# *IN VITRO* UBIQUITINATION OF AN ARRESTIN-RELATED TRAFFICKING ADAPTOR BY THE *SCHIZOSACCHAROMYCES POMBE* E3 LIGASE PUB1

A Thesis

Presented to the Faculty of the Graduate School

of Cornell University

in Partial Fulfillment of the Requirements for the Degree of

Master of Science

by

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August 2019

ABSTRACT

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The ubiquitination of proteins at the plasma membrane (PM) serves as an endocytosis signal in the downregulation of PM protein levels and cellular responses to their environment. In *Saccharomyces cerevisiae*, the E3 ubiquitin ligase Rsp5 ubiquitinates its PM targets by forming complexes with arrestin-related trafficking (ART) adaptor proteins that enable Rsp5 to recognize specific sets of PM proteins. The range of specificity for some of the ARTs has been uncovered in yeast models, but the molecular mechanisms by which the ART proteins present substrates to the Rsp5 catalytic domain for ubiquitination remains poorly understood. The ubiquitination of Art1 on lysine residue K486 by Rsp5 has been shown to play a critical role in the Art1-Rsp5 complex's function, which suggests that this adaptor ubiquitination likely plays a role in stabilization of the Art1-Rsp5 complex or in forming the correct complex structure to enable recognition of substrates. To better understand the mechanism by which Art1 presents PM substrates to Rsp5, and to uncover the functional significance of Art1 ubiquitination, our lab seeks to structurally characterize the Art1-Rsp5 complex. Here I report the purification and biochemical characterization of Pub1 and Any1, the homologous E3-adaptor pair from *S. pombe*, and an approach to generate the ubiquitinated form of Any1 *in vitro* for the purposes of structural studies and further biochemical investigation.

## BIOGRAPHICAL SKETCH

Paul Burrowes began his science education early in high school thanks to a week-long crash-course in genetics and molecular biology taught by Dr. Johnathan Whetstine. This experience seeded his interest in the biological sciences and lead him to pursue a science-focused college career. Paul entered Worcester Polytechnic Institute in 2013 as a major in biotechnology but switched to the biochemistry program in his second year to better suit his research interests. During summer terms, Paul worked in Johnathan Whetstine's lab at Massachusetts General Hospital's Cancer Center where he gained invaluable mentorship and hands-on experience with biological research. He conducted his senior research at WPI under Dr. Destin Heilman after having volunteered in the lab for two years, where he focused on the functions of apoptosis-inducing viral proteins and their intracellular trafficking behavior. Paul graduated from WPI with a bachelor's degree in biochemistry in the spring of 2017, and moved to Ithaca, NY to further study biochemistry and structural biology at Cornell University starting in the fall of 2017. Paul has spent one year in the Mao lab working in collaboration with the Scott Emr lab to biochemically and structurally characterize the yeast E3 ubiquitin ligase Rsp5 and its adaptor protein Art1 to develop a better understanding of how specificity in ubiquitination and degradation of membrane proteins is achieved and regulated.

ACKNOWLEDGEMENTS

Special thanks to Matthew Baile and Evan Guiney. Their previous work and invaluable feedback made this collaboration possible. I also thank my committee members—Dr. Yuxin Mao, Dr. Holger Sondermann, and Dr. Scott Emr—for their support and critical feedback throughout this project. Thank you to all Mao Lab and Hu Lab members for thoughtful discussions and questions during lab meetings. Lastly, thank you to Qing Zhang, who will joining the project and continuing this collaboration.

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# 1. BACKGROUND

The plasma membrane (PM) serves as a crucial barrier between the cytosol and the cell's environment. Proteins embedded in the PM can act as sensors for molecules in the environment, scaffolds for cellular movement, and channels for uptake or export of nutrients and signaling molecules, among other things. Cells regulate levels of PM proteins in part through the endocytic pathway, a process by which PM proteins are internalized as intracellular vesicles, which fuse with the endosome. The early endosome serves as a sorting hub; proteins destined for degradation will be delivered to the lysosome, while proteins to be recycled are trafficked from the endosome membrane back to the PM. Ubiquitination is a common endocytosis signal for membrane cargoes and is important in determining whether cargoes are recycled or sent to the lysosome.

