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Directed evolution of SUMO and Ubiquitin proteases to generate high affinity substrate traps

A thesis submitted in partial satisfaction of
the requirements for the degree Master of Science

in

Biology

By

Benjamin Wang

Committee in charge:

Professor Hulin Zhou, Chair
Professor Eric Allen, Co-Chair
Professor Randolph Hampton

2020

The thesis of Benjamin Wang is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California San Diego
2020

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ABSTRACT OF THE THESIS

Directed evolution of SUMO and Ubiquitin proteases to generate high affinity substrate traps

By

Benjamin Wang

Master of Science in Biology

University of California San Diego, 2020

Professor Huilin Zhou, Chair

Professor Eric Allen, Co-Chair

Post translational modifications such as ubiquitin and the Small Ubiquitin-Like Modifier (SUMO) regulate numerous cellular processes and are evolutionarily conserved in all eukaryotes. Studies on these post translational modifications often require the enrichment of their endogenous conjugates prior to analysis because the modified proteins exist transiently and are low in abundance. Current approaches for isolating these modified proteins contain several unaddressed issues that question the reliability of these approaches. Remarkably, it has been recently demonstrated that the catalytically inactive form of the SUMO protease Ulp1 can be used as a purification tool to purify SUMOylated proteins from yeast cell extracts. In the first

chapter of this thesis, we used yeast surface display-based directed evolution to engineer the binding affinity of Ulp1 to further improve the purification strategy. In the second part of this thesis, we identified CthUbp15 as the ubiquitin protease most suited for directed evolution in order to create a similar purification tool for ubiquitin studies.

Chapter 1:

Introduction to the Ubiquitin and SUMO pathways

1.1 General Overview of the SUMO and Ubiquitin pathways

The ubiquitin system is a well characterized post translational modification (PTM) that is evolutionarily conserved and regulates almost all cellular process in eukaryotes. Originally discovered as a trigger for selective protein degradation in reticulocytes (Hershko, Ciechanover, Heller, Haas, & Rose, 1980), ubiquitin has since then been identified to additionally serve other non-proteolytic functions (Komander & Rape, 2012). Protein modification by ubiquitin is crucial to the proper progression of many cellular processes, including cell-cycle progression (Joo et al., 2007), endocytosis (L. Hicke & Riezman, 1996; Kolling & Hollenberg, 1994), intracellular protein trafficking (Katzmann, Babst, & Emr, 2001; Raiborg et al., 2002), transcriptional regulation (Robzyk, Recht, & Osley, 2000), and protein-quality control (Sommer & Wolf, 1997). In light of the numerous roles that ubiquitin plays in the cell, it is not surprising that disruptions to this intricate system has been closely linked to the pathology of many diseases and cancer (Ciechanover & Brundin, 2003; Nakayama & Nakayama, 2006; Ross & Pickart, 2004).

The covalent attachment of the essential 76 amino acids ubiquitin onto the lysine residues of other proteins, a process known as ubiquitination, occurs through an enzymatic cascade of three enzymes: the E1 activating enzyme, E2 conjugating enzymes and E3 ligases. Ubiquitin is initially synthesized in an inactive precursor form which requires cleavage at its C-terminus to expose the diglycine motif that is the site of substrate conjugation. The conjugation begins with the activation of the mature ubiquitin in an ATP-dependent reaction, resulting in the attachment of ubiquitin's C terminal glycine onto a cysteine residue in the E1 activating enzyme. This activated ubiquitin is then transferred onto the active-site cysteine on the E2 conjugating enzyme. Finally, with the help from the E3 ligases, ubiquitin is relayed from the E2 enzyme onto the targeted substrates through the formation of an isopeptide bond between its C-terminal glycine

and the ε-amino group of substrate's lysine residue (Hershko & Ciechanover, 1998). While there is only one E1 enzyme in human, several E2s and a vast diversity of E3s together catalyze the ubiquitination of many different cellular substrates.

Ubiquitin itself can also be ubiquitinated at its N-terminus or any one of its seven lysine residues, leading to the formation of polyubiquitin chains with distinct linkage topologies. It is now known that different lengths of the chain and their linkage topologies altogether signals for distinct outcomes in the cell, forming a complex signaling network commonly referred to as the Ubiquitin Code (Komander & Rape, 2012). Although all possible ubiquitin linkages have been identified *in vivo* (Peng et al., 2003; P. Xu et al., 2009), the function and significance of most linkages are poorly understood. This is in part due to the fact that most of the lysine residues on ubiquitin are non-essential, except for Lys48. Lys48 chains are best known for signaling for protein degradation by the 26S proteasome whereas the functions of linked chains through Lys6, Lys27, Lys29 and Lys33 requires further investigation.

Like ubiquitin, Small Ubiquitin-like Modifier (SUMO) is a highly conserved eukaryotic PTM that is also essential for cell viability. There are four paralogs of SUMO in mammalian cells, SUMO-1, -2, -3 and -4 (Chen, Mannen, & Li, 1998; Lapenta et al., 1997; Matunis, Coutavas, & Blobel, 1996), whereas only a single SUMO protein, Smt3, exist in yeast (Meluh & Koshland, 1995). Despite sharing minimal sequence homology, SUMO and ubiquitin occupy the same three-dimensional structure, the ubiquitin grasp fold, and are conjugated onto cellular substrates utilizing similar enzymatic mechanisms (Mossessova & Lima, 2000). However, SUMO serves drastically different functions in the cell and has been implicated in processes distinct from ubiquitin. Initially discovered as a modification on the GTPase activating protein RanGAP1 that signals its localization to the nuclear pore complex (NPC) (Lapenta et al., 1997;

Matunis et al., 1996), SUMO is now identified to be involved in many nuclear processes including transcription control (Lin et al., 2006), nuclear transport (S.-J. Li & Hochstrasser, 2000), chromosome segregation (Suhandynata et al., 2019), protein-protein interaction (Namanja et al., 2012), DNA repair (Hardeland, Steinacher, Jiricny, & Schär, 2002), and ribosomal DNA silencing (Liang et al., 2017).

Ubiquitin and SUMO conjugation statuses are dynamic and change constantly in response to cell cycle stages and various environmental stimuli. The removal of SUMO and ubiquitin from protein conjugates are catalyzed by their respective family of proteases. More than 100 deubiquitinating enzymes (DUBs) are encoded in the human genome for ubiquitin deconjugation. These deubiquitinating proteases are categorized into five subfamilies based on their sequence similarities and mechanisms of action: with four of the subfamilies being cysteine proteases and the fifth a specialized group of metalloproteases (Amerik & Hochstrasser, 2004). Interestingly, out of the 17 DUBs identified in yeast, none are essential for cell viability, suggesting functional overlaps among various DUBs. (Amerik, Li, & Hochstrasser, 2000). In contrast, the deSUMOylation enzymes constitute a much simpler system, with only two SUMO proteases in yeast, Ulp1 and Ulp2, and six in human, SENP1-3 and SENP5-7 (Gong, Millas, Maul, & Yeh, 2000; Erica S. Johnson, 2004; S.-J. Li & Hochstrasser, 1999, 2000). Mass spectrometry analysis of the substrates of Ulp1 and Ulp2 in previous study have revealed that they regulate distinct targets; with Ulp1 deSUMOylating the bulk of SUMO conjugates whereas Ulp2 specifically deSUMOylates the RENT complex, the inner kinetochore, and several subunits of the MCM helicase (de Albuquerque, Liang, Gaut, & Zhou, 2016).

Together, the SUMO and ubiquitin systems constitute two major regulatory pathways involved in the maintenance of many aspects of the cell. Given the significance of SUMO and

ubiquitin, it is of great scientific and therapeutic interests to gain further understanding of the two systems.

