Introduction

The tumor suppressor p53 plays a significant role in a variety of cellular functions including DNA repair, cell cycle modulation, and programmed cell death, also called apoptosis.\(^1\) Mutations or functional inactivation of p53 is a hallmark in many cancer types, such as breast and colorectal cancer, affecting greater than 22 million people.\(^1\) Approximately half of these patients display mutant p53 that has lost its tumor suppressor function, while the other half exhibit decreased p53 levels due to overexpression of the E3 ubiquitin ligases murine double minute 2 (MDM2) and MDMX. MDM2 decreases p53 levels by acting as a transcriptional inhibitor and as an E3 ligase facilitating degradation of p53 via the proteasome.\(^1\) In fact, there is overwhelming molecular and genetic evidence that the major role of MDM2, and its related complexes, is the targeting of p53 for proteasomal degradation.\(^4\) Conversely, overexpression of p53 has been linked to the up-regulation of genes involved in cellular growth and apoptosis. Due to this, p53 has become an attractive therapeutic target by being the most frequently mutated gene in human cancer that not only plays a vital role in cellular function, but also has been proven to rescue apoptotic pathways in cancer cells.\(^5\)\(^6\)

In eukaryotic cells, the pathway for protein degradation via ubiquitination is commonly referred to as the ubiquitin proteasome system (UPS).\(^7\) Ubiquitination requires an increasingly complex enzymatic cascade involving E1 ubiquitin activating enzymes, E2 ubiquitin conjugating enzymes, and E3 ubiquitin ligases (Figure 1). First, free ubiquitin becomes conjugated to an E1 enzyme via a high-energy thioester bond (Figure 1B). Next, the ubiquitin is transferred from E1 to an E2 conjugating enzyme, forming an E2--Ub complex (Figure 1C). Finally, an E3 ubiquitin ligase facilitates the transfer of ubiquitin from E2 to a lysine residue on the target protein (labeled substrate, Figure 1D). The E3 ligase recognizes and ubiquitinates the protein through the recognition of a short amino acid sequence termed a degradation sequence, or degron.\(^8\) Finally, multiple ubiquitins

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Figure 1. Overview of the ubiquitin proteasome system (UPS). Protein ubiquitination requires a series of enzymatic reactions starting with free ubiquitin (A) binding an E1 enzyme (B). Ub is then transferred from E1 to an E2 enzyme (C) and then finally to an E3 ligase (D) resulting in a polyUb chain. Ub is removed from the protein by a DUB (E). Ubiquitinated proteins then move on to impact signaling pathways (F) or are degraded by the 26S proteasome to small peptide fragments (G). Reproduced from Melvin et al.\(^2\)
are sequentially added to the protein to form a polyubiquitin chain (a process termed polyubiquitination), effectively targeting the protein for degradation by the 26S proteasome complex (Figure 1G).

The development of therapeutic methods relating the numerous biomolecular interactions of p53 in oncogenic signaling pathways has been steadily progressing in recent years. For instance, efforts to restore the deregulated p53 pathway, such as small-molecule inhibitors of the p53-MDM2 interaction, p53 chaperone drugs, p53 gene therapy, and MDM2 inhibitors, are currently in clinical trials. Although a myriad of therapeutic opportunities to restore p53 function have been attempted, the most common approach has been to inhibit the function of proteins that reduce p53 amount and activity in cells. A majority of efforts and clinical trials are focused around small drugs that block the protein-protein interaction between p53 and HDM2, the human protein analog of MDM2. Drug discovery research has found peptides with higher affinities for the MDM2/HDM2 binding domain than p53, reinforcing the notion that the binding pocket of MDM2 can be inhibited effectively, as exemplified in Figure 2. The vast library of MDM2 inhibitors includes nutlin, benzodiazepinedione, chromenotriazolopyrimidine, terphenyls, chalcones, MI-219, RG7112, and many others that exhibit good pharmokinetic and pharmacodynamics properties. Additionally, a class of novel compounds termed stapled peptides has been developed to inhibit the p53-MDM2 interaction with more comprehensive and potent effects. The stapled peptide sMTide-02 was proven to display greater specificity and potency in its biological mechanism of MDM2 inhibition than previously identified inhibitors like nutlin and SAH-8. Molecules such as these stapled peptides are still under biochemical investigation in the hopes of reaching clinical trials and becoming powerful therapeutic tools. In addition to expanding the p53 related therapeutics library, research has progressed toward therapeutic characterization and evaluation, demonstrating that p53-targeted therapeutics must be sensitively controlled in order to achieve maximum tumor cell eliminations without bringing about toxicity to normal proliferating tissues. For instance, using murine models, researchers have indicated that therapies restoring wild-type p53 function can lead to apoptosis in normal, healthy tissues. Accordingly, one must control the intensity and duration of p53 response via precise activation or rescue p53 function.