In order to respond to changes in the extracellular environment and maintain cellular homeostasis, the selection membrane proteins to be endocytosed must be under exquisite regulatory control. This regulation is achieved in part by the attachment of ubiquitin to the PM proteins via the various classes of ubiquitin ligases. Ubiquitination of proteins is achieved through an enzymatic cascade that begins with the hydrolysis of ATP by an E1 ubiquitin activating enzyme to facilitate the formation of a thioester bond between a cysteine residue of the E1 and the carboxyl-terminus of Ubiquitin (E1~Ub). E1-linked ubiquitin can then be transferred to E2 ubiquitin conjugating enzymes through the formation of a new thioester-linkage (E2~Ub). The attachment of ubiquitin to a substrate protein is ultimately achieved by E3 ubiquitin ligases. RING-type E3's act as scaffolds that spatially coordinate an E2~Ub and a substrate protein to facilitate transfer of Ubiquitin directly from the E2 to the substrate lysine residue. In the case of the HECT-type E3's (homologous to E6AP C-terminus), the E3 plays a catalytic role and forms a covalent E3~Ub intermediate prior to ubiquitination of the substrate (Huang et al., 1999).

In yeast, the HECT-type E3 ubiquitin ligase Rsp5, the only member of the NEDD4-family of ligases in yeast, is currently the only known enzyme responsible for the ubiquitination of yeast PM

proteins. NEDD4-family proteins typically feature a C2 domain and 2-4 WW domains in their N-terminal tail, and all feature a C-terminal HECT catalytic domain. Rsp5 contains three WW domains that enable binding to PPXY motifs found in its substrates (Kanelis et al. 2001). Upon substrate binding and ubiquitin transfer from an E2, ubiquitination is catalyzed by the C-terminal HECT domain, which has been shown to prefer formation of K63-linked polyubiquitin chains (Kim and Huibregtse, 2009). Structural studies of the NEDD4-family E3's have revealed that the C-lobe of the HECT domain, which harbors the catalytic cysteine, can rotate to occupy different conformations. After the transfer of ubiquitin from E2 to E3, the C-lobe rotates to orient the thioester-linked ubiquitin towards substrates bound, in the case of Rsp5, to the WW3 domain (Kamadurai et al., 2009; Kamadurai et al., 2013). The Rsp5 HECT domain also contains a noncovalent ubiquitin binding site (or "exosite") in its N-lobe which has been shown to stimulate Rsp5 activity when occupied by free ubiquitin (Kim et al., 2011; Zhang et al., 2016). This exosite has been shown in crystal structures of the Itch E3 (a 4 WW-containing NEDD4-family E3) to be occupied by its WW2 domain and a helical linker, forming what is considered to be an autoinhibited state of the NEDD4 E3's (Zhu et al., 2017), though this has yet to be directly observed with Rsp5.

Rsp5's WW domains allow it to bind to PPXY-containing substrate proteins, but many Rsp5 PM substrates have been identified that do not contain PPXY motifs, and instead require a PPXY-containing adaptor protein to recruit Rsp5. In yeast, numerous soluble adaptor proteins for Rsp5 belonging to the α-arrestin family have been identified (Alvarez, 2008). The yeast α-arrestins allow Rsp5 to target PM proteins for ubiquitination, which facilitates their endocytic trafficking, so this family of proteins is referred to as arrestin-related trafficking adaptors, or ARTs (Lin et al. 2008). This family includes the Art proteins 1 through 10 (Lin et al., 2008; Nikko and Pelham, 2009), as well as Bul1,2, and 3 (Merhi and Andre, 2012). Arrestins are characterized by N- and C-terminal arrestin domains connected by a flexible hinge and are most commonly associated with the roles of β-arrestins in the signal dampening of phosphorylated GPCRs (Aubry and Klein, 2013). However, the yeast α-arrestins feature extended C-

terminal regions that harbor PY-motifs and enable binding to Rsp5. Each of the ARTs enables Rsp5 to target a specific set of PM cargoes (Lauwers et al., 2010). For example, Art1 enables Rsp5 targeting of the PM proteins Can1 and Mup1, among others, which transport arginine and methionine, respectively (Lin et al., 2008).