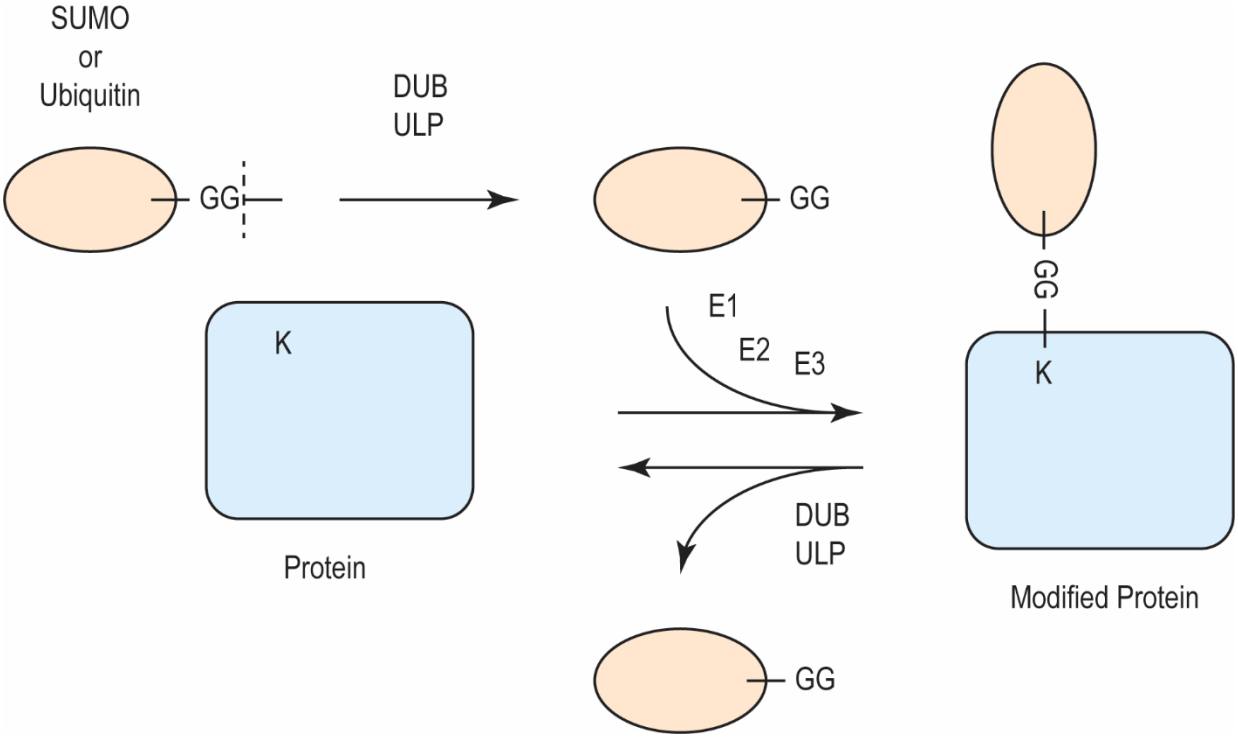

Figure 1.1 The reversible modification of proteins by Ubiquitin and SUMO. Protein modifications by ubiquitin and SUMO occur through the sequential actions of the E1, E2, and E3 enzymes. The removal of ubiquitin and SUMO from modified proteins are catalyzed by their respective groups of isopeptidases (DUB for ubiquitin, ULP for SUMO).

Chapter 2:

Directed Evolution of Ulp1 to enhance its SUMO binding affinity to optimize SUMO substrate trap

2.1 Introduction

Studies on SUMOylated proteins are inherently challenging, as they appear transiently and constitute only a small fraction of the total protein abundance in the cell. Therefore, the enrichment of these endogenous modified proteins is fundamental to their downstream analysis. Because purifications with antibodies raised against SUMO are typically ineffective and produce relatively high backgrounds of non-specific contaminants (Becker et al., 2013), conventional methods often rely on epitope tagging the N-terminus of SUMO so that commercially available purification reagents, such as Nickel affinity resin, can be used for the enrichment of SUMO conjugates (Figure 2.1) (Erica S Johnson, Schwienhorst, Dohmen, & Blobel, 1997; Wohlschlegel, Johnson, Reed, & Yates, 2004). However, this approach has one key caveat. Genetic evidences from a previous study have indicated that the epitope tags can disturb the native SUMOylation pattern and compromise SUMO's cellular function to an unknown extent (de Albuquerque et al., 2016). While the loss of the SUMO isopeptidase Ulp2 is known to cause the accumulation of polySUMOylated proteins and slow cell growth (Bylebyl, Belichenko, & Johnson, 2003), these phenotypes are partially alleviated in cells additionally expressing epitope tagged SUMO (de Albuquerque et al., 2016). This observation suggests that the results of studies which also utilized the epitope tagging approach may have been confounded in a similar fashion. For these reasons, an enrichment approach that can capture the modified proteins while preserving the natural functions of SUMO is critical for future studies.

One possible alternative is by using proteins with intrinsically strong affinity for SUMO to capture the modified proteins, an approach known as SUMO substrate trap (Figure 2.1). SUMO Interacting Motif (SIM), a short consensus sequence defined by V/I-X-V/I-X/I, is a widely shared enzyme domain that mediates SUMO-dependent protein interactions (Namanja et

al., 2012). Prior studies have reported the use of proteins containing tandem repeats of SIM to purify SUMOylated proteins. Bruderer et al. has developed the use of RNF4-coupled resins, which contains 4 SIM motifs, to purify the endogenous polySUMO conjugates from mammalian cell extracts. Indeed, this approach has successfully purified the SUMOylated proteins, with up to 80% recovery of the polySUMOylated species, and have confirmed the validity of such tools (Bruderer et al., 2011). However, it is important to note that the modest affinity of SIMs (Kd in 2-3μM range) was insufficient to enrich for the monoSUMOylated proteins.

Further search for other potential SUMO binders identified Ulp1 as a promising candidate. The crystal structure of Ulp1 in complex with SUMO revealed an extensive contact surface of 2400 $Å^2$, and that its constricted but shallow active site tunnel is key to its broad specificity for SUMOylated substrates (Mossessova & Lima, 2000). Moreover, the catalytically inactive form of Ulp1 has been measured to have nanomolar affinity for SUMO (Kd = 12.8nM) (Elmore et al., 2011), approximately 200 times greater than that of SIM, all of which indicate that the application of Ulp1 can greatly enhance the sensitivity the of SUMO substrate trap approach. Remarkably, recent studies have reported that this Ulp1 pulldown approach is capable of enriching for approximately 50% of the total SUMOylated proteins from yeast cell extracts and can even detect the low abundance SUMO conjugates, including the kinetochore subunits (Elmore et al., 2011; Suhandynata et al., 2019).

While Ulp1 pulldown has greatly improved the detection for SUMO conjugates, it remains to be further optimized. As mentioned above, Ulp1 makes extensive contact with SUMO over several domains, suggesting that potential mutations improving its binding affinity likely exist**.** Although making modifications based on computational designs have been successful in some cases, current understanding of the complex relationship between enzyme structures and

functions is far from perfect to accurately predict improving mutations. Directed evolution can easily bypasses this issue by making gradual improvements over multiple generations; by accumulating the beneficial mutations through iterative rounds of recombination and selection to ultimately create a novel protein with significant improvements in the desired traits. This evolutionary approach to enzyme engineering has proven to be a powerful strategy for optimizing various enzyme parameters including thermostability(Giver, Gershenson, Freskgard, & Arnold, 1998), substrate specificity(Zhao & Arnold, 1999), binding affinity (E. T. Boder, K. S. Midelfort, & K. D. Wittrup, 2000; Yi Li et al., 2005), and catalytic efficiency (Branon et al., 2018; Lam et al., 2015).

Many organisms have been utilized as platforms for the creation of proteins libraries, allowing directed evolution studies. In particular, directed evolution using yeast surface display has demonstrated numerous success in enhancing protein affinity and offers several advantages over other reported scaffolds (Boder & Wittrup, 1997; Daugherty, Chen, Olsen, Iverson, & Georgiou, 1998; Packer & Liu, 2015). Yeast, being an easily cultured single cell eukaryote with simple genetics, provides the best expression system with its eukaryotic protein-folding machinery. Furthermore, the high level of protein copy number ($10^4 \sim 10^5$) displayed on the yeast cell surface allows for sensitive detection of proteins with low binding affinity. Finally, its compatibility with Fluorescence Activated Cell Sorting (FACS) allows for fine discrimination between mutants based on quantitative measurements of their equilibrium binding kinetics.

In this study, we present a directed evolution strategy for improving the binding affinity of Ulp1. We first established and optimized the detection of SUMO binding on yeast cell surfaces, then proceeded to the directed evolution of Ulp1. To additionally create a thermostable high affinity SUMO binder, we also subjected the Ulp1 ortholog in the thermophilic fungi *C.*

thermophilum to enzyme evolution. To overcome limitations in FACS selection, we have also established the use of Magnetic Activated Cell Sorting (MACS) as a possible alternative to flow cytometric sorting.

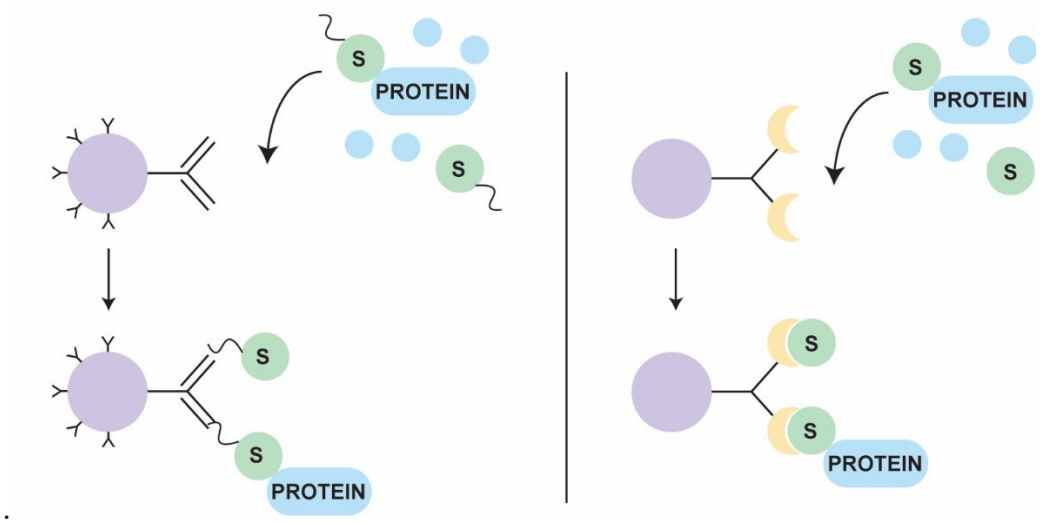

Figure 2.1 Epitope tagging and the SUMO substrate trap can be used to purify endogenous SUMOylated proteins. Cartoon schematic showing that SUMOylated proteins and unconjugated SUMO can be purified from complex protein mixtures by using commercially available affinity resins that bind to the epitope tag on SUMO. Antibody-coated resin is shown as a representative here. Alternatively, SUMO binding proteins can also be used as a purification tool following the same concept.