This expanding library of MDM2 inhibitors, combined with ongoing efforts to further explore the inhibitory mechanism of the MDM2-p53 binding interaction demonstrates how the ubiquitin-mediated degradation of p53 via MDM2 activity has become the focal point of p53 related cancer drug discovery. The establishment of this biomolecular target niche, combined with the potential issues and restraints regarding therapeutic mechanism sensitivity, highlights the dire need for precise analytical
tools to evaluate the p53-MDM2 interaction in the UPS. In order to address this need, this project aims to develop a new biomolecular probe able to measure MDM2 activity. Specifically, the biosensor will be based on a degradation sequence of p53, allowing for recognition and binding by the E3 ligase MDM2.

Previous work in our lab has focused on the identification and characterization of novel recognition substrates based on naturally occurring degrons from various proteins involved in the UPS related to oncogenic signaling pathways. These degron-based substrates incorporated naturally occurring degrons to facilitate E3 ligase recognition, binding, and ubiquitination. The degron-based substrates consist of four essential components: the degron for E3 ligase recognition and binding, an ubiquitination site lysine, a linking region to prevent steric hindrance issues, and a fluorescent tag for visualization (Figure 3). The fluorescent tag, normally 5,6 carboxyfluorescein, allows for visualization of the reporter through either gel electrophoresis, HPLC, or capillary electrophoresis. This previous study resulted in the optimization of a previously described synthetic degron, termed Bonger, to identify the minimal amino acid sequence still capable of ubiquitination. We found that a four amino acid sequence (RRRG) could be efficiently ubiquitionated, which exhibits the potential to be incorporated into a novel proteasome reporter. This work was recently published in PLOS ONE, but was not included in this document due to size considerations. Additionally, as part of this study, we developed a degron-based substrate based on amino acids 92 to 112 from p53, as a reporter for evaluating MDM2 activity. This specific sequence was demonstrated to be necessary for MDM2 mediated degradation. Our previous work demonstrated that the p53 degron-based substrate exhibited a high degree of ubiquitination as well as rapid reaction kinetics in comparison to other degron-based substrates, which has prompted further exploration of this region of p53 for use in an E3 ligase MDM2 reporter.

A successful reporter for MDM2 activity needs to be easily synthesizable and resistant to intracellular peptidases, enzymes that hydrolyze the peptide bonds of small proteins, which requires that the degron-based substrate consists of a minimal sequence of amino acids capable of binding to the target protein. This is important because the substrate needs to incorporate a peptidase-resistant sequence, termed a protectide, to prevent rapid degradation by intracellular peptidases. Previous work in the Waters lab has identified several protectides and utilized them to stabilize peptide-based kinase reporters. To address these goals, we aim to identify the minimal functional degron sequence based on the previously tested p53 degron-based substrate by utilizing a shotgun synthesis approach to create a library of substrates of varying lengths, which will be tested for MDM2-mediated ubiquitination. The shotgun method involves synthesizing smaller, overlapping sequences of the known p53 degron and testing them with an in vitro ubiquitination assay using multiple analytical techniques. Novel information about the minimal functional degron and its subsequent ubiquitination by MDM2 in an in vitro assay will provide the basis for the design of a novel MDM2 reporter.
Materials and Methods

Substrate synthesis and purification.
Substrates were synthesized as previously described. Briefly, peptides were synthesized by solid phase peptide synthesis (SPPS) on a Creosalus Thuramed Tetras Automated Peptide Synthesizer using 150 mg of CLEAR-amide resin. Amino acids were activated with HBTU (4 eq), HOBT (4 eq), and DIPEA (5 eq) in DMF and NMP. Deprotection of the peptide N-terminus was conducted in 2% DBU and 2% piperidine in DMF for 2 x 15 min each. Amino acid coupling steps were performed twice for 30 min or 1 hr for natural amino acids and once for 4 hr in the case of Fmoc-Lys(ivDde)-OH. Acetylation of peptide N-termini was accomplished with 5% acetic anhydride and 6% 2,6-lutidine in DMF for 40 minutes. The Fmoc-Lys(ivDde)-OH side chain was deprotected with 3% anhydrous hydrazine in DMF for 3 cycles of 5 min each. Removal of the protecting group was confirmed by a Kaiser test. Conjugation of the fluorescent tag was performed with either 5,6-carboxy-fluorescein (4 eq) or 6-carboxy-fluorescein (4 eq) combined with PyBOP (4 eq), HOBT (4 eq), and DIPEA (4 eq) in DMF reacting in a dark environment overnight. The peptide was cleaved from the resin using a mixture of TFA, TIPS and H$_2$O in a 95:2.5:2.5 ratio, reacting for a minimum of 3 hr. After the TFA was evaporated, the product was precipitated with cold ether, extracted into water, and lyophilized.

