Summer Scholar Report

The Targeted Charging of Ubiquitin to E2: Developing a New Tool to Study the Ubiquitin Proteasome System

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Abstract:

Here we present initial in vitro work on a new tool to study the ubiquitin-proteasome system (UPS), named "The Targeted Charging of Ubiquitin to E2" or "tCUbE." This approach will allow us to uncover the complex enzymatic cascades of the UPS by connecting ubiquitinated target proteins to the activity of a specific ubiquitin-conjugating enzyme, or E2, of interest. While developing this assay in mammalian cells, we worked to purify each engineered protein construct for use in complementary in vitro studies, as well as mutated constructs for control experiments. These in vitro assays allow us to confirm that each component of tCUbE is functioning as designed and can interact with native UPS enzymes.

Introduction:

The ubiquitin-proteasome system (UPS), most well-known for its role in protein degradation, is also a dynamic signaling pathway that regulates a variety of cellular processes, including cell cycle control and the immune response.[1,2] The UPS mediates these essential functions by modifying target proteins with ubiquitin, a small 76 residue protein, through a three-enzyme cascade (E1-E2-E3).[1] Proper function of the UPS requires the coordination of hundreds of enzymes and thousands of specific protein-protein interactions. Due to its importance and complexity, malfunction of this system has been linked to multiple cancers, heart disease, and neurodegenerative disorders.[2] A variety of anti-cancer therapeutics that target the UPS have been approved or are in development, but these are limited to a few well characterized E3-target interactions.[3,4] A better understanding of the protein network in the UPS would give insight into the mechanisms and limitations of current cancer therapies, as well as uncover opportunities for novel, specific drug targets.

A UPS cascade begins with an E1 enzyme activating ubiquitin in an ATP dependent process.[1,2] Then, a ubiquitin-conjugating E2 enzyme catalyzes a transthioesterification reaction to carry ubiquitin to an E3 ligase, which recognizes a variety of both E2 and target proteins, resulting in the transfer of ubiquitin to a final substrate.[1,2] A protein can be mono-ubiquitinated, or poly-ubiquitinated through an iterative process, leading to ubiquitin chains which can vary in both length and topology.[1,2] In humans, there are 2 main E1s, about 40 E2s, and well over 600 known E3s, making it difficult to link final ubiquitinated products to specific E1-E2-E3 pathways.[2] At the center of each ubiquitination cascade, E2 enzymes are known to play an essential role in determining

substrate specificity, multiplicity, and chain topology. Further study of this key point is needed to deconvolute the complexity of the UPS.[5,6] Here, we propose a new tool that will allow us to study the UPS from the center, by targeting a tagged ubiquitin to an E2 of interest in vivo and monitoring the products that arise from the activity of this E2.

Our tool, called "The Targeted Charging of Ubiquitin to E2" (tCUbE), will allow us to delineate specific E2-E3-substrate interactions that lead to distinct ubiquitinated products in living cells. This approach differs from traditional methods because it focuses on the upstream components of the UPS and introduces a new point of control. As seen in Figure 1A, using the addition of a small molecule, specific E2-ubiquitin interactions can be induced *in vivo.*

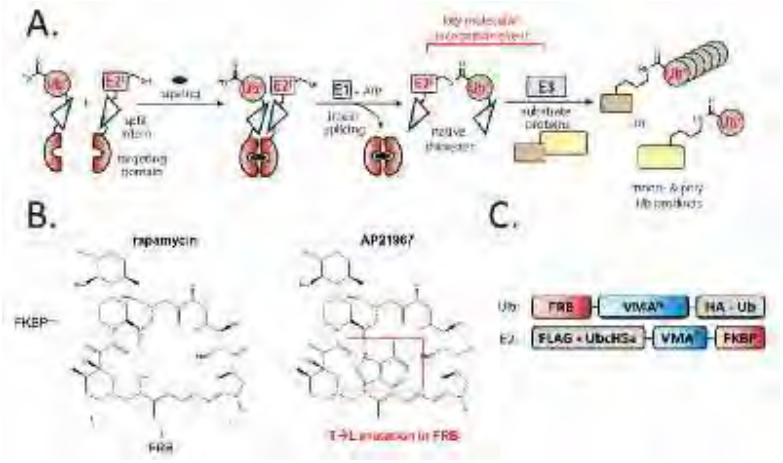

Figure 1: *The Targeted Charging of Ubiquitin to E2. (A) Overview of tCUbE and designed constructs interacting with the native UPS enzymes. (B) Structures and binding properties of small molecule dimerizers rapamycin and AP21967 (Rapalog). (C) Design of the protein constructs]*