2.2 Results

2.2.1 Establishing and optimizing the detection of SUMO binding on yeast cell surface with Fluorescence Activated Cell Sorting

In our directed evolution strategy, we capitalized on the femtomolar affinity of streptavidin for biotin as the basis of our detection scheme for SUMO binding (Figure 2.2A), similar to a previously published protocol (Chao et al., 2006). Briefly, we displayed the protein of interest, in our case Ulp1, on the yeast surfaces as a fusion between a C-terminal myc tag and the mating protein Aga2p, which is covalently linked to the anchorage subunit Aga1p. The expression of Ulp1 on cell surfaces and its binding with SUMO can each be detected by labeling with fluorophores, thus allowing quantitative screening with FACS. For labeling with SUMO, yeast cells were incubated with biotinylated SUMO to initiate binding with the surface displayed Ulp1. Then, the cells were stained with streptavidin-phycoerythrin (SAPE) to fluorescently label the surface-bound SUMO.

To first generate the substrates that we used for SUMO labeling (Figure 2.2B-C), recombinantly expressed SUMO and biotin ligase BirA were each purified from bacteria using Nickle affinity chromatography. The bacterial cell lysates, the unbound fraction after resin binding and the elution fraction were all analyzed via SDS-PAGE and Coomassie staining. As seen in their elution fractions, both proteins were relatively pure with minimal contaminants after a single step affinity purification. It is important to note that the recombinant SUMO was C-terminally tagged with a 15 residues peptide sequence known as Avitag which can be site-specifically biotinylated *in vitro* by incubation with BirA. BirA can catalyze the transfer of biotin onto the lysine within Avitag.

SUMO was biotinylated following previously published protocol (Y. Li & Sousa, 2012). Recombinant SUMO was enzymatically biotinylated using BirA by mixing SUMO and BirA at a

1 to 100 molar ratio, in an ATP-dependent reaction. To confirm the biotinylation of SUMO, the reaction mixture was dialyzed to remove the excess biotin before aliquots of the mixture were each incubated with various molar concentrations of SAPE, up to 1:4 SUMO to SAPE molar ratio. Notably, samples were not boiled before loading into SDS-PAGE in order to retain the binding interaction between SAPE and biotin. Binding of SUMO (15kDa) to SAPE (278kDa) caused a delayed electrophoretic mobility shift via SDS-PAGE, resulting in the depletion of the low molecular weight SUMO band. The depletion of the SUMO band in lane 5 confirmed that SUMO is biotinylated and that each molecule of SAPE can bind to approximately four molecules of SUMO (Figure 2.2D).

At first, SUMO binding by the surface displayed Ulp1 were too low to be clearly detected (Figure 2.2E, lower panel). FACS analysis of cells labeled with 1uM of monomeric SUMO showed no distinct signal apart from the negative, baseline peak that represented yeast cells with no SUMO binding either due to plasmid loss or denaturation of the surface displayed proteins. To overcome this issue without simply increasing the ligand concentration which can potentially lead to excessive nonspecific binding, we preloaded SUMO onto SAPE before labeling to increase the ligand avidity. One key advantage that the detection scheme offers is that streptavidin is a homo-tetramer that can bind to four molecules of biotin, thus forming a tetravalent ligand complex when preincubated with biotinylated SUMO (Figure 2.2D, upper panel). Labeling of cells with 200nM of the tetramerized SUMO showed drastic improvement in SUMO binding, indicated by the shifted shoulder relative to baseline. This technique, known as "streptavidin preloading", increased the local concentration of SUMO by several hundred folds, thus allowing detection of mutants possessing modest affinity. For these reasons, all subsequent FACS analysis were performed with streptavidin-preloaded ligands.

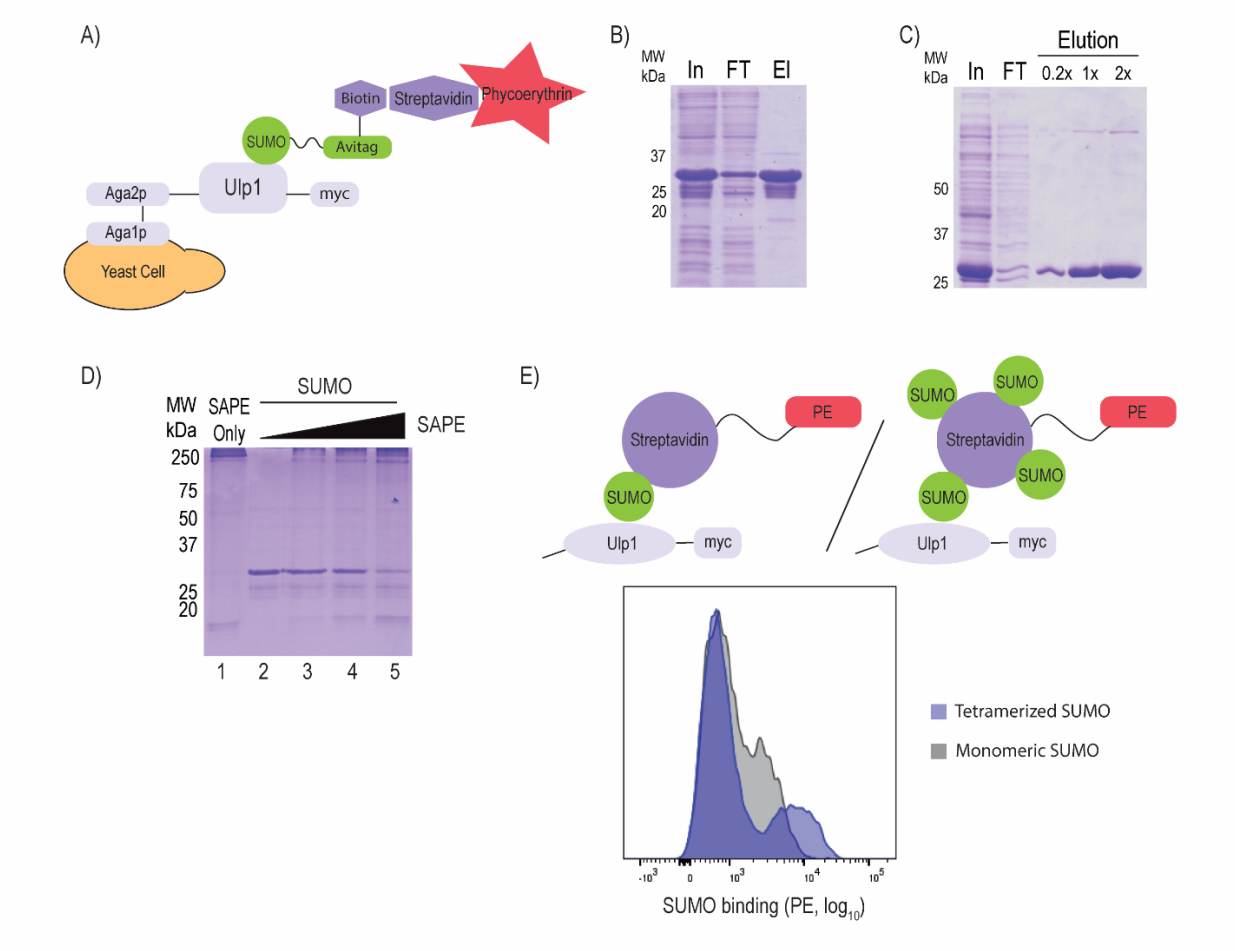

Figure 2.2 Stable detection of SUMO binding on yeast cell surfaces requires the use of a tetravalent SUMO complex as ligand. (A) Schematic illustration of yeast surface display and detection for SUMO binding events. (B) Ni-NTA affinity purification of recombinantly expressed SUMO-Avitag from IPTG-induced Rosetta (DE3) cell lysate. In represents input, the whole cell lysate, FT represents flowthrough, the unbound cell lysate after incubation with Ni resins, El represents elution, the eluate collected after elution with 200mM imidazole. (C) Ni-NTA affinity purification of recombinant BirA. (D) Binding test of biotinylated SUMO-Avitag with SAPE. Lane 1 - SAPE only, lane 2 - SUMO-Avitag only, lane 3 - 1 to 1 molar ratio of SUMO to SAPE, lane 4 – 1 to 2 molar ratio, lane 5 – 1 to 4 molar ratio. (E) FACS data comparing the difference in signal intensity between Ulp1 cells labeled with SUMO monomer versus preloaded SUMO.