Primary purification of peptides was performed via Reverse Phase HPLC using a C-18 semi-preparative column in a Waters 2998 HPLC with photo iodide ray detector running absorbance detection at 214 nm and 490 nm. Gradients were run from polar, hydrophilic Solvent A (95% water, 5% acetonitrile 0.1% TFA) to non-polar/hydrophilic solvent B (95% acetonitrile 5% water 0.1% TFA). The lyophilized peptide was reconstituted in 10-15 mL of Solvent A and filtered using a Millex 0.22 μL syringe driven filter. A trace was run using a 60 minute gradient of 0-100% B with a 200 μL sample injection. Traces were analyzed in collaboration with Adam Melvin to identify which peaks to collect. Peaks present in both 214 and 490 nm that were substantially greater than background noise, indicating the presence of fluorescein in combination with a peptide backbone were determined to be of importance and thus collected. The peptide was then purified via the above method using 1.0 mL injections and collecting the significant peaks. Eluent fractions containing substrate from primary purification were rotovapped using a Buechi Rotovapor R-200, lyophilized, reconstituted in solvent A, and subjected to secondary purification via reverse phase HPLC on the Waters 2998 HPLC. For secondary purification, a trace was run using a 100 minute gradient of 0-100% B with a 200 μL sample injection, and then analyzed according to peak presence at 214 nm and 490 nm. Subsequent injections were increased to 1 mL and the remaining peptide was purified collecting only the top 50% of desired peaks to ensure purity. Eluent fractions containing the desired substrate were rotovapped and lyophilized. The presence of the desired peptide in first and second purification HPLC eluent fractions was verified using ESI or MALDI mass spectrometry. The mass spectrometry analysis was performed by either Gregory Woss or Brendan Peacor at the UNC Proteomics facility (MALDI) or the UNC Department of Chemistry Mass Spectrometry Facility (ESI), using an AB SCIEX 4800 PLUS MALDI-TOF/TOF mass spectrometer. The purified peptide substrate was quantified using a Nanodrop 2000 spectrophotometer, measuring the fluorescent activity of the FAM tag, to determine the concentration of peptide. The peptides were reconstituted in 50 mM phosphate buffer and quantified by Beers Law, \( A = \varepsilon l C \), where \( A \) is the absorbance, \( \varepsilon \) the molar absorptivity, \( l \) the path length, and \( C \) the concentration). For our purposes \( l = 0.1 \) cm and \( \varepsilon = 72,000 \).
Ubiquitin pull down assay
Peptide ubiquitination was evaluated using HeLa S100 cytosolic lysate-based ubiquitination assays combined with an antibody pull down specific for ubiquitin as previously described. The assay was carried out at 37°C for 2 hrs at a total reaction volume of 100 µL. The assay consisted of buffer (10mM Tris-HCl pH7.6 and 5mM MgCl₂), 2mM DTT, 20 µg/mL ubiquitin aldehyde (Boston BioChem), 400 µg/mL methylated ubiquitin (MeUb) (Enzo Life Sciences), 1X ATP-ERS (Boston BioChem), 100 µM proteasome inhibitor MG-132 (EMD Chemicals), 2mg/mL HeLa S100 cytosolic lysates as the source of E1, E2, and E3 enzymes, 4.2 µg of peptide substrate, and the inhibitors Complete ULTRA and PhosSTOP (Roche). After incubation, samples were incubated with Control-Agarose beads (LifeSensors), diluted in TBS-T buffer (20mM Tris-HCl pH 8.0, 150mM NaCl, and 0.1% Tween-20), for 60 min on a tube rotator at 4°C. Samples were then centrifuged to pellet the Control beads at 1800g for 5 min. The supernatant was transferred to a new tube to which a solution of Agarose-TUBES1 (LifeSensor), diluted in TBS-T buffer, was added and then incubated overnight on a tube rotator at 4°C. The purpose of these beads was to bind ubiquitin and anything conjugated to ubiquitin following a standard pull down or immunoprecipitation assay. Ubiquitin-bound beads were washed 5X with TBS-T buffer and samples were cleaved from the bead with 50 µL 2X tricine sample buffer, heated for 5 min at >90°C, and isolated by centrifugation at 13000g for 5 min. Subsequently, samples were analyzed by gel electrophoresis (SDS-PAGE) using precast Bio-RAD Mini-PROTEAN® 16.5% Tris-Tricine Gels. A fluorescent protein ladder (Benchmark, Life Technologies) was used as a means of comparing the molecular weight of samples run in the gel to predetermined values. Gels were run in 1X Tris-Tricine Running Buffer (at 120V for ~ 90 minutes) and imaged at the Lineberger Cancer Center using a GE Typhoon Imager. Scans were run with a photo multiplier tube (PMT) of 600 V and at 800 V using the Green 526 SP filter set and a pixel size of 100 microns was used.

In vitro ubiquitination assay.
Peptide ubiquitination was further evaluated using an in vitro ubiquitination assay. The reaction consisted of 10 mM Tris-HCl (pH 7.6), 2 mM MgCl₂, 2 mM DTT, 300 µM ubiquitin, 1X ATP-ERS (Boston Biochem), 1 µM Ube1 (E1, Boston Biochem), 10 µM UbcH5b (E2, Boston Biochem), 1 µM HDM2 (E3, Boston Biochem), and varying concentrations of peptide substrate (1 µM, 5 µM, 10 µM, 20 µM, and 30 µM) in a total reaction volume of 20 µL at 30°C for 2 hrs. The reaction was halted by the addition of 40 µL Tricine Sample Buffer (BioRad), and samples were loaded onto SDS PAGE gels, electrophoresed and visualized as previously described using a GE Typhoon Imager.