To accomplish this, we use the conditional protein splicing method developed by Mootz et al . that combines complementary binding domains with self-splicing "split inteins."[7,8] Upon addition of the small molecule rapamycin, binding domains fused to the E2 of interest and tagged ubiquitin construct associate. This subsequently causes conditional protein splicing to remove the bulky binding domains, allowing the charged E2 to continue through the native UPS cascade to interact with endogenous E3s and transfer the tagged ubiquitin to substrate proteins. [7,8] These products can be monitored, identified, and connected to specific E2-E3 interactions.

tCUbE will overcome many drawbacks of current methods to study the UPS, which are limited by its complexity, redundancy, and the transient nature of these protein-protein

interactions in vivo . One main advantage is the ability of tCUbE to operate within cells, enabling us to monitor the UPS in a native environment. Another advantage is the the power to “turn on” tCUbE, through the inducible targeting step. Finally, tCUbE focuses on the upstream components of UPS cascades, allowing us to visualize more complete and previously unstudied pathways. Long term, tCUbE can be used to profile the activity of all known human E2’s.

Currently, we are developing tCUbE in mammalian cells. However, because of the complexity of the tCUbE system and the UPS overall, we also created a version of this system that can be used for complementary studies in vitro. This will allow us to to better analyze how tCUbE functions in the context of a cell, and confirm that each component of tCUbE is working as designed. Here we present the design, expression, and purification process of our novel ubiquitin and E2 constructs, as well as in vitro studies that support the initial steps of tCUbE.

Results:

Construct Design & Cloning:

When designing our parent constructs (Figure 1C), we chose UbcH5a as our initial E2 to study. This enzyme has been previously studied and is known to promote ubiquitination of p53, a clinically relevant target.[9] The split intein halves for conditional protein splicing are derived from the vacuolar ATPase subunit of S. Cerevisiae (VMA), and have little native affinity for each other. Their association is then reliant on the Rapamycin-dependent dimerization of complementary FRB and FKBP binding domains, fused to each half of the intein. The binding domains are positioned as exteins, so that they are removed and linked together by a peptide bond after the inteins become active.[8-9]

The ubiquitin construct contains the FRB binding domain at its N-terminus, one half of the split VMA intein, an HA epitope tag for detection, then ubiquitin, leaving the C-terminus free for conjugation. The E2 construct contains UbcH5a with a FLAG epitope tag at its N-terminus, followed by the other half of the VMA intein, and then the FKBP binding domain. To avoid any off target effects of rapamycin interacting with the human m-TOR protein (target of rapamycin), we decided to use a synthetic rapamycin derivative, rapalog, as our dimerizing agent (Figure 1B). This required a complementary mutation in the FRB domain at threonine 2098 to leucine for binding compatibility.[8] We termed this construct rbUb (rapalog binding ubiquitin). The original ubiquitin construct could then be used as a targeting control, as the wtFRB domain cannot accommodate rapalog. We also designed intein inactive control constructs by mutating the key intein residues that catalyze the splicing reaction to alanine (Cys in VMAN and Asn in VMAC). Finally, we made a catalytically inactive E2 variant, replacing the cysteine used for thioester linkage to ubiquitin with alanine.

We cloned the two parent constructs into the vector pDEST17™for bacterial protein expression using Gateway™ cloning. This vector contains a T7 promoter for IPTG induction and an N-terminal $(His)_6$ tag for affinity purification by Nickel column. Each mutant construct was made using PCRbased mutagenesis with the parent constructs as templates.