2.2.2 Directed evolution of budding yeast Ulp1

With the detection for SUMO binding established, we then began the directed evolution of Ulp1. We used error prone PCR to mutagenize Ulp1's catalytic domain (403-621) which yielded a mutant library that consisted of several million clones each containing 1-3 mutations. The mutant library initially showed no binding with SUMO (Figure 2.3C), presumably because most mutations were detrimental to the binding interaction. The mutagenesis also disrupted the expression of most mutants on the cell surface, as indicated by the low expression level of the mutant library (Figure 2.3B). To ensure that changes in SUMO binding are not due to host-expression biases, a preliminary selection was performed to normalize for the protein expression level (Figure 2.3B). Fluorescently labeled cells with similar expression level as wildtype Ulp1 cells were isolated with FACS.

The myc-sorted mutant library was then subjected to multiple rounds of selection to isolate cells exhibiting the strongest binding to SUMO (Figure 2.3A). We gradually increased the selection stringency over four rounds of selection (Figure 2.3C), which ultimately led to a distinct improvement in SUMO binding relative to wildtype Ulp1 (Figure 2.3D). Although the selections were only performed once, the improvement in SUMO binding for the 4^{th} sorted library have been confirmed with at least three replicates and a representative data shown in Figure 2.3D. To explore whether binding by this sorted library cells were specific to SUMO or nonspecifically to the secondary reagent SAPE, a negative selection was performed on the 4^{th} sorted library by labeling the cells with SAPE only (Figure 2.3E). FACS analysis of the negative selection confirmed that the previous observed binding was indeed specific to SUMO.

We thus sequenced 8 cells randomly selected from the 4^{th} sorted library, with the results shown in Table 1. With the exception of p6, which contained a I519V mutation, all other mutants

shared three identical mutations at F542S, D570N and L597F, thus validating our evolution strategy.

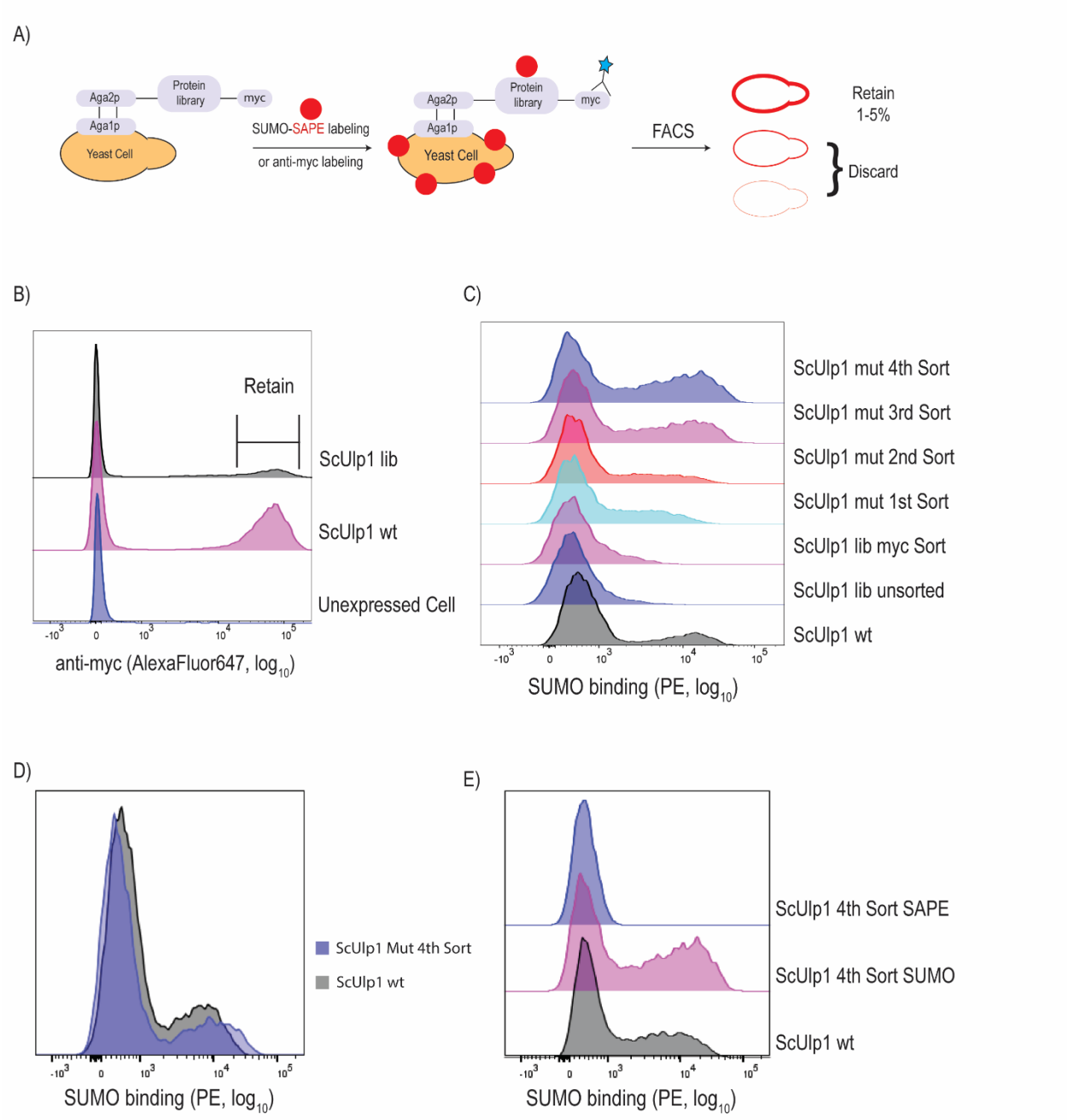

Figure 2.3 Directed Evolution of budding yeast Ulp1 (A) Schematics showing the overall selection process for better SUMO binders from the mutant library. (B) FACS analysis of the expression level for Ulp1 mutant library in side-by-side comparison with Ulp1 wt. Unexpressed cell is the negative control that confirms the expression is specifically induced by culturing in galactose media. (C) FACS analysis of the SUMO binding signal of the mutant library after each stages of sorting. (D) FACS analysis of the difference in SUMO binding signal between Ulp1 wildtype and the enriched mutants after 4 rounds of FACS selection. (E) FACS analysis comparing the SUMO binding signal of the enriched mutants when incubated with SUMO or with SAPE only.

Table 1 Mutations in selected mutant clones.

Residue Position #	WT	p1	p2	p3	p4	p5	p6	p7	p8	
519	I						V			519
542	F	S	S	S	S	S		S	S	542
570	D	N	N	N	N	N	N	N	N	570
597	L	F	F	F	F	F	F	F	F	597

Red: Mutation in >5 clones

2.2.3 Establishing Magnetic Activated Cell Sorting with CthUlp1

From our experiences, one major disadvantage to Ulp1 is its poor solubility at cold temperature. Interestingly, a recent study has identified a putative ortholog of Ulp1 in the thermophilic fungi *Chaetomium thermophilum* that exhibits greater solubility and thermostability than budding yeast Ulp1(Lau et al., 2018). We thus also subjected this thermostable Ulp1 to directed evolution to further enhance its binding affinity. We will henceforth refer to this thermostable Ulp1 as CthUlp1.

Because there have been no reports on the binding affinity of CthUlp1, the concentration of SUMO needed to label CthUlp1 cells had to be determined empirically. Cells displaying either CthUlp1 or budding yeast Ulp1 were each incubated with various concentrations of SUMO before FACS analysis (Figure 2.4A). Our data identified 0.4nM of SUMO as the ideal concentration for labeling, as it is the minimal concentration that binding can be detected without reaching saturation. Remarkably, there is an apparent difference in binding between CthUlp1 cells and Ulp1 cells when the cells were labeled with 0.4nM SUMO. To determine whether the difference in binding could be caused by differences in expression level, we measured the expression level of both cells through anti-myc staining. Our FACS data showed that both cells have comparable expression level (Figure 2.4B). Taken together, these observations suggested that CthUlp1 has weaker binding affinity than Ulp1, although further investigation with a more quantitative measurement is required to confirm this observation.

As flow cytometry can typically sort up to 10^8 cells per hour, the size of the library is limited by the feasibility of the FACS screening time. To overcome this limitation, Magnetic Activated Cell Sorting (MACS) is often utilized to reduce the size of the initial library. Here we hypothesized that MACS can be used as an alternative to FACS screening. We generated a

mutant library of CthUlp1 following the same procedures described above and used MACS to perform the selection (Figure 2.4C). Cells expressing CthUlp1 mutants were first incubated with monomeric biotinylated SUMO before labeling with streptavidin-coated magnetic resin. The resin-coated cells were subsequently passed through a magnetic column to isolate cells that were labeled with SUMO. As seen in Figure 2.4C, SUMO binding for the mutant library cells gradually improved over two MACS selections. Interestingly, while the first round of MACS screening only led to a minor improvement in binding, cells after the second round of screening displayed drastic improvement in binding, suggesting that the selection stringency of MACS sorting is variable and requires further optimization.