Analysis of peptide ubiquitination by analytical HPLC
Subsequent analysis of peptide ubiquitination was performed by analytical HPLC for more precise quantification. For this analysis, samples from the in vitro ubiquitination assay were immediately stored at -20°C to terminate the assay. Analytical HPLC sample analysis utilized an Agilent 100 HPLC system, a Phenomenex Jupiter 300 C-18 Column, and a fluorescence detector. Gradients were run from polar, hydrophilic Solvent A (99.9% water 0.1 % TFA) to non-polar/hydrophilic solvent B (99.9% acetonitrile, 0.1%TFA) for 15 minutes. Assay samples were thawed to room temperature and then directly injected into the column for analysis. Additionally, samples were supplemented with a 10µM fluorescein internal standard to aide in the quantification process, also termed “spiking” the analyte. A trace was performed using a 15 minute gradient of 0-100% B to identify the peaks found in the chromatogram. Traces were examined in Origin to determine peak identity. Significant peaks including: unmodified, or parent, peptide, ubiquitinated peptide, and internal standard, were integrated to obtain their peak areas. Relative peak areas were calculated to determine the relative concentration.
of each component present in the sample by a calculated comparison of the peak area of the sample to the 10 \( \mu M \) FAM standard.

\[
W_{1/2\text{AVG}} = \frac{W_{1/2A} + W_{1/2B}}{2} = \frac{(0.134 + 0.131)}{2} = 0.133
\]  
\text{(Eq. 1)}

\[
R = \frac{0.589 \Delta t_R}{W_{1/2\text{AVG}}} = \frac{0.589(9.60 - 8.74)}{0.133} = 3.81
\]  
\text{(Eq. 2)}

\[
N = \frac{5.55 t_R^2}{w_{1/2}^2} = \frac{5.55(8.74^2)}{(0.134)^2} = 23611
\]  
\text{(Eq. 3)}

\[
H = \frac{L}{N} = \frac{250nm}{23611} = 0.11nm
\]  
\text{(Eq. 4)}

Analytical parameters included resolution (R), number of theoretical plates (N), and plate height (H) and were calculated as follows. Using the average width, \( W_{1/2\text{AVG}} \), of two peaks of interest at half height (Eq. 1), values for resolution, \( R \), were determined by multiplying the difference in resolution of the two peaks, \( \Delta t_R \), by a constant and dividing by \( W_{1/2\text{AVG}} \) (Eq. 2). The number of theoretical plates was determined by taking the square of the retention of one peak \( t_R \) and multiplying by a constant and dividing by its width at half height, \( W_{1/2} \) (Eq. 3). Finally, theoretical plate height was calculated by dividing the column length, \( L \), by the number of theoretical plates (Eq. 4).

**Results**

**Iterations of the p53 degron-based substrate are differentially ubiquitinated**

All peptide substrates synthesized and tested in this study (Table 1) are based on the p53 degron identified by Gu and colleagues found to be necessary for MDM2 mediated degradation.\(^\text{12}\) We previously developed a substrate based on this degron and found that it exhibited pronounced ubiquitination kinetics.\(^\text{2}\) Further, we found that it could be multi-monoubiquitinated when multiple lysine residues were incorporated into the N- and C-termini (e.g., p53 v2 and v3, Table 1).\(^\text{8}\) Mono-

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**Table 1. p53 substrate library.** A comprehensive list of all substrates synthesized for this study. Bold lysine residues correspond to the ubiquitination site lysine. The first sequence, p53 v2, represents the initial p53 sequence proven to be a strong candidate for degron reporter use.\(^\text{2}\)
ubiquitination refers to the conjugation of one ubiquitin molecule to one lysine residue on the substrate while multi-monoubiquitination refers to the conjugation of one ubiquitin molecule to multiple lysine residues within the substrate. Full length substrates (p53v2-v5, Table 1) all exhibited varying degrees of mono-ubiquitination (Figure 5, lanes 1-5), and those with multiple free lysine residues exhibited multi-monoubiquitination (Figure 5, lanes 1-2). Due to the multi-monoubiquitination of the full length substrate with multiple ubiquitination site lysines, shortened overlapping fragments based on the degron sequence were designed with a single ubiquitination site lysine to assess if they could be mono-ubiquitinated using a pull down assay incorporating HeLa cytosolic lysates. Three substrates (p53 v6-v8, Table 1) were designed for this study to isolate the minimal portable degron, the smallest functional region of the degradation sequence to be used for reporter targeting. Substrate p53 v6 was comprised of the amino acids immediately following the internal lysine residue and exhibited the strongest degree of ubiquitination of the shortened sequences (Figure 5, lane 5). Other abridged substrates, p53 v7 and v8, were also ubiquitinated to a lesser extent (Figure 5, lane 6-7). Due to the differential ubiquitination of these primary fragments in the ubiquitination pull down assays, it was decided to break these sequences into further fragments to continue the characterization of the minimal functional degron.