Given that the ARTs guide Rsp5 to specific target proteins, questions remain surrounding both the regulation of the ARTs themselves and the mechanism by which the ARTs present substrates to Rsp5 for ubiquitination. The activity of the Art1-Rsp5 complex depends on a Rsp5-catalyzed ubiquitination of lysine residue K486 on Art1. The Art1 K486R mutant cannot be ubiquitinated, resulting in defective Can1 receptor endocytosis (Lin et al., 2008). Despite the importance of this modification, the functional role that it plays in Art1-Rsp5 activity remains poorly understood. The functional dependency of the Art1-Rsp5 complex on adaptor ubiquitination suggests that this ubiquitination could play a role in stabilization of the complex or in forming the correct complex structure to enable recognition of substrates. Additionally, this ubiquitin-dependent activation is not exclusive to Art1. Both Art4 and Art8 have been shown to rely on activating ubiquitination (Becuwe et al., 2012; Hovsepian et al. 2017), and other ART proteins have been identified in screens as

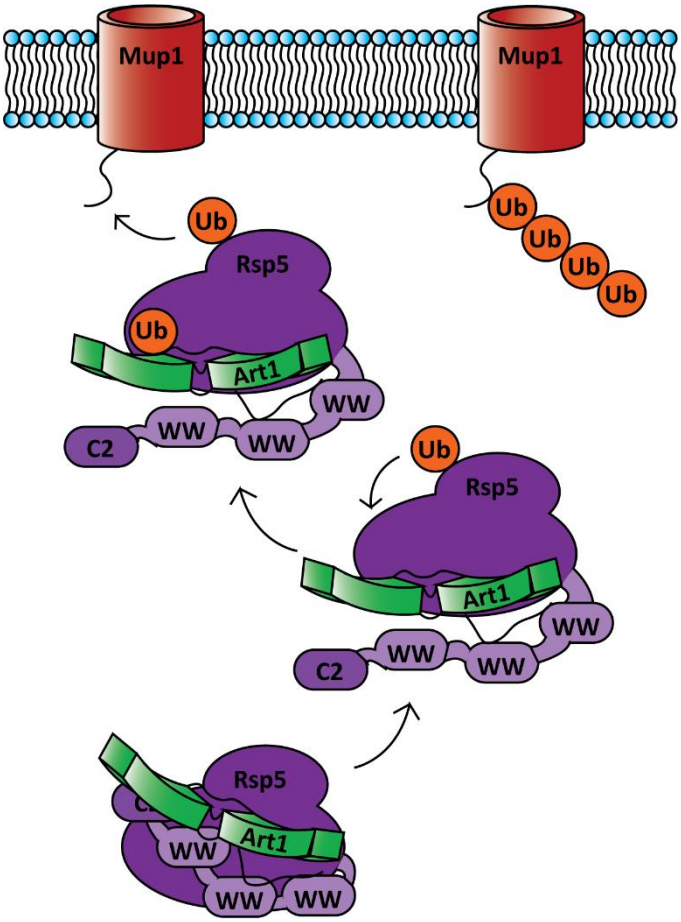



Figure 1: (Model) Rsp5 binds to PY motifs of Art1 via WW2 and WW3. Rsp5 catalyzes ubiquitination of Art1 K486, which results in the formation of fully-functional Rsp5-Art1 complex capable of ubiquitinating PM substrates.

Rsp5 substrates (Gupta et al. 2007), which suggests that adaptor ubiquitination could be part of the general ART-Rsp5 regulation mechanism. This raises the possibility that once an adaptor has been ubiquitinated by Rsp5, the complex may occupy a conformation in which the ART-Ub rests on the HECT-domain exosite and stimulates the catalytic activity of the HECT domain (Figure 1). Following the formation and subsequent activation of the Art1-Rsp5 complex, PM cargoes can be recognized and ubiquitinated. In the case of the methionine transporter Mup1, Art1-Rsp5 recognizes an acidic patch on the N-terminal tail of the protein near lysine residues 27 and 28, both of which are ubiquitinated (Guiney, Klecker, and Emr, 2016).

In *S. pombe*, the Pub1 E3 ligase and its adaptor Any1 exhibit analogous WW domain/PY-motif binding interactions as well as adaptor ubiquitination that plays a critical role in the complex's function. Any1 lysine residue K263 is ubiquitinated by Pub1, and this ubiquitination event was found to be critical for targeting of the Cat1 arginine transporter (Nakashima et al. 2014). The Pub1 protein bears roughly 75% sequence similarity to its homolog Rsp5, and features the same number of WW domains. Any1, however, bears less direct similarity to Art1. The entirety of the Any1 sequence can be computationally predicted to form an arrestin fold, while Art1 with over double the number of residues, 818, has been found to contain many poorly conserved "loop" regions which lie between the more highly conserved portions of the protein sequence that model as an arrestin (Baile et al., 2019). Despite the differences between these ARTs, their common features suggest that studies of ART-E3 complexes found in both organisms will improve the broader understanding of NEDD4-family E3's and their arrestin binding partners. To better understand the mechanism by which ARTs present PM substrates to Rsp5, and to uncover the functional significance of Art1 ubiquitination, our lab seeks to structurally characterize the Art1-Rsp5 complex. Here I report the purification and biochemical characterization of Pub1 and Any1, the homologous E3-adaptor pair from *S. pombe*, and an approach to generate the ubiquitinated form of Any1 *in vitro* for the purposes of structural studies and further biochemical investigation.

## 2. METHODS

**Protein Expression & Purification**

Rsp5, Pub1, and Any1 constructs were generated using overlap extension PCR and cloned into a pET28a vector containing an N-terminal 6xHis-SUMO tag. The resulting plasmids were transformed into Rosetta *E. coli* for expression of recombinant proteins. Rosetta cell cultures were grown to 0.6-0.8 OD600 and protein expression was induced by addition of 250µM IPTG. After addition of IPTG, Rosetta cultures were incubated at 18°C for 16-20 hours before being harvested for protein purification.

Cells were harvested from culture media by centrifugation at 4000x*g*. Cell pellets (from 1L of culture) were resuspended in 30mL of SEC-compatible buffer containing 20mM Tris-HCl, pH 7.5 and 150mM NaCl. Cells were lysed by sonication. Soluble lysates were separated from cell membranes and debris by centrifugation at 20,000x*g*, 4°C for 45 minutes. After separation, soluble lysates were incubated with ~4mL of $Co^{2+}$ chelated IDA-agarose bead suspension in SEC buffer (2mL packed bed) at 4°C for 1.5 hours, with gentle rotation. Lysate/bead mixtures were filtered by gravity column. Protein present in the filtrate was monitored using a mini-Bradford assay in 96-well dishes. To ensure purity of the His-SUMO proteins bound to the beads, the column was washed with SEC buffer until no proteins were detectable in the flow-through by mini-Bradford assay. After washing, the beads were suspended in a 4mL slurry with SEC buffer and were treated with recombinant His-Ulp1 to specifically cleave the His-SUMO tag of proteins bound to the beads. Ulp1 treatment was conducted either for 3 hours at room temperature (protein stability permitting) or overnight at 4°C. Proteins were eluted from the beads by washing with SEC buffer. Protein purity was assessed by SDS-PAGE and further purification was conducted using a HiLoad 16/600 Superdex 200 pg sizing column.

**Protein Binding Assay & Analytical SEC**

Use of a Superdex 200 Increase 10/300 GL column enabled the separation of small volumes of low-concentration protein samples. To screen pairs of recombinant proteins for stable protein-protein

interactions, solutions of each protein were made at 25µM in SEC buffer in addition to a solution containing both proteins, each at 25µM. The mixtures were made at a total volume of 250µl. Typically, these mixtures are incubated on ice until injection. However, the yeast proteins Pub1 and Any1 were incubated at 30°C for up to 15 minutes to promote their canonical binding. After mixing and incubation, these samples were individually loaded on the sizing column and collected into 500µl fractions. The approximate molecular weight of protein contents in any peaks shown on the chromatogram were confirmed by SDS-PAGE.

**Crystal Screening and Optimization**

Commercially available crystal screens were organized into 96 deep-well block trays. An ARI – Art Robbins Instruments Crystal Phoenix robot was used to mix screening conditions with protein. Screen conditions were dispensed into an IntelliPlate 96 screening tray as 100nl drops, which were mixed with 100nl of Any1 protein at a concentration 5mg/ml. Protein complexes were mixed such that both Rsp5 and Any1 were equimolar and resulted in a final complex concentration of ~8mg/mL. The addition of ubiquitin to complex mixtures was conducted adding 2-6 molar equivalents of ubiquitin to the protein mixture. Reservoirs for each condition were filled with 100ul of each screen. Plates were sealed and drops were monitored for crystal formation every 24 hours after initial seeding, and every 48 hours after the first week.

Optimization of Any1 crystals was conducted in 1mL reservoir hanging-drop plates. To form drops for crystallization, 1µl of Any1 protein was mixed 1:1 with the well condition and dispensed onto glass coverslips. The coverslip was inverted and sealed to the reservoir using vacuum grease. Drops were monitored for crystal formation every 24 hours after seeding, and every 48 hours after the first week.

***In Vitro* Ubiquitination of Any1**

Ubiquitination of Any1 was conducted using purified recombinant proteins to reconstitute a ubiquitination cascade *in vitro*. Human UBA1 and UbcH5c were purified to serve as the E1 and E2

enzymes, respectively. Reactions were conducted in which either Rsp5 or Pub1 was used as the E3. Reaction mixtures (adapted from Luo et al. 2015) were made using 100nM E1, 250nM E2, 500nM E3, and 30µM Any1 in a reaction buffer containing 20mM Tris (pH 7.0) 5mM $MgCl_2$, 50mM creatine phosphate, 3 U/mL of pyrophosphatase, 3 U/mL of creatine phosphokinase, and 2.8mM ATP . The ubiquitination reaction was catalyzed by the addition of 30µM Ubiquitin and incubated for 30 minutes at room temperature. Total ubiquitination was assessed using Coomassie-stained SDS-PAGE.

**Experimental Nickel Affinity separation of Any1-Ub From *In Vitro* Ubiquitination Reaction**

Any1 was reacted with His-TEV-Ubiquitin K0 (all lysines mutated to arginine) in 20mM Tris pH 7.0, 150mM NaCl mixed with the same concentrations of enzymes and reagents described above. The reaction was initiated by the addition of Ubiquitin-K0. After reacting for 30 minutes, the entire reaction mixture was loaded by FPLC onto a HisTrap FF 1mL nickel column. Proteins were eluted from the column using an imidazole gradient of 0-500mM over 20 column volumes. Fraction contents were assessed using SDS-PAGE and Coomassie staining.

**Experimental Cation Exchange of Any1-Ub From *In Vitro* Ubiquitination Reaction**

Any1 was reacted with His-TEV-Ubiquitin K0 in 50mM HEPES pH 7.0, 50mM NaCl mixed with the same concentrations of enzymes and reagents described above. The reaction was initiated by the addition of Ubiquitin-K0. After reacting for 30 minutes, the entire reaction mixture was loaded by FPLC onto a cation exchange (CIEX) HiTrap SP HP column (1mL). Proteins were eluted from the column using a salt gradient of 50-500mM NaCl over 20 column volumes. Fraction contents were assessed using SDS-PAGE and Coomassie staining.

# 3. RESULTS

## 3.1 Purification of Rsp5 and the *S. pombe* ART Any1

To facilitate our study of Art1's ubiquitination by Rsp5, I optimized an approach to conduct *in vitro* ubiquitination using catalytically active recombinant proteins. N-terminal truncations were made to Rsp5 to facilitate the expression of stable protein in *E. coli* while retaining the WW2 and WW3 domains which have been shown to be critical for specific interactions between Rsp5 and Art1 and are required for ubiquitination of Art1 (Lin et al., 2008). Rsp5 217-809 is a deletion of the N-terminal C2 domain, and Rsp5 331-809 features the additional deletion of the first WW domain (Fig.2A). These truncations of Rsp5 were fused to 6xHis-SUMO and expressed in *E. coli* for the purpose of protein purification. Both constructs were successfully purified and exhibited yields of approximately 10 milligrams of protein per liter of culture (Fig.2B,C).

Efforts to purify Art1 were unsuccessful, likely due to poor stability of Art1 during expression in *E. coli*. However, the Art1 homolog found in *S. pombe*, Any1, was found to be amenable to our protein purification methods. Any1 17-361 fused to 6xHis-Sumo was successfully purified to concentrations above 5mg/ml (Fig.3A). This was achieved with the addition of 5mM DTT to purification buffers, after elution of the protein from $Co^{2+}$beads, to partially prevent the aggregation of Any1 during the concentration process. Rsp5 WW1-C and WW2-C were both screened for complex formation with purified Any1 using size exclusion chromatography (Fig3.B,C). Both Rsp5 constructs were shown to stoichiometrically bind to Any1 when incubated in equimolar quantities at 30°C prior to separation on the sizing column, which indicates that a stable complex between Rsp5 and Any1 can form that does not require ubiquitination of Any1. Given that the complex of Rsp5 WW2-C and Any1 appeared to be more stable than that with Rsp5 WW1-C, I selected Rsp5 WW2-C as a candidate for co-crystallization with Any1.

**3.2 Crystallization of Any1**

Based on alignments of Any1 structural predictions to known protein structures using HHpred (Zimmermann at al. 2018), Any1 is predicted to contain arrestin folds similar to many structurally characterized visual or β-arrestin proteins (Fig.4A). Purified Any1 17-361 was screened for crystallization using a panel of 8 commercially available 96-condition screens in the presence or absence of equimolar Rsp5 WW2-C. Crystallization was observed for free Any1 protein (Fig.4B), and optimization of crystallization conditions resulted in the formation of bundled sheets of many Any1 crystals (Fig. 4B). Any1 failed to crystallize in the presence of Rsp5 WW2-C. Free ubiquitin was also added in an attempt to aid in the formation of a stable complex, as a previous Rsp5 structure was solved with ubiquitin occupying the HECT domain exosite (Kim et al., 2011), but this also did not promote the crystallization of Any1-Rsp5 complex in our screening panel.

**3.3 Rsp5 Ubiquitinates Any1 *In Vitro***

In the interest of generating ubiquitinated Any1 for further crystallographic and biochemical studies, I tested the ability of purified Rsp5 WW2-C to conjugate ubiquitin to Any1 *in vitro*. Any1 was incubated with UBA1 (human E1), UbcH5c (human E2), and Rsp5 WW2-C in buffer containing ATP. Reactions were initiated by the addition of ubiquitin. The ubiquitination of Any1 by Rsp5 WW2-C was monitored for 30 minutes after the addition of ubiquitin, and I found that Rsp5 (500nM) appears to convert less than 50 percent of the available Any1 (30μM) to a ubiquitinated form (Fig.5). The formation of poly-Ub chains can be observed throughout the reaction, the longest of which become apparent after 10 minutes of reaction. This indicates that Rsp5 favors poly-Ub chain extension over the initial ubiquitination of Any1, which appears to be consistent with prior biochemical studies of Rsp5. To prevent the formation of poly-Ub chains, mutations were made to ubiquitin such that each lysine residue was mutated to arginine (Ub-K0), Reactions conducted with Ub-K0 exhibited a dramatic increase in Rsp5's utilization of the Any1 pool, coupled with an increased yield of monoubiquitinated Any1

(Fig.5). As expected, bands indicative of polyubiquitination were lost, but Rsp5 was still able to attach ubiquitin to numerous lysine residues in Any1, rather than the single K263 residue which has been previously identified as the biological target for ubiquitination by Pub1.

**3.4 Purification of the *S. pombe* HECT E3 Pub1, and *In Vitro* ubiquitination of Any1**

To test the performance of Any1 ubiquitination using its true biological E3, Pub1, two truncations of Pub1, 201-767 and 287-767, were fused to 6xHis-SUMO and purified (Fig.6A). Pub1 WW1-C (201-767) is a C2 domain deletion (analogous to Rsp5 217-809), and Pub1 WW2-C (287-767) is a further deletion of the WW1 domain (analogous to Rsp5 331-809). Both constructs were successfully purified to final concentrations >12mg/ml (Fig.6B,C). As with Rsp5, the Pub1 constructs were examined for their ability to bind to Any1 using size exclusion chromatography. Both constructs of Pub1 were each mixed with Any1 in equimolar ratios and incubated at 30°C before separation by SEC. Pub1 WW1-C and Pub1 WW2-C both exhibit a shifted elution volume and co-elution with Any1, indicating that both are able to form a binary complex with Any1 in the absence of Any1 ubiquitination (Fig.7A,B). In similar fashion as with Rsp5, the complex between Any1 and Pub1 WW2-C appears to be more stable than that with Pub1 WW1-C. Both Pub1 constructs were tested for ubiquitination of Any1 in the presence of either WT-Ub (Fig.8A) or Ub-K0 (Fig.8B). When conjugating WT-Ub, both Pub1 constructs exhibit lower polyubiquitination activity than Rsp5, but they still fail to ubiquitinate most of the Any1 pool (Fig.8A). In the presence of Ub-K0, however, monoubiquitinated Any1 is formed as the most abundant product, followed by Any1-Ub(2) (Fig.8B). The attachment of additional ubiquitin beyond the first indicates that Pub1 is still able to catalyze ubiquitination of Any1 lysine residues other than K263 *in vitro*, despite no other sites having been previously identified or associated with a biological function.

**3.5 Experimental Purification of Ubiquitinated Any1**

I attempted to purify monoubiquitinated Any1 by using affinity chromatography and ion exchange approaches to discriminate between the properties un-reacted Any1 and its various

ubiquitinated forms. By linking a poly-histidine tag to the N-terminus of Ub-K0, nickel affinity chromatography can be used to separate the ubiquitinated forms of Any1 from the unreacted form and other reaction components (Fig.9A). As expected, this method enables the removal of the unreacted Any1, but it fails to separate monoubiquitinated Any1 from those with two or more ubiquitin attached. Use of the nickel column separates the ubiquitinated products from the non-ubiquitinated reactants, but the reaction still generates multiple products which require further separation. In an attempt to separate the ubiquitinated forms of Any1 from each other and enable the specific isolation of monoubiquitinated Any1, I conducted Cation Exchange chromatography (CIEX) of the Any1/Ub-reactions (Fig.9B). I found that the Any1 species in the reaction are weakly separated by the salt gradient, but not to a degree suitable for the isolation of pure, monoubiquitinated Any1. CIEX separations of these reactions conducted in a pH range of 6.0 to 7.0 result in Any1-Ub co-eluting with trace amounts of di-ubiquitin Any1. While this may be due to the similar properties of these species, it is possible that the weak Any1-Any1 interactions observed in nickel affinity separations contribute to the co-elution of these proteins and render them difficult to separate in these conditions.

## 4. DISCUSSION

Ubiquitination of ART-family proteins by Rsp5 is important for the biological function of Rsp5-Art complexes. Here I have reported the successful purification of Pub1 and Any1 using *E. coli*, as well as *in vitro* ubiquitination of Any1 by Pub1. Both proteins are amenable to straightforward purification methods and produce yields suitable for crystallographic studies. Free Any1 was successfully crystallized, and conditions for crystallization are being optimized for the purpose of structural determination. At the time of writing, this would represent the second α-arrestin structure following TXNIP (Hwang et al., 2014), and the first structure of an ART-family arrestin. Efforts to co-crystallize Any1 and Pub1 as a binary complex have proven unsuccessful despite the apparent stability of their binding (Fig. 7). A broader panel of crystallization conditions, in conjunction with cryoEM methods, may yet reveal

structural information about the ART-E3 complexes' conformational states prior to adaptor ubiquitination. CryoEM in particular is a promising avenue due to the small quantities of protein needed relative to crystallography, and the ability to image the ART-E3 complexes in multiple conformational states, if present. For this particular application, EM presents a distinct advantage over crystallography, which depends on stable interactions between protein complexes occupying identical conformations in order to promote both crystal formation and structural determination.

Towards the larger goal of uncovering the role of Any1 ubiquitination in the function of the Pub1-Any1 complex, I have developed an *in vitro* reaction system to conjugate 6xHis-Ubiquitin to Any1 using Pub1 as the E3 ligase. In its current state, the reaction produces linkages between Any1 and Ub at several lysine residues, rather than the discrete monoubiquitination of Any1 K263 observed *in vivo*, resulting in multiple ubiquitin attachments to individual Any1 proteins. Given that *in vivo* ubiquitination of Art1 and Any1 is only observed on a single lysine residue, it is likely that these further ubiquitination events are artifacts of the *in vitro* system that either are too unfavorable to occur prominently *in vivo* or are regulated against by yeast deubiquitinases (Kee, Lyon, and Huibregtse, 2005). Discrete ubiquitination of Any1 K263 may be achievable by mutating the off-target sites observed *in vitro*. The reactions generate sufficient quantities of ubiquitinated products for mass spectrometry analysis, so empirical determination of the ubiquitinated lysine residues in Any1 is both feasible and a likely route to Any1 mutants that retain their overall stability while blocking off-target ubiquitination.

My attempts to specifically isolate the monoubiquitinated form of Any1 generated by *in vitro* ubiquitination with 6xHis-Ub resulted in poor separation of the proteins, if at all. Nickel affinity chromatography provides a one-step removal of any unreacted Any1, but the effective separation of Any1-Ub from Any1-Ub(2) remains a technical challenge. The observation that free Any1 appears to bind to nickel columns until the addition of low imidazole suggests that Any1 may be exhibiting weak binding to the ubiquitinated Any1 species bound to the column. This may also explain the propensity of Any1-Ub

and Any1-Ub(2) to co-elute when conducting size exclusion- or ion exchange-based methods. Separation of Any1-Ub from free Any1 by nickel column still serves as a promising approach for isolating protein in quantities sufficient for crystallographic studies. Future experiments of this type may be able to implement Any1 mutants featuring K/R mutations, such that K263 is the only remaining lysine site for ubiquitination, to specifically generate large quantities of Any1 K263-Ub while removing the potential for promiscuous ubiquitination of off-target sites. Additionally, mass spectrometry may be used to determine which lysine residues in Any1 are being ubiquitinated by Pub1 *in vitro*, and could provide a list of specific residues to mutate. This approach, rather than the complete K/R mutagenesis of the full protein, is more likely to yield a stably expressed protein for structural studies.

Reconstitution of the ubiquitination cascade in *E. coli* has been previously reported as a method for more faithfully recapitulating ubiquitination of various proteins than canonical *in vitro* methods (Keren-Kaplan et al., 2012), and may render isolation of monoubiquitinated Art1 or Any1 possible. Simultaneous expression of compatible E1, E2, and Pub1 in addition to Ubiquitin and Any1 in a single strain of *E. coli* could enable both the synthesis and isolation of a monoubiquitinated Any1 population. Additionally, this method may serve as a means of stabilizing ARTs during expression via stable complex formation with the E3, and could enable biochemical studies of other ARTs, including Art1, that have proven so far to be poor candidates for single protein overexpression.

5. FIGURES

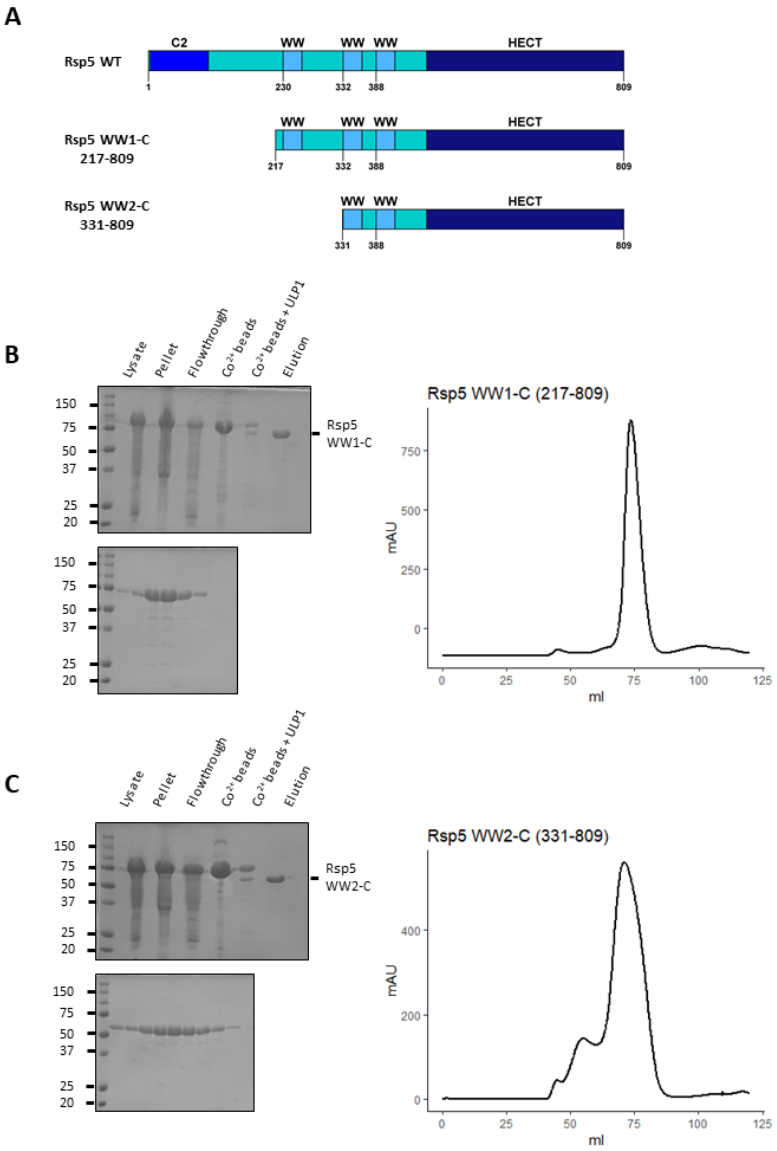



Figure 2: Generation and purification of Rsp5 truncations. (A) Domain map of full-length Rsp5 from *S. cerevisiae*. Truncations were made to the N-terminal region of the protein to generate Rsp5 WW1-C (217-809) and Rsp5 WW2-C (331-809). Both truncations were cloned into a pET28a vector containing an N-terminal His-SUMO tag for expression and purification of protein from *E. coli.* Purification of (B) Rsp5 WW1-C and (C) WW2-C was conducted using Cobalt affinity chromatography followed by removal of the His-SUMO tag by His-ULP1 (Top Left). Proteins recovered from the column were subjected to size exclusion chromatography (Right) and fractions were analyzed by SDS-PAGE (Bottom Left).