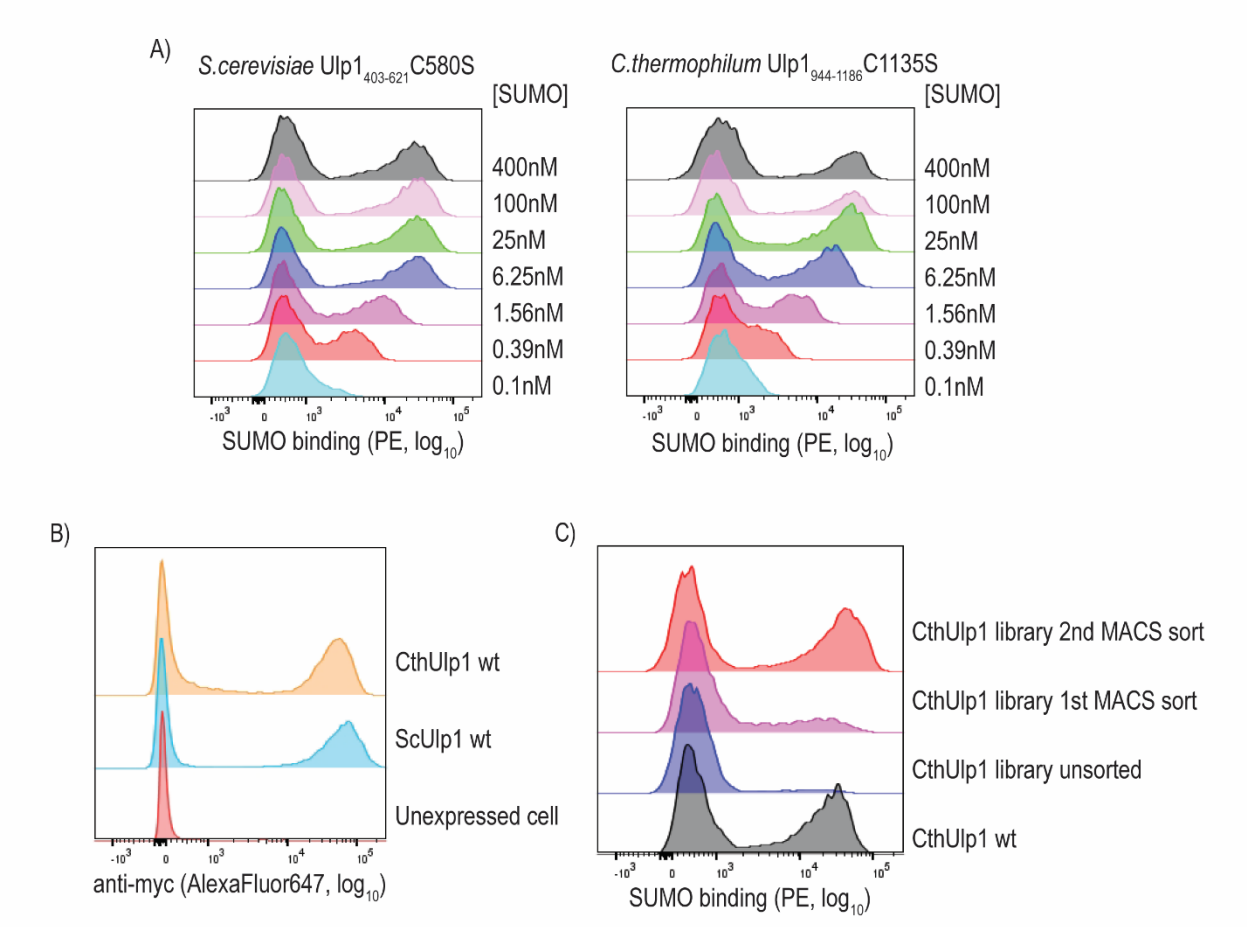

Figure 2.4 Characterizations of CthUlp1 and MACS sorting of CthUlp1 mutant library. (A) Yeast displaying CthUlp1 were incubated with various concentrations of SUMO and analyzed by FACS to determine empirically the ideal SUMO concentration for labeling CthUlp1 cells. The same experiment were done with budding yeast Ulp1 both as positive control and as reference. (B) The expression level of CthUlp1 were determined by anti-myc staining prior to FACS and compared to yeast Ulp1. (C) FACS analysis of the enriched CthUlp1 mutants after each rounds of MACS selection.

2.3 Discussion

Until recently, studies on SUMOylated proteins have mostly depended on epitope tagging the N-terminus of SUMO to purify the endogenous conjugates (Wohlschlegel et al., 2004). As previously mentioned, the potential disruption in SUMO conjugation caused by epitope tagging, especially in polySUMO chain formation, has raised concerns over the method. Researchers have been forced to resort to epitope tagging despite of this caveat, as other reported alternatives have failed to provide the same level of sensitivity. Luckily, recent reports of using the catalytic domain of Ulp1 as a purification reagent has presented a technological breakthrough in the detection of SUMOylated proteins (Elmore et al., 2011; Suhandynata et al., 2019). Here, we began optimizing this approach by using directed enzyme evolution via yeast surface display to enhance the binding affinity of Ulp1. Although binding by Ulp1 and the mutant library on the yeast surface were initially too weak to be detected, streptavidin preloading was able to overcome this technical hurdle by increasing the ligand avidity by several folds (Figure 2.2D). Four consecutive rounds of FACS screening enriched for mutants with improved binding led to the identification of three recurrent mutations (Figure 2.3D, Table 1). Interestingly, none of the three mutations are positioned at the binding interface with SUMO when mapped onto the structure of Ulp1, consistent with previous observations that beneficial mutations can occur at positions distal to the binding site (Eric T Boder, Katarina S Midelfort, & K Dane Wittrup, 2000; Mossessova & Lima, 2000). Although the improvement in binding after the initial round of evolution is modest, our results nevertheless validated our evolutionary strategy.

Unlike in published protocols, we did not utilize a two-dimensional labeling scheme during our screening process (Eric T Boder et al., 2000). Unfortunately, simultaneous labeling with SAPE and anti-myc antibodies has not worked in our hands, potentially due to steric

interference between the two. Therefore, it can be argued that some unique clones which may have possess stronger binding but also exhibited variations in expression level could have been excluded from our mutant pool after the initial anti-myc selection.

It is important to note that the discrepancy in SUMO binding apparent from comparing Figure 2.2E and 2.4A is due to the positional difference of the Avitag on SUMO: with the former data obtained with the Avitag positioned at the C-terminus and the latter at the N-terminus. Evidences on the substrate specificity of Ulp1 from biochemical assays has suggested that besides from the active site tunnel, the catalytic domain of Ulp1 contains additional elements that imparts its specificity for SUMO conjugates and SUMO molecules without side chains C-terminal to the diglycine motif (Reverter & Lima, 2004). Indeed, cells incubated with N-terminally tagged SUMO exhibited stronger binding signal even at low SUMO concentration (compare signal in Figure 2.2E with 200nM C-terminally tagged SUMO with signal from Figure 2.4A with 6.25nM N-terminally tagged SUMO). This observation indicated that N-terminally tagged SUMO, with an unobstructed C-terminus, is the more ideal ligand for Ulp1 and was thus used instead in the following experiments. Interestingly, the improvement in binding by the enriched mutants (Figure 2.3D) was not seen when N-terminally tagged SUMO were used as ligand instead (data not shown), indicating that the identified mutations had modified Ulp1's substrate specificity. We believe that this issue can be easily resolved with the replacement of N-terminally tagged SUMO as ligand in our following evolution experiments and should not undermine the validity of our established methods.

The recent report of a thermostable Ulp1 ortholog (CthUlp1) has presented an interesting candidate for our directed evolution (Lau et al., 2018). The initial analysis presented here suggests that the binding affinity of CthUlp1 is potentially worse than that of budding yeast Ulp1

(Figure 2.4A), although a more direct, quantitative measurement is needed to confirm this observation. In addition, we have empirically determined the ideal SUMO concentration for labeling CthUlp1 cells, such that any changes in binding affinity by CthUlp1 mutants can be detected. Because MACS does not offer the advantage of selecting specific cell population based on equilibrium binding kinetics, the improvement in binding after each rounds of selection cannot be predicted and the concentration of ligand for labeling for MACS had to be determined empirically. From our experience so far, we did not see any improvement in binding for the CthUlp1 mutants beyond the 2nd MACS sort, which demonstrated the same binding signal as wildtype from FACS analysis. This can be due to 1) the ligand concentration was too high such that the selection stringency is not enough to differentiate better binders from those exhibiting similar affinity as wildtype, or 2) the labeling condition used for FACS cells had oversaturated the displayed clones with SUMO, suggested by the saturated signal (Figure 2.4C).

2.4 Methods

Cloning

Standard yeast genetics techniques were used in the construction of the yeast strain. The yeast strain used in surface display experiments have the following genotype: G418::pGAL-Aga1, ura3-52, leu2delta1, trp1delta63, his3delta200, lys2deltaBgl, hom3-10, ade2delta, ade8, MATa.