It was decided to move away from the previously discussed pull down assay towards an in vitro ubiquitination assay designed to incorporate only the E1, E2, and E3 enzymes to minimize off target effects from the array of proteins and enzymes found in cellular lysates. Instead of using HeLa S100 cytosolic lysates as a source enzymes, this new design incorporated purchased E1, E2, and E3 enzymes as the functional reaction components, eliminating the possibility of substrate degradation by intracellular proteases. By using purchased enzymes instead of cytosolic lysates, we were able to ensure that the only E3 ligase present in our assay was HDM2, providing specificity for the interaction of p53 and HDM2, which is of prime interest for our reporter. The entire library of substrates was subjected to this assay and exhibited varying degrees of ubiquitination (Figure 6). By comparing relative band intensities (Figure 6), it was concluded that p53v6 and p53v11 exhibited the highest degrees of ubiquitination. Although SDS-PAGE analysis of the assay samples demonstrated reproducible results, proving the successful ubiquitination of library substrates, the established analytical limitations of this method prevented it gauging substrate ubiquitination at the desired level of precision.
Characterization of HPLC separation conditions to quantify p53-based substrate ubiquitination

Based on the results obtained from the SDS-PAGE analysis of the library of degron-based substrates (Figure 6), it was decided to utilize a more precise analytical tool to provide more quantifiable data on the degree of peptide ubiquitination. We decided to employ reverse phase analytical HPLC because it is a well-established, reliable analytical technique that also offers a high degree of precision measurement and the ability to quantitatively compare the degree of substrate ubiquitination. To effectively utilize HPLC as an analytical metric, we had to first identify the separation conditions for unmodified (or parent) peptide from ubiquitinated peptide for an octadecyl carbon chain (C18)-bonded silica analytical column. Separation conditions were determined using ubiquitination assay samples from the p53 v11 peptide, a potent substrate based on our previous results. Based on previously established results from studies using HPLC to separate peptides and proteins, we employed a gradient of 99.9% water, 0.1%TFA to 99.9% ACN, 0.1%TFA over a 15 minute time course using fluorescence detection to distinguish vital components of the assay sample. Parent peptide and ubiquitinated peptide (Ub~peptide) both contain a fluorescein tag, therefore fluorescence detection serves as the most effective method of identifying peaks of interest.

Three peaks were present on chromatographic traces of assay sample corresponding to parent peptide (Figure 7A, 2), Ub~peptide (Figure 7A, 3), and an unidentified non-reactive species (Figure 7A, 1). Multiple control experiments were performed (Figure 7B) removing E1 enzyme, E2 enzyme, ubiquitin, or parent peptide, in order to identify the peaks corresponding to parent peptide and ubiquitinated peptide. We determined that peak 1, with a retention time of ~4 minutes, was due to auto-fluorescence of a non-reactive assay component which did not affect the migration and analysis of the ubiquitinated product. Peak 2, with a retention time of ~9 minutes, was identified as the parent peptide, while the smaller peak three, with a retention of ~10 minutes was determined to be ubiquitinated substrate. While we are confident of this identification, further analysis using mass spectrometry of peak eluent is planned to completely characterize the...
identity of each peak. However the results are pending due to backlog at the UNC Proteomics Facility. After successful characterization of peak identities in each assay sample, it was decided to incorporate an internal standard of 10 µM fluorescein. Comparison of assay sample traces spiked with internal standard to traces of pure fluorescein identified and confirmed that our chosen internal standard peak migrated with a retention time of ~12 minutes (Figure 8 A). We selected fluorescein as an internal standard because it has a different retention time than any peaks of interest from our assay sample, and it is important to correlate peak area to product concentration. The resolution between two adjacent peaks, indicative of the ability of HPLC conditions to separate a mixture, was calculated to be 3.81 (Eq. 2). Additionally, a theoretical plate number of the separation was found to be 23,611 (Eq. 3) with a theoretical plate height of 0.11 nm (Eq. 4). These parameters are evidence of the quality and efficiency of the experimentally derived separation conditions. The successful discovery of analytical HPLC separation conditions allowed us to move forward with the project and more quantitatively screen the p53 substrate library for degree of ubiquitination.

**Quantification of p53-based substrate ubiquitination using HPLC**

With functional and effective separation conditions for the analysis of the *in vitro* ubiquitination assay samples, we set out to characterize substrate ubiquitination using analytical HPLC. To properly characterize peptide ubiquitination we selected to calculate Michaelis-Menten kinetics by evaluating ubiquitin conjugation of the p53 based substrates of varying concentrations. When chromatography is performed with an internal standard of known concentration, peak areas can be used to calculate the concentration of each species of interest. Through integrating the desired signal peak, shown by the boundary bars in Figure 8B, one can obtain the selected peak’s area. Subsequently, the peak area of the known internal standard can be used to calculate a relative concentration for the peak of interest. Preliminary data extrapolated from HPLC analysis of substrates p53 v6 and p53 v7 subjected to *in vitro* assay conditions are listed in Table 2. Data was obtained by quantifying peak area relative to known internal standard in order to determine the concentration of parent peptide and ubiquitinated peptide. In getting numerical values for the concentration of ubiquitinated peptide and parent peptide, we will be able to more accurately see the degree of ubiquitination of various substrate library members. Additionally, this will