Protein Expression:
To start, we performed small scale pilot expressions in BL21(DE3) E. Coli cells. The initial attempts showed expression of each parent construct at the correct molecular weight, but almost exclusively in the total lysate rather than soluble fraction, as seen in Figure 2A. To optimize for expression of soluble proteins, first we screened a variety of cell lines for higher levels of each protein in the soluble fraction. Cells with increased expression of tRNAs used for difficult codons (BL21(DE3) RIL), with a different induction system (BL21 A.I.), or even enhanced expression systems (C41(DE3)), all resulted in high levels of insoluble protein for both constructs.

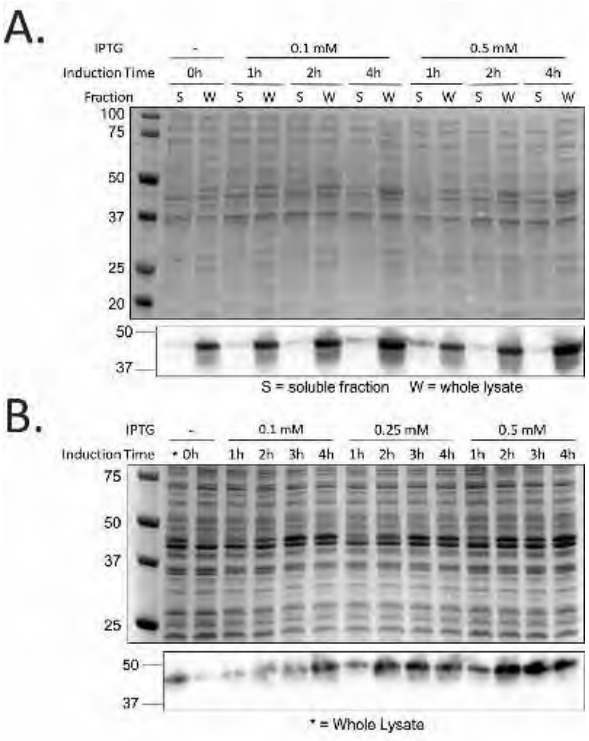

Figure 2: *Protein Expression (A) Expression of insoluble rbUb in BL21(DE3) cells. Expected MW: 47.5 kDa. Top: Coomassie stained gel, Bottom: Anti-HA WB. (B) Expression of rbUb in the soluble fractions of BL21(DE3) PLysS cells, Top: Coomassie stained gel, Bottom: Anti-HA WB.]*

We noticed that these experiments showed very high levels of basal protein expression, which could result in subsequently overexpressed protein after induction remaining

insoluble as well. This led us to try BL21(DE3) pLysS cells, which co-express T7 lysozyme to inhibit leaky expression of the T7 RNA polymerase. This resulted in much higher levels of soluble protein (Figure 2B). Using this cell line, the induction was optimized for the highest level of soluble protein. For both constructs, inducing at 16-18 °C improved solubility when compared to induction at room temperature (25°C) or 37 °C. We varied IPTG concentrations, finding 0.5 mM IPTG showing the greatest expression for both constructs. Finally, we tried induction times ranging from 1 hour to overnight. We found that overexpression peaked between 2 and 4 hours, then declined with longer expression times.

Protein Purification:
Using the N-terminal $(His)_6$ tag added by pDEST17™, we aimed to purify each protein construct using immobilized metal affinity chromatography (IMAC).