Recombinant protein expressions and purifications

Each of the recombinant proteins used (SUMO-Avi, Avi-SUMO and BirA) were cloned into Ligation Independent Cloning (LIC) vector containing C/N-terminal His_6- FLAG-Avitag. BirA was cloned into LIC-2BT vector with N-terminal His_6 – Tobacco Etch Virus (TEV) cleavage site. and transformed into Rosetta^{TM}2 (DE3)pLysS competent cells (EMD Millipore) and grown into 1 liter of Luria Broth(LB) media with 100µg/mL of ampicillin and 25µg/mL of chloramphenicol at 37°C until optical density (OD_{600}) of the culture reaches around 0.5. Then, protein expression was induced by the addition of 0.2mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and switched to 18°C for an additional 16 hours incubation. The cells were then pelleted and lysed with sonication in phosphate buffered saline (PBS) with protease inhibitors (2mM phenylmethylsulfonyl fluoride, 200µM benzamidine, 0.5µg/mL leupeptin, 1 µg/mL pepstatin A). The recombinant proteins were purified using Nickel nitrilotriacetic acid (Ni-NTA) chromatography. Elution fractions were pooled and dialyzed into PBS with 10% glycerol before storage at -80°C freezer.

In vitro biotinylation with BirA

Avitag-ligands were each biotinylated in a reaction mixture containing 67μM of ligand, 0.5μM of BirA, 160μM of biotin, 3mM of ATP and 3mM of magnesium chloride. The reaction mixture was incubated at room temp on a rocking platform for 1 hour before quenching the reaction by heating the mixture at 60°C for 10 minutes. The reaction mixture was centrifuged at 20,000xG for 30 minutes at 4°C to remove precipitated proteins before it was dialyzed into ice cold PBS with 10% Glycerol. The dialyzed protein was stored in -80°C freezer.

Generation of mutant libraries for yeast display

Mutant libraries for Ulp1 and CthUlp1 were generated by error-prone PCR. Briefly, 1.6μg of the temple in vector pCTCON2 were amplified for 20 rounds with 0.4μM of forward and reverse primers, 2μM of dPTP and dGTP (Invitrogen). The PCR products were then gel extracted and electroporated into electrocompetent HZY398 with the BamHi-PstI linearized pCTCON2 vector (4μg insert/1μg vector). The transformed yeast cultures were rescued in 50mL of -Trp Glu media for 2 days at 30°C.

Yeast Cell culture

For yeast-display, culturing conditions and media components were adapted from previously published protocols (Chao et al., 2006). Yeast cells freshly transformed with the yeast display plasmid pCTCON2 were cultured in standard Tryptophan dropout media supplemented with 38mM Anhydrous Dibasic Sodium Phosphate, 71mM Anhydrous Monobasic Sodium Phosphate and 2% Dextrose (-Trp Glu) in 30°C shaker for 2 days. Protein expression was

induced by inoculating the saturated yeast culture into -Trp Gal media (-Trp Glu medium with 2% dextrose replaced with 2% Galactose and 0.2% Dextrose) at a 1:200 dilution and incubated at 30°C for 20 hours. The final OD of -Trp Gal culture must be between 0.5 to 1.0 for optimal protein expression and protein folding after displaying on cell surface.

After FACS sorting, yeast cells were collected in 5mL of -Trp Glu media supplemented with 100μg/mL of Penicillin-Streptomycin (Gibco) and 10μg/mL of Tetracycline (Sigma-Aldrich) and incubated to saturation at 30°C. The saturated cells were then used to inoculate 50mL of -Trp Glu media and grown to saturation before protein expression is induced following the same procedures described above.

Cells collected after MACS sorting follows a similar protocol, only that the cells were directly collected from the magnetic column and grown into 50mL of the antibiotics-supplemented -Trp Glu media. If contamination persisted, the cultures were additionally supplemented with 1mg/mL of 5-Fluoroorotic acid before inoculating into the Galactose media for protein expression.

Yeast display selections

For preloading SUMO onto streptavidin-phycoerythrin (ThermoFisher Scientific), 80μM of biotinylated SUMO were incubated with 3.6μM of SAPE at room temperature for 1 hour.

To label yeast cells, cells were harvested by centrifuging 400μL of an OD0.5 culture (corresponding to 2 million cells) at 4,000xG for 2 minutes. The cell pellet was then washed three times, each time with 500uL of PBS with 0.1% bovine serum albumin (1mg/mL BSA, referred to as PBSB) before it was incubated with the labeling mixture. For analyzing the expression level of surface displayed proteins, the harvested cells were incubated with anti-c-

myc antibodies (chicken anti-c-Myc IgY from Invitrogen) at a 1:100 dilution in 50μL of PBSB for 1 hour at room temperature. After incubation, the primary antibody mixture was aspirated, washed twice with 125μL of PBSB and incubated with Alexa Fluor 647-goat anti-chicken IgG (Invitrogen) at a 1:200 dilution in 50μL of PBSB for 1 hour at 4°C. For analyzing SUMO binding, the harvested cells were incubated with 50μL of 200nM of preloaded SUMO for 1 hour at room temperature. Finally, the labeling mixture (SAPE-SUMO or Alexa Fluor 647) were aspirated and cells were resuspended in 1mL of PBSB for FACS analysis. For the sample incubated with SAPE only (Figure 2.3E), cells were incubated with SAPE at a 1:100 dilution in 50μL of PBSB for 1 hour at room temperature.

All characterizations of clones were done on a BD FACS LSR Fortessa X-20 (BD Biosciences) with the appropriate lasers and emission filters (488nm and 575/26 for PE, 640nm and 670/30 for Alexa Fluor 647). To analyze single yeast cells, cells were plotted by forward scatter width (FSC-W) and side scatter width (SSC-W) and a gate was drawn to isolate the population between 50 to 100 FSC-W, 50 to 100 SSC-A for population 1 (P1). Cells from P1 were then plotted by forward scatter height (FSC-H) and forward scatter width (FSC-W) and a second gate was drawn to isolate the population between 50 to 170 FSC-H and 50 to 100 FSC-W for population 2 (P2). Cells from P2 were then plotted by side scatter height (SSC-H) and side scatter width (SSC-W) and a third gate was drawn to isolate the population between 50 to 170 SSC-H and 50 to 100 SSC-W for population 3 (P3).

All FACS sorting were done with a Sony SH800S Cell Sorter and the gating setup for singlet discrimination is the same as described above. Labeling of cells for FACS sorting also follows the same procedures as above, only 10 million cells were used, and the volume of the labeling mixture adjusted proportionally. At least a ten-fold excess of yeast cells relative to the

original library size was labeled each round to ensure the library was not under-sampled. The sorting gate for the initial selection to normalize for the variation in protein expression level in the mutant library was set such that only cells with the same Alexa Fluor 647 signal as the wildtype were selected. The sorting gate for isolating the strongest SUMO binders during each rounds of selection were drawn such that only the top 5% of cells with highest PE signal were selected. This gate was further restricted to isolating the top 1% of cells with highest PE signal after the second round of selection.

Magnetic Activated Cell Sorting

The procedures used for MACS follows the protocol provided with the product (Miltenyi Biotechnology). Briefly, 100ODmL of induced cells (corresponding to 1 billion cells) are harvested by centrifuging at 4,000xG for 2 minutes. The harvested cell pellet was then washed once with 25mL of PBS with 0.5% BSA, 2.5mM EDTA (referred to as PBSM) before incubating with 1mL of 200mM biotinylated SUMO in PBSM for 1 hour at room temperature. After incubation, the SUMO mixture was aspirated, and the cells were washed once with 5mL of ice cold PBSM. After washing, the cells were incubated with 5µL of streptavidin-coated MACS microbeads (Miltenyi Biotechnology) in 500µL of PBSM for 10 minutes at 4°C. Then, the mixture was aspirated, and the cells were resuspended in 5mL of PBSM before loading into the magnetic MS column (Miltenyi biotechnology).

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Dr. Huilin Zhou designed the strategy to use directed evolution to improve Ulp1's binding affinity.

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Chapter 3:

The ubiquitin protease Ubp15 from *C. thermophilum* is a suitable candidate for the development of ubiquitin substrate trap via directed enzyme evolution

3.1 Introduction

Like SUMO, studies defining the ubiquitin system are also hampered by the low abundance level of ubiquitin conjugates. This issue is commonly addressed via the same approach used in SUMOylation studies: by epitope tagging the N-terminus of ubiquitin (Ellison & Hochstrasser, 1991). However, epitope tagging ubiquitin faces one critical issue in addition to the caveats mentioned in the previous chapter. Because multiple genes encode ubiquitin in most eukaryotes, it is almost impossible to tag all ubiquitin genes at their chromosomal loci (Özkaynak, Finley, & Varshavsky, 1984; Wiborg et al., 1985). While this problem can be more easily addressed in organisms with facile genetics such as yeast, studies in other higher eukaryotes require the overexpression of tagged ubiquitin to outcompete the expression level of endogenous ubiquitin (Peng et al., 2003; Tagwerker et al., 2006), causing concerns over whether the overexpression would result in artifactual ubiquitination patterns. For these reasons, the reliability of this approach is questionable despite its success in previous studies.