![Figure 8. HPLC quantification of substrate ubiquitination](image-url)
allow for the precise characterization of reporter ubiquitination due to varying the initial concentration of parent peptide. Data representative of the concentrations of parent peptide and Ub–peptide will subsequently allow us to calculate the Michaelis-Menten constants, $K_m$ and $k_{cat}$, to precisely evaluate substrate performance. Preliminary experiments using p53 v6 and p53 v7 demonstrate that the concentration of Ub–peptide appears to increase in direct correlation to the initial concentration of parent peptide (Table 2). Unfortunately, it appears that the concentrations of parent peptide vary to some degree and do not correspond to the amount of parent peptide substrate originally used in the assay (Table 2). We are currently investigating this phenomenon to explore this inconsistency and determine the cause and its analytical implications. Nevertheless, the preliminary data obtain here provides the basis for a successful analysis of substrate ubiquitination to identify the minimal peptide sequence capable of MDM2-mediated ubiquitination. Continued collection and analysis of peptide ubiquitination is currently underway to analyze the entire p53 substrate library.

**Discussion**

In this project we attempted to identify and characterize the minimal amino acid sequence based on the naturally occurring degron found to be necessary for MDM2-mediated ubiquitination of p53. Based on the previous success of work with a p53 degron-based substrate, we employed a shotgun sequencing approach to synthesize a library of substrates of varying lengths and ubiquitination site lysine locations to find an ideal, yet minimized, recognition sequence for MDM2. Substrates were subjected to a HeLa S100 cytosolic lysate-based pull down assay and an enzyme only *in vitro* ubiquitination conditions, both analyzed by SDS-PAGE, to evaluate peptide ubiquitination. Subsequent analysis of the *in vitro* ubiquitination assay was performed by analytical HPLC to perform a more precise quantification about the degree of peptide ubiquitination. Initial examination of the p53 peptide-based substrates via the pull down assay showed successful mono-ubiquitination and multi-monoubiquitination of full length substrate sequences (Figure 5), validating the functionality of the p53 degron sequence proposed by Gu et al. for use in a degron-based substrate to assess MDM2 activity. Next, the p53-based sequence, relatively large to be successfully utilized as a peptide-based reporter, was further broken down to identify if smaller iterations could retain the same degron potential. Evaluation of smaller fragments (p53 v6-8) by the ubiquitin pull down assay showed that these substrates exhibited varying degrees of ubiquitination (Figure 5), proving their functionality for use as a smaller functional degron. Substrate p53 v6, which was shown to be more strongly ubiquitinated than the other two fragments, was hypothesized to contain the smallest functional degron. With this successful minimization of the p53 degron, efforts were made to further minimize this sequence to isolate the smallest portable degron.

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**Table 2. Preliminary data from HPLC analysis of *in vitro* ubiquitination assay samples.** Concentrations of parent peptide and ubiquitinated peptide product in samples of substrates subjected to *in vitro* assay conditions. Values were determined by peak area comparison to known internal standard. Concentrations of parent peptide not subjected to assay conditions are also included. Preliminary analysis explored the substrates p53v6 and p53v7 and their degree of ubiquitination.
The ubiquitin pull down assay, using cell lysates as a source of E1, E2, E3 enzymes, provides functionality while also providing specificity to ubiquitinated product via the antibody pull down to ensure that what is seen on gel analysis is ubiquitinated peptide only. Alternatively, this type of assay poses certain limitations, such as the addition of unwanted cell lysate components like proteases and peptidases which can rapidly degrade the peptide-based substrate, and an unspecified number of functioning E3 ligases. Therefore, for continued analysis of the shotgun sequenced substrates (p53 v6-13, Table 1) an in vitro enzyme only assay was employed. In replacing the cell lysate source of UPS enzymes with purchased, specific E1, E2, and E3 enzymes, these assay conditions provided a more controlled environment, ensuring that observed E3 ligase activity was from HDM2 and was specific to its interaction with p53, while also removing the risk of substrate degradation via cytosolic peptidases. Substrates were analyzed via these new assay conditions and shown to be ubiquitinated to varying degrees (Figure 6). Further observation suggested that p53 v6 and p53 v11 exhibited the highest degrees of ubiquitination, suggesting that the smallest portable p53 degron of interest lies within this region. Additionally, the lack of observable ubiquitination of the negative control substrate p53 v13 (Table 1, Figure 6), which lacks any free lysine residues for ubiquitination validates that the lysine residue is essential for substrate ubiquitination. However, one limitation of using SDS-PAGE to analyze substrate ubiquitination is high signal-to-noise- ratio inherent in quantifying images of gels. In fact, analysis of the negative control, p53 v13, failed to capture the observable results due to the high degree of background noise from using the Typhoon imager (data not shown). Overall these results from the gel electrophoretic analysis of assay samples point to p53 v6 and p53 v11 as being the most potent portable degrons of p53. However, due to the limitations of quantifying gels using ImageJ, a more precise and quantifiable method of assay samples analysis was explored.

To perform a more precise analysis of the library of substrates, analytical HPLC analysis coupled with fluorescence detection was chosen. This method not only provided a more numerical, quantifiable data analysis than SDS-PAGE, but was also a well-established, reproducible, stable analytical tool that can be applied to the detection of fluorescently tagged substrates. In order to proceed toward more quantitative analysis via analytical HPLC, separation conditions had to be experimentally determined for the separation of ubiquitinated peptide from parent peptide in the assay samples. Drawing from previous knowledge of peptide separation via reverse phase HPLC, it was decided to use a gradient from water to acetonitrile across a 15 minute interval to determine if the two desired analytes could be effectively separated. Preliminary traces using fluorescence detection showed three peaks of unknown identity, suggesting that some assay component in addition to parent peptide and Ub~p peptide all eluted off the column. In order to classify these unknown peaks, control experiments (Figure 7B) were run removing individual assay components to ascertain the identities of each peak from the trace. In comparing a complete assay sample (Figure 7A), with various controls that eliminated peptide ubiquitination, such as lack of E1, E2, and ubiquitin, established that peak 3 (Figure 7A) was present in all samples where the peptide was expected to be ubiquitinated, but absent in control experiments (Figure 7B). Therefore, we hypothesized that peak 3 was the ubiquitinated peptide product from in vitro assays. Additionally, a control experiment (data not shown) was performed where only parent peptide was run on the column to further verify that peak 2 (Figure 7A) corresponded to the peak observed in parent only control runs, further reinforcing the peak selection and identification. Having characterized peaks 2 & 3, we were still curious about the unknown peak labeled 1 (Figure 7A). This peak is seen to be present in all sample traces, but not in control traces of parent peptide only, E1 only, E2 only, and E3 only (data not shown). Therefore, we concluded that peak 1 must be due to auto-fluorescence of a non-reactive component of the in vitro ubiquitination assay. With the retention time
of peak 1 being so distinct from the retention times of the desired analyte peaks, there is no concern about this non-reactive species being present in the HPLC trace and has since been deemed unnecessary for further analysis.

Further, to confirm the characterization of eluent peaks in the HPLC analysis of the assay sample, fractions were collected and sent off to be analyzed via MALDI Mass Spectrometry. High traffic and use of the core facility at the UNC Proteomics facility has caused delay in obtaining these results, and the spectra for eluent fractions are still pending. Further, this type of analysis cannot be performed using the ESI in the Department of Chemistry due to the relatively high molecular weight of the Ub~peptide species (~8700 Da). In addition to successful peak identification, the calculation of analytical parameters such as resolution, number of plates, and plate height (Figure 7C) verify the quality of separation that is obtainable from these experimentally derived separation conditions. A high resolution, 3.81 (Figure 7C), demonstrates the degree and cleanliness of separation between the two desired peaks (2&3) on a chromatogram, thus verifying the ability of the overall procedure to separate and resolve the two solute compounds. A theoretical plate number of 23,611 (Figure 7C) further reinforces the quality of the experimentally determined separations conditions because in liquid chromatography, the separation of two compounds is enhanced by the increase in the number of theoretical exchange plates where solute and stationary phase interact increases. Finally, a small theoretical plate height, 0.11 nm, for the separation (Figure 7C) lends further credit to its quality because a small plate height is desirable for a chromatographic separation. The successful development of a procedure to separate parent and ubiquitinated peptide on an analytical column allowed us to move forward with more quantitative analysis of our p53 substrate library.

Preliminary application of this experimentally determined HPLC procedure on samples using substrates p53 v6 and p53 v7 demonstrated an overall trend of increasing concentration of ubiquitinated peptide coupled with an increase in the initial concentration of parent peptide (Figure 9). This suggested that these two degron-based substrates adhere to some type of characterizable kinetic behavior. Unfortunately, it was observed that the calculated concentration of parent peaks was significantly lower than the concentration of peptide used in assay conditions (Figure 9). This caused speculation of whether sample was being lost on the column during HPLC separation. The supposed loss of peptide seemed random and inconsistent across experiments using the same peptide substrate (Figure 9, p53 v6) and brought into question the functionality of HPLC as a tool for our desired analysis. If the amounts of peptide are lost inconsistently across different analytical runs for identical samples, it will be impossible to compare relative concentration across different traces and thus we would not be able to compare the degree of ubiquitination between substrates with this analytical approach. Due to this concern, experiments are currently underway using the p53 v6 substrate to determine if the ratio of parent peptide and ubiquitinated peptide seen in the eluent are consistent or variable. If loss is consistent due to a systematic error, then it can be compensated for and the analytical technique can be utilized with minor corrections.

Another theory for the variation in calculated concentration of parent peptide and Ub~peptide originates from the calculation procedure using the internal standard peak area to quantify the other peak concentrations. Fluorescein is known to change its quantum fluorescent state sensitively to its surrounding environment; therefore it is likely that the pure FAM internal standard fluoresces stronger than FAM bound to ubiquitin. If this is the case, the direct one-to-one calculation using the peak area of the internal standard to calculate the concentration of parent peptide and Ub~peptide species is
incompatible because the FAM-tag for each species is emitting varying degrees of fluorescence. Accordingly, further control experiments are planned to assess the difference in FAM fluorescent signal between the pure FAM internal standard and FAM bound to peptide. If the internal standard FAM is found to fluoresce more prominently than the FAM bound to peptide then the difference in signaling will propagate through the calculation of concentration, making the value lower than it should be. Therefore, we intend to proceed by no longer using the internal standard to calculate the concentration of product and parent peptide, but rather compare ratios of product to parent peptide across different analytical traces to compare their respective degree of ubiquitination. This eliminates the analytical problem due to possible inconsistencies from the FAM internal standard. Further speaking to the environment of the fluorescein tag and internal standard, we are aware that FAM fluorescence is highly pH sensitive, which is why we utilize pH controlled buffers in assay conditions, maintain a consistent amount of TFA in all HPLC solvents, and plan to run control experiments on fluorescence reproducibility. Once the corrected analytical procedure is determined from these further control studies, the entire substrate library will be subjected to in vitro enzymatic assay conditions combined with analytical HPLC analysis (Figure 8) to determine the ratio of ubiquitinated peptide to parent peptide. It is important to note that all of the data collected thus far can still be used for the subsequent analysis, so it is not necessary to repeat any of the above experiments.

Using the ratios of parent to Ub−peptide for each substrate at varying initial peptide concentrations, we will be able to numerically compare the degree of ubiquitination of each substrate and use these values to assess the Michaelis-Menten (MM) kinetics for each of our substrates in the library, specifically the MM rate constants. Michaelis-Menten kinetics is the most common biochemical technique to model enzyme-catalyzed alterations of a target protein that relates the reaction rate to the initial concentration of substrate. Through modeling the reaction rate over varying initial concentrations of substrate, this biochemical model allows for the determination of the maximum rate, \( v_{\text{max}} \), of the enzymatic reaction as well as the Michaelis constant, \( K_m \), which is the value of substrate concentration when the reaction rate is at \( \frac{1}{2} v_{\text{max}} \). By modeling the data obtained from HPLC analysis of assay samples with varying starting concentrations of parent peptide, we can determine the reaction rate, or degree of ubiquitination. By obtaining the \( K_m \) and \( v_{\text{max}} \) for each member of the substrate library, we can compare the ubiquitination kinetics to determine which substrate is the most strongly ubiquitinated and contains the ideal portable degron sequence. This kinetic analysis allows us to further supplement the previous conclusion about the identity of the smallest portable degron of p53 based on the results from the SDS-PAGE analysis for the identify of a potential MDM2 reporter.

Once we have successfully obtained conclusions about the identity of the smallest portable degron, we have the opportunity for future work involving the quantitative exploration of inhibitors to MDM2. We intend to utilize the peptide-based reporter with the smallest functional degron sequence to assess the performance of certain MDM2 inhibitors to evaluate how these inhibitors limit the activity of MDM2 on reporter ubiquitination by competitive inhibition for the p53 binding pocket in MDM2. Such proposed work would provide a way of testing and comparing various MDM2 inhibitors to determine which are most effective. Such novel biomolecular and kinetic data would be of great value to investigators exploring the inhibition of the MDM2-p53 interaction as therapeutic drug target. Although a sparse number of reports relating to the UPS already exist\(^{10}\), most of these analytical tools are not compatible with single cell analysis and require lager populations of cells and complex genetic engineering.\(^2\) Recently, the analysis of single cells, especially primary cells obtained directly from patient biopsy, has provided a vast amount of information that other types of analysis, therefore there is
a need for single cell reporters, to which degron based substrates provide the perfect complementation. Combining the proposed degron-based substrates into full single-cell reporters, incorporating β-hairpins as protecting motifs to stabilize the peptide-based reporter, opens up a spectrum of UPS system to target in active cancer and tumor tissues.

In conclusion, this study has demonstrated the effective development of a p53 degron-based substrate and performed preliminary analysis into the identification of a minimal portable degron that can be effectively incorporated into an analytical probe for MDM2 activity. Additionally, we have detailed the development of various analytical techniques including biochemical assays coupled with SDS-PAGE or analytical HPLC to assess the degree of ubiquitination of the proposed degron-based substrates. By uncovering more information about p53, including new techniques of analyzing its interaction with its E3 ligase MDM2 and the precise sequence of the smallest effective degron, we are providing essential information of this essential enzymatic interaction. Future work based on this study includes MDM2 inhibitor performance analysis and the foundation for a new reporter of MDM2 activity in single cells, both of which will expand the database of information about p53-MDM2 related therapeutic research as a whole.

References