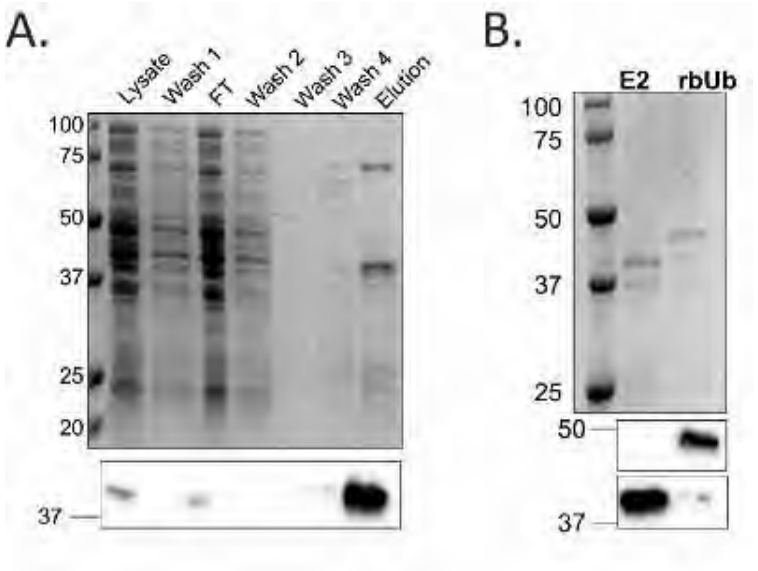

Figure 3: Protein Purification (A) Analysis of early protein purification of E2 construct by gravity flow IMAC. Expected MW: 40 kDa. Top: coomassie stained gel, Bottom: Anti-FLAG WB. (B) Purified protein constructs during optimization process. Top: coomassie stained gel, Middle: Anti-HA WB, Bottom: Anti-FLAG WB]

We attempted purification using two nickel affinity column methods: a gravity flow column and a column for fast protein liquid chromatography (FPLC). Initial attempts showed the gravity flow nickel column to have purer protein and higher yields. Continuing with this method, we optimized the lysis, wash, and elution conditions of the purification to remove as many contaminants as possible without sacrificing protein yield

(Figure 3). High levels of protein have been continuously seen in the flow through from each affinity column, leading us to believe that the His tag is not properly exposed in the native protein structures of our protein constructs. While we obtained pure enough samples to begin in vitro work and have identified nonspecific bands, we are continually working to optimize the purification for purer product.

Activation of rbUb by E1 In Vitro:

The first step of a UPS cascade is the ATP-dependent activation of ubiquitin by an E1 enzyme, forming a reducible thioester linkage between an active site cysteine and the Cterminus of ubiquitin. Before testing the targeted charging steps of tCUbE, we wanted to ensure that the fused intein and binding domain would not interfere with the recognition or activation of rbUb. UbE1 is the ubiquitin-activating enzyme for most UPS cascades in humans, so we purchased active, His-tagged, recombinant UbE1 to test with rbUb.

The active E1~rbUb complex can be observed as the appearance of a high molecular weight band only when ATP is included in the reaction and the gel is run under non-reducing conditions (Figure 4). While the band appears higher than the molecular weight that we expected, both the dependence on ATP and non-reducing conditions combined with the observed decrease in unreacted E1 and rbUb signal support our identification of E1~rbUb. We predict that a different gel will better resolve this high molecular weight protein complex.

Wild type ubiquitin has been shown to be fully activated in 30 minutes in similar experiments, so we included a longer time point to see if the added domains slowed this reaction.[10] Interestingly, we observed the most active E1~rbUb signal after 30 minutes, decreasing slightly in two hours. Further experiments will be done to test the competition between wild type ubiquitin and rbUb in E1 activation, as both will be present in the cellular environment, and background ubiquitin transfer can potentially interfere with tCUbE.