Many alternative approaches for capturing ubiquitin conjugates developed by prior studies have primarily focused on either the use of ubiquitin binding domains (UBDs) or ubiquitin-specific antibodies. Purification strategies utilizing the UBDs faced the same issues as the SIM pulldown approach described in the previous section. More than 20 families of UBDs have been characterized to date, with their affinities typically in the range of 100 micromolar and bind to a single hydrophobic region on ubiquitin (Linda Hicke, Schubert, & Hill, 2005; Raasi, Varadan, Fushman, & Pickart, 2005). Purification strategies capitalizing on these proteins have been successful in detecting polyubiquitinated proteins, with reports of isolating over 200 and 127 distinct ubiquitin substrates (Mayor, Lipford, Graumann, Smith, & Deshaies, 2005; Weekes et al., 2003). However, its modest affinity for monoubiquitin again suggests that monoubiquitin

conjugates are potentially under-represented in studies utilizing this approach. On the other hand, enrichment strategies based on ubiquitin-specific antibodies have provided a more complete perspective on the ubiquitin system. The development of antibodies that specifically recognize the diglycine remnant of trypsin-digested ubiquitin conjugates by Xu et al has enabled the identification of thousands of ubiquitylation sites in human and murine cells (Kim et al., 2011; Wagner et al., 2012; G. Xu, Paige, & Jaffrey, 2010). It is important to note, however, that proteins modified by two related Ubiquitin-like proteins (Ubls), NEDD8 and ISG15, can also generate the identical diglycine remnant after trypsin cleavage, thus complicating analysis.

Meanwhile, there has been no reports on methods analogous to Ulp1 pulldown suitable for ubiquitin studies. Given the success with Ulp1 pulldown and the vast diversity of deubiquitinating enzymes (DUBs), we reasoned that the DUBs can be exploited in a similar fashion. With more than 60 members, the Ubiquitin Specific Proteases (USPs) constitute the largest and most diverse subfamily of the DUBs and are involved in the regulation of numerous biological processes (Hochstrasser, 1996). Structural analysis on the catalytic core domain of human USP7 revealed that all USPs adopts a conserved three-domain architecture (the Fingers, Palm, Thumb) which highly complements the shape of ubiquitin (Min Hu et al., 2002), and covers approximately 30% of the ubiquitin surface. These findings provided the first mechanistic insight into the substrate specificity of the USPs. Furthermore, characterizations on the enzyme kinetics of several USPs has shown that the affinity of most USPs is generally in the range of 1 micromolar, approximately 100-fold stronger than the affinity of the UBDs (Amerik & Hochstrasser, 2004). Taken together, these findings suggest that an affinity purification tool based on the USPs, once developed, could easily overcome the limitations in previous methods.

Our search for potential candidates for the development of ubiquitin substrate trap led to the identification of two enzymes: human Usp2 and Usp4. Usp4 has been shown to regulate a variety of substrates that are involved in many different cellular processes. Usp4 has been implicated in the endoplasmic reticulum-associated degradation (ERAD) pathway by preventing the degradation of the A2a receptor (Milojevic et al., 2006). At the spliceosome, Usp4 is guided by Sart3 to facilitate the maturation of the spliceosome active site by promoting its rearrangements (Song et al., 2010). Furthermore, Usp4 has generated significant therapeutic interests for its role in inhibiting p53-mediated apoptosis and cell cycle checkpoints by targeting ARF-BP1(Zhang, Berger, Yang, & Lu, 2011). Structural analysis on Usp4 have revealed three additional domains to the catalytic core that are all required for its full catalytic activity, including the N-terminal DUSP-Ubl domain, the linker sequence that connects the N-terminal domain to the catalytic domain, and the Ubl-insert domain within the catalytic core (Clerici, Luna-Vargas, Faesen, & Sixma, 2014). While most USPs binds to ubiquitin with micromolar affinity, the Usp4's catalytic domain was reported with a remarkable affinity of 44 nanomolar. This affinity can be further enhanced by many folds if the catalytic cysteine is inactivated by alanine substitution (Kd = 0.6nM), as a result of reduced steric hindrance between the cysteine sulfhydryl and the C-terminal carboxylate of ubiquitin (Morrow et al., 2018). The extraordinary Kd of Usp4 suggests that subjecting Usp4 to enzyme evolution is unnecessary, as it is potentially readily applicable as a purification reagent.

On the other hand, Usp2 does not possess strong affinity like Usp4 (Kd = 2.8uM), but instead forms an extensive binding interface of 3850 $Å^2$ with ubiquitin, corresponding to 40% of the ubiquitin surface (Renatus et al., 2006). Usp2 is known to target a fewer number of substrates that have also been implicated in cancer progression, including fatty acid synthase, MdmX and

Mdm2 (Allende-Vega, Sparks, Lane, & Saville, 2010; Graner et al., 2004; Stevenson et al., 2007). In addition to its large binding interface, as many as 30 Usp2 residues are positioned within 4 angstrom proximity to ubiquitin (Renatus et al., 2006). These characteristics suggest that Usp2 is potentially more suited for directed enzyme evolution by offering greater margin for improvement.

In this chapter, we continued to apply the evolutionary strategy presented in the previous chapter and began the development of a ubiquitin substrate trap based on USPs. To identify a suitable USP for directed enzyme evolution, we evaluated several USPs from different organisms for their expression level and ubiquitin binding on yeast cell surfaces. Our results showed that CthUbp15, one of the putative orthologs to Usp4, has the highest expression level and ubiquitin binding on the yeast surface and thus the best starting enzyme for directed evolution. We then empirically determined the ideal ubiquitin concentration for labeling CthUbp15-displayed cells.

3.2 Results

3.2.1 CthUbp15 is a suitable candidate for the development of ubiquitin substrate trap via directed evolution

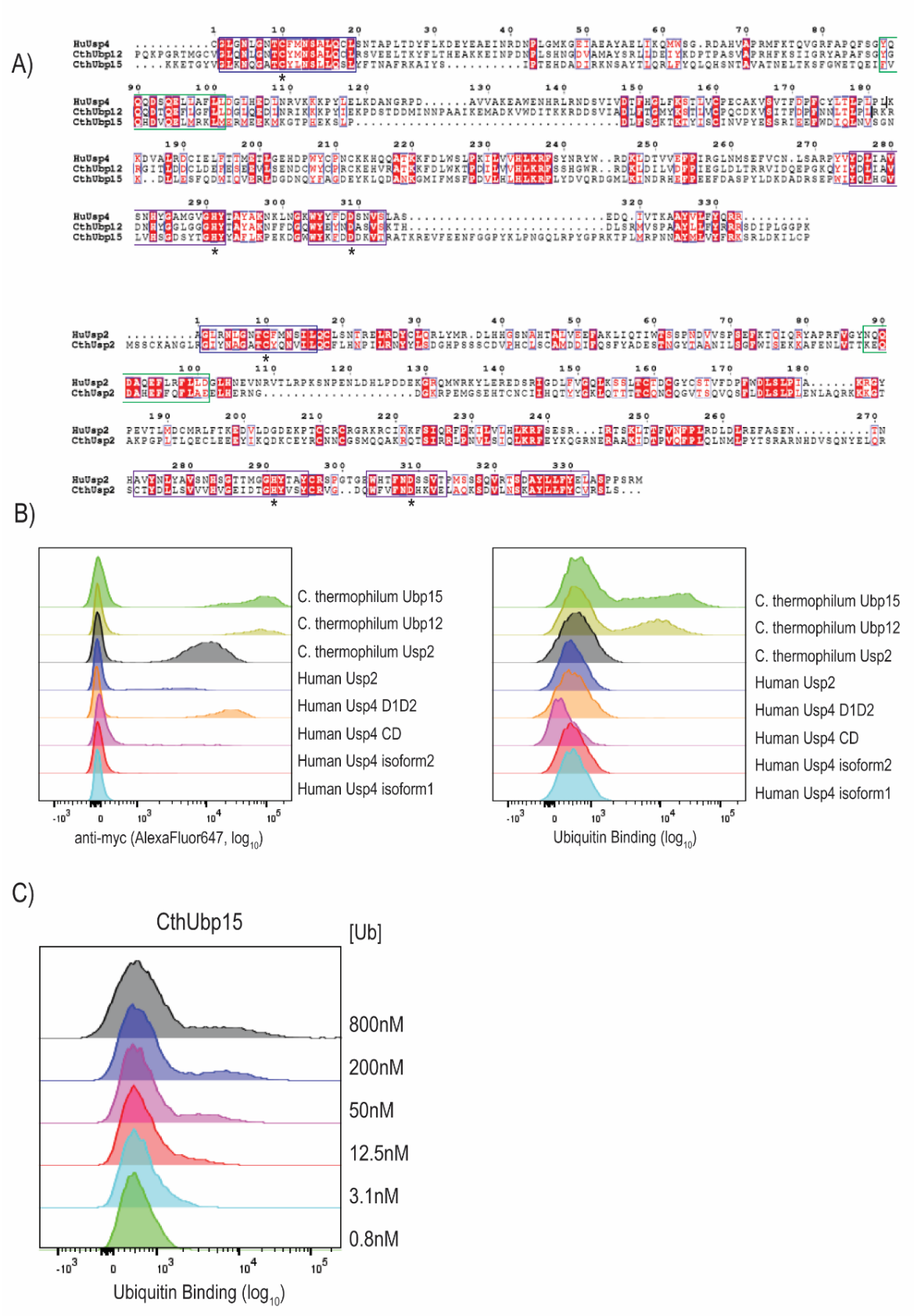
In the previous chapter, we showed that directed evolution can be used to further improve the efficiency of the Ulp1 pulldown approach. In order to develop a similar tool for purifying ubiquitin conjugates, our first objective was to identify a USP suitable for enzyme evolution. In addition to Usp2 and Usp4, we also sought to identify several thermostable ubiquitin proteases following similar logic underlying the discovery of CthUlp1. We performed two BLASTp searches through the genome of *C.thermophilum* using the catalytic domains of human Usp2 and Usp4 as query sequences (Figure 3.1A). Our search for Usp4 orthologs led to two candidates: the top scoring alignment, which we referred to as CthUbp12, showed 44% sequence identity whereas the second candidate, CthUbp15, showed 32% sequence identity. On the other hand, our search for Usp2 orthologs led to one candidate, CthUsp2, which showed 29% sequence identity. According to their respective sequence alignments, all three enzymes shared high sequence homology in regions that corresponding to the Cys, QDE and His boxes, thus confirming their identity as putative USPs.

