in K562 Lysates. (A) Electropherogram showing the formation of ubiquitinated products from a cell lysate ubiquitination assay. The peak indicated by the blue arrow corresponds to ubiquitinated product. Data is zoomed in for clarity, peak (1) does not maximize the signal (not shown). (B) Spiking experiment confirm the indicated peak as ubiquitinated OWRWR.

Table 5.1 List of Buffer Salts and Additives Screened

Buffer Salts	Additives
Borate	2-propanol
CAPS	3-(N,N-Dimethylpalmitylammonio) propanesulfonate[17]
Glycine	Sodium dodecyl sulfate (SDS)
HEPES	Cetrimonium bromide (CTAB)
MOPS	Cyclodextrin
Sodium Bicarbonate	Dextrin
Sodium Citrate	N,N-dimethylacrylamide
Sodium Phosphate	Polyethylene glycol (PEG)
Sodium Tetraborate	Polyvinylpyrrolidone
Tricine	Putrescine
Tris	Sodium deoxycholate (SDC)
	Spermine
	Sulfobutylether-β-cyclodextrin
	Tween 20
	Ubiquitin

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