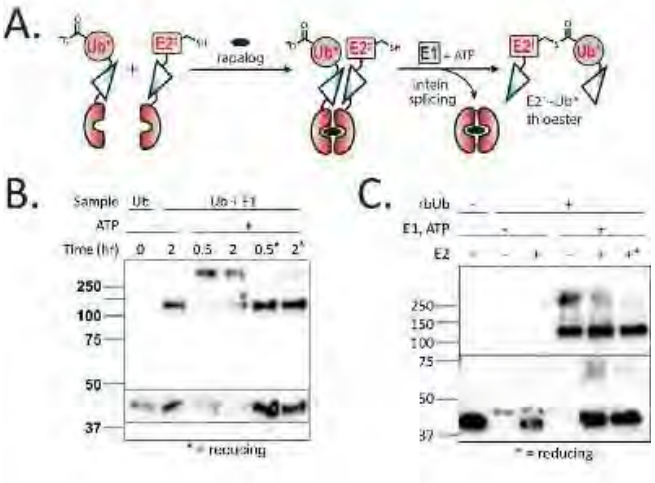

Figure 4: *Initial In Vitro work (A) Overview of initial steps of the UPS and tCUbE. (B) rbUb activation by E1. Anti-His tag WB, with higher exposure inlay. High molecular weight bands, in addition to a decrease in E1 (121 kDa) and rbUb (47.5 kDa) are observed with ATP under non-reducing conditions. (C) rbUb transfer to E2. Anti-His tag WB, with higher exposure inlay. Spliced E2-rbUb appears under non-reducing conditions at 60 kDa.]*

Transfer of rbUb to E2 in vitro :
After activating ubiquitin, E1 enzymes recognize multiple E2 enzymes and transfer ubiquitin to another active site cysteine through a transthioesterification reaction. To assess this step, we included our E2 construct with the activated E1~rbUb complex. This also allows for the analysis of the splicing functionality induced by proximity, rather than Rapalog treatment. We observed a decrease in the high molecular weight signal from the E1 activation, as well as the appearance of a band at the molecular weight of the post-splicing rbUb~E2 complex under only non-reducing conditions. While the FRB-FKBP extein fragment was not observed, additional experiments are underway to confirm the identity of the rbUb~E2 complex and test the splicing functionality by Rapalog treatment.

Conclusion:
Here we present the design, expression, and purification, and initial in vitro testing of protein constructs used tCUbE, a new tool to study the UPS. We have screened expression conditions to optimize expression, and used IMAC to purify our ubiquitin and E2 constructs. Initial results have showed that our rbUb can be activated by recombinant E1, and can be transferred to our E2 construct. Moving forward, additional *in vitro* experiments will be run to demonstrate conditional protein splicing by Rapalog, transfer to E3 and target proteins, and finally competition between rbUb and wtUb. We will also express and purify the mutated constructs for additional control experiments.

By confirming and characterizing the functionality of each step of tCUbE in vitro , we will be better able to interpret and support our results in vivo . The development of tCUbE will provide us with a powerful new tool to study ubiquitination cascades in living cells. By connecting ubiquinated products with the activity of specific E2 enzymes, we will better understand the regulation of vital cellular processes, as well as uncover new opportunities for drug development targeting many cancers and chronic diseases.

Methods:
Cloning:
All plasmids were prepared using Gateway™ cloning, and listed protocols. Entry clones were generated in the donor pDONR221 from either attB flanked PCR fragments or expression clones. attB recombination sites were added by PCR using specially designed primers and Phusion© DNA polymerase. Expression clones were generated in the destination vector pDEST17.

PCR Site Directed Mutagenesis:
All mutations were created in expression clones using the QuikChange II Site-Directed Mutagenesis Kit™according to provided protocols, and primers designed according to given guidelines.

Protein Expression:
50 ng of plasmid was transformed into each cell line according to the given protocol, and plated on agar plates with Ampicillin for all cell lines except for Bl21(DE3)PLysS, which required Ampicillin & Chloroamphenicol. 5 mL cultures for pilot expressions and 1L cultures for full-scale expressions were grown in LB with the appropriate antibiotics. Cultures were grown until an OD of 0.5 – 0.6, then induced with IPTG and moved to the desired induction temperature. All cultures were spun down and frozen at -20 °C until lysis. Cells pellets were thawed on ice, resuspended in 50 mM phosphate buffer with 150 mM NaCl, 1 mM DTT, ó Pierce™ Protease Inhibitor Tablet, and Benzonase™ Nuclease. Samples were lysed by 10 minutes sonication (15s on, 15s off).