To determine the best suited enzyme for ubiquitin substrate trap from our list of candidates, we expressed each of the USPs on yeast cell surfaces and compared their expression levels and ubiquitin binding. The detection scheme for ubiquitin binding and protein expression were the same as described in the previous section, only C-terminally biotinylated ubiquitin was used as ligand instead (See Figure 2.2 A and E). In addition to the catalytic domains of Usp2, CthUsp2, CthUbp12 and CthUbp15, 4 mutants of Usp4 reported in the structural study by Clerici et al were evaluated (D1D2, CD, Isoform1 and Isoform2) (Clerici et al., 2014). Usp4 CD

contains the entire C-terminal catalytic domain whereas the Ubl insert domain which divides the catalytic core is removed in Usp4 D1D2. Usp4 isoform1 represents the full length Usp4 whereas isoform 2 has the linker sequences which connects the N-terminal DUSP-Ubl domain to the catalytic domain shortened from ~70 residues to ~20 residues. Only Usp4 D1D2 and the three CthUSPs were expressed on yeast surfaces, with CthUbp12 and CthUbp15 exhibiting high expression level (Figure 3.1B, left panel). This observation is consistent with the notion that the thermostable enzymes generally possess better solubility, thus leading to higher expression levels. On the other hand, binding was only detectable in CthUbp12 and CthUbp15, with CthUbp15 showing the strongest binding signal (Figure 3.1B, right panel). Taken together, these observations indicated that CthUbp15 was the best starting enzyme for directed evolution.

To experimentally determine the ideal ubiquitin concentration for labeling cells displaying CthUbp15, we incubated the cells with various concentrations of ubiquitin and compared the binding via FACS. As seen in figure 3.1C, samples incubated with 800nM or 200nM showed minimal differences in binding, suggesting that CthUbp15 were saturated with ubiquitin starting at 200nM. However, ubiquitin binding virtually undetectable after 12.5nM. These observations suggest that 50nM of ubiquitin is the ideal ligand concentration that allows sensitive discrimination of binding improvements in our future directed evolution experiments.

Figure 3.1 CthUbp15 is a suitable candidate for the development of ubiquitin substrate trap via directed enzyme evolution. (A) Sequence alignments of human Usp2 and Usp4 with their putative orthologs identified in *C. thermophilum*. Vertical black lines in the Usp4 and CthUbp12 sequences denote the insert domains within their catalytic domains which were removed prior to alignment. The Cys, QDE and His boxes are indicated in blue, green and purple, respectively. (B) Yeast displaying each of the identified USP candidates were assayed for their protein expression level and their binding to ubiquitin by FACS. (C) Ubiquitin concentration best suited for labeling CthUbp15 cells were determined empirically. CthUbp15 cells were incubated with various concentration of ubiquitin before FACS analysis.


A)
HuUsp4
CthUbp12
CthUbp15
HuUsp2
CthUsp2
B)
C. thermophilum Ubp15
C. thermophilum Ubp12
C. thermophilum Usp2
Human Usp2
Human Usp4 D1D2
Human Usp4 CD
Human Usp4 isoform2
Human Usp4 isoform1
anti-myc (AlexaFluor647, log10)
Ubiquitin Binding (log10)
C)
CthUbp15
[Ub]
800nM
200nM
50nM
12.5nM
3.1nM
0.8nM
Ubiquitin Binding (log10)

3.3 Discussion

Due to the nature of the ubiquitin proteome, studies on ubiquitin requires the initial enrichment of endogenous ubiquitin conjugates. Yet, conventional methods used to detect the ubiquitinated proteins have several issues that limits the global characterization of the system. Recent successes in Ulp1 pulldown has presented the possibility of exploiting the natural affinity of USPs to capture ubiquitinated proteins. Because most USPs binds to ubiquitin with modest affinity, the USPs are unlikely to be readily applicable as a purification tool. Thus, we planned to further improve its binding affinity through directed enzyme evolution once the suitable USP candidate is identified. We found that CthUbp15, an ortholog of Usp4 in the thermophilic fungi, exhibits both high expression on yeast cell surface and strong ubiquitin binding. Interestingly, although prior studies have shown that the Usp4 D1D2 mutant binds to ubiquitin with nanomolar affinity, with a Kd of 0.6 nanomolar (Ward et al., 2018), we could not detect any binding from our FACS analysis. It is possible that due to its poor solubility, the surface displayed D1D2 protein is unstable and sensitive to its environment, suggested by its detectable expression and lack of binding. Whether this is indeed the case requires further investigation. Nevertheless, this does not exclude the possibility that Usp4D1D2 can be used as a ubiquitin substrate trap without the need of enzyme evolution.

From our observations in the previous chapter, one question remains to be answered: Does the C-terminal biotin tag on ubiquitin compromises its binding by our USP candidates? While we are currently in progress of addressing this question, structural data on the USPs suggests that interference is most likely minimal. The overall structure of USPs are known to possess remarkable conformational flexibility, allowing the enzyme to constantly switch between an unproductive conformation and a catalytically active state upon ubiquitin binding (Min Hu et

al., 2002). The realignment of the catalytic cleft can sometimes involve a distance change of more than 8 angstrom, as seen in the case of HAUSP, allowing the accommodation of large protein structures C-terminal to the ubiquitin diglycine. Furthermore, biochemical assays on the substrate specificity of Usp14 has revealed that the enzyme preferentially cleaves the distal ubiquitin of polyubiquitin chains, suggesting that additional factors within the N-terminus of ubiquitin also contributes to the enzyme's specificity(M. Hu et al., 2005). Consistent with this finding, the N-terminal residues on ubiquitin were found to form up to 12 direct hydrogen bonds with the Finger domain of the enzyme. Collectively, these characteristics of the USPs suggest that the binding observed in our FACS data are likely a true reflection of the affinity of the candidates.

One aspect regarding the ubiquitin system that remains mostly clear is how the various ubiquitin chain topologies encode for different outcomes, forming what is known as the ubiquitin code (Komander & Rape, 2012). Although substantial progress has been made in identifying the different participants in the maintenance of this code, such as the enzymes that synthesize and disassemble specific chains and the receptors/binding proteins that elicit specific cellular responses, many aspects remain unclear. For example, while it is known that most proteins modified by Lys48-linked ubiquitin chains are targeted for proteasomal degradation, the ubiquitinated Met4 is surprisingly long-lived (Kaiser, Flick, Wittenberg, & Reed, 2000). Met4 is a transcription factor that regulates methionine biosynthesis and is rapidly polyubiquitinated when the cell is saturated with methionine (Thomas & Surdin-Kerjan, 1997). However, rather than signaling for degradation by the 26S proteasome, Met4 ubiquitination inhibits its activity as transcriptional activator (Flick, Raasi, Zhang, Yen, & Kaiser, 2006). This finding strongly suggests that the topology of the ubiquitin chain is not the only determinant to its cellular

consequences. Furthermore, the substrates and functions of chains linked through Lys6, Lys27, Lys29 and Lys33 are poorly understood (Komander & Rape, 2012). In order to better understand the ubiquitin code, a tool that can isolate polyubiquitinated substrates of distinct linkage topologies is necessary. Besides from the USPs, other subfamilies of the DUBs include many linkage specific ubiquitin proteases, such as the JAMM proteases and the OTU proteases. The strategy presented in this chapter can be similarly applied to these linkage specific DUBs to develop a purification tool that can specifically capture polyubiquitinated substrates of a distinct linkage type.

Going forward, we are currently in progress of further evaluating CthUbp15's binding through biochemical assays. We have expressed and purified recombinant CthUbp15 which is needed for conjugating onto resin in order to test whether it can be used to purify ubiquitinated proteins from cell extracts. Although the development of the ubiquitin substrate trap is still in progress and its efficiency has yet to be determined, its creation can nevertheless revolutionize ubiquitin studies.

3.4 Methods

The protocols for yeast cell culturing and labeling for FACS analysis are the same as described in previous section, only that biotinylated ubiquitin was used instead of SUMO. The purification of Ubiquitin-Avitag are the same as described in the previous section.

3.4 Acknowledgements

Dr. Huilin Zhou designed the approach to use ubiquitin protease to purify ubiquitinated proteins and determined the candidate proteases to develop this approach.

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