Gravity Flow Affinity Purification:
Bacterial pellets were lysed as described above and cleared by centrifugation. 20 mM imidazole was added directly to the supernatant. 10 mL of binding buffer (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.5) was used to equilibrate the column (GE Healthcare His GraviTrap ™) then the lysate was applied to the column. 10 x 10 mL wash buffer (binding buffer with 65 mM imidazole) was used to wash the column, then 2.5 mL elution buffer (binding buffer with 500 mM imidazole) was used to elute bound protein. A GE Healthcare Nap25™column was equilibrated with 5 x 5 mL 50 mM Tris-HCl, 300 mM NaCl, pH 7.5. The eluate from the His GraviTrap column was applied to the Nap25 column. 3.5 mL equilibration buffer was used to elute the purified protein, which was quantified by Bradford Assay, 5% glycerol was added, then samples were aliquoted and flash frozen.

In Vitro Assays:
Protein samples were thawed on ice. E1 activation experiments were run in 50 mM Tris-HCl with 300 mM NaCl, using 0.25 μ M E1, 2-4 μ M rbUb, 0.5 mM DTT, 5 mM ATP, 10 mM $MgCl_2$ and incubated at 37 °C for 30 – 120 minutes. E2 transfer experiments were run with the above conditions plus 2 μ M E2. Reactions were quenched with SDS-PAGE loading dye, with or without DTT.

SDS-PAGE & Western Blotting:
Samples were boiled at 95°C for 5 minutes, then centrifuged for 2 minutes. Samples were loaded onto BioRad Mini-Protean TGX™ any kD gels, and run for 35 minutes at 200 V. For Coomassie staining, gels were exposed to Coomassie blue for 1 hour to overnight,

then destained until minimal background was left. These were imaged on a ChemiDoc XRS+ system. For western blotting, gels were transferred onto PVDF membranes at 4 °C for 1 hour at 100 V. Membranes were blocked in 5% milk in TBST, then all primary antibodies were added to a final concentration of 1:1000 and incubated at 4 °C overnight. Membranes were washed 3 x 5 min with TBST. Secondary antibody, either anti-Mouse IgG or anti-Rabbit IgG was added to a final concentration of 1:2000 and incubated at room temperature for 1-2 hours. Membranes were washed 3 x 5 min with TBST, then exposed to BioRad Clarity Max Western ECL Blotting Substrates™, then imaged on a Chemi-Doc XRS+ system.

References:

1. The Ubiquitin Code. *Annu. Rev. Biochem*. **81**, 203–229 (2012).
2. Bedford, L., Lowe, J., Dick, L.R., Mayer, R.J. & Brownell, J.E. Ubiquitin-like protein conjugation and the ubiquitin–proteasome system as drug targets. *Nat. Rev. Drug Discov*. **10**, 29–46 (2011).
3. Weathington, N.M. & Mallampalli, R.K. Emerging therapies targeting the ubiquitin proteasome system in cancer. *J. Clin. Invest*. **124**, 6–12 (2014).
4. Stewart, A.K. How Thalidomide Works Against Cancer. *Science* **343**, 256–257 (2014).
5. David, Y., Ziv, T., Admon, A. & Navon, A. The E2 Ubiquitin-conjugating Enzymes Direct Polyubiquitination to Preferred Lysines. *J. Biol. Chem.* **285**, 8595–8604 (2010).
6. Wenzel, D.M., Stoll, K.E. & Klevit, R.E. E2s: Structurally Economical and Functionally Replete. *Biochem. J.* **433**, 31–42 (2010).
7. Mootz, H.D. & Muir, T.W. Protein Splicing Triggered by a Small Molecule. *J. Am. Chem. Soc.* **124**, 9044–9045 (2002).
8. Mootz, H.D., Blum, E.S., Tyszkiewicz, A.B. & Muir, T.W. Conditional Protein Splicing: A New Tool to Control Protein Structure and Function in Vitro and in Vivo. *J. Am. Chem. Soc.* **125**, 10561–10569 (2003).
9. Honda, R., Tanaka, H. & Yasuda, H. Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS Lett.* **420**, 25–27 (1997).
10. Mulder, M. P. C. et al. A cascading activity-based probe sequentially targets E1-E2-E3 ubiquitin enzymes. *Nat. Chem. Biol.* **12**, 523– 530 (2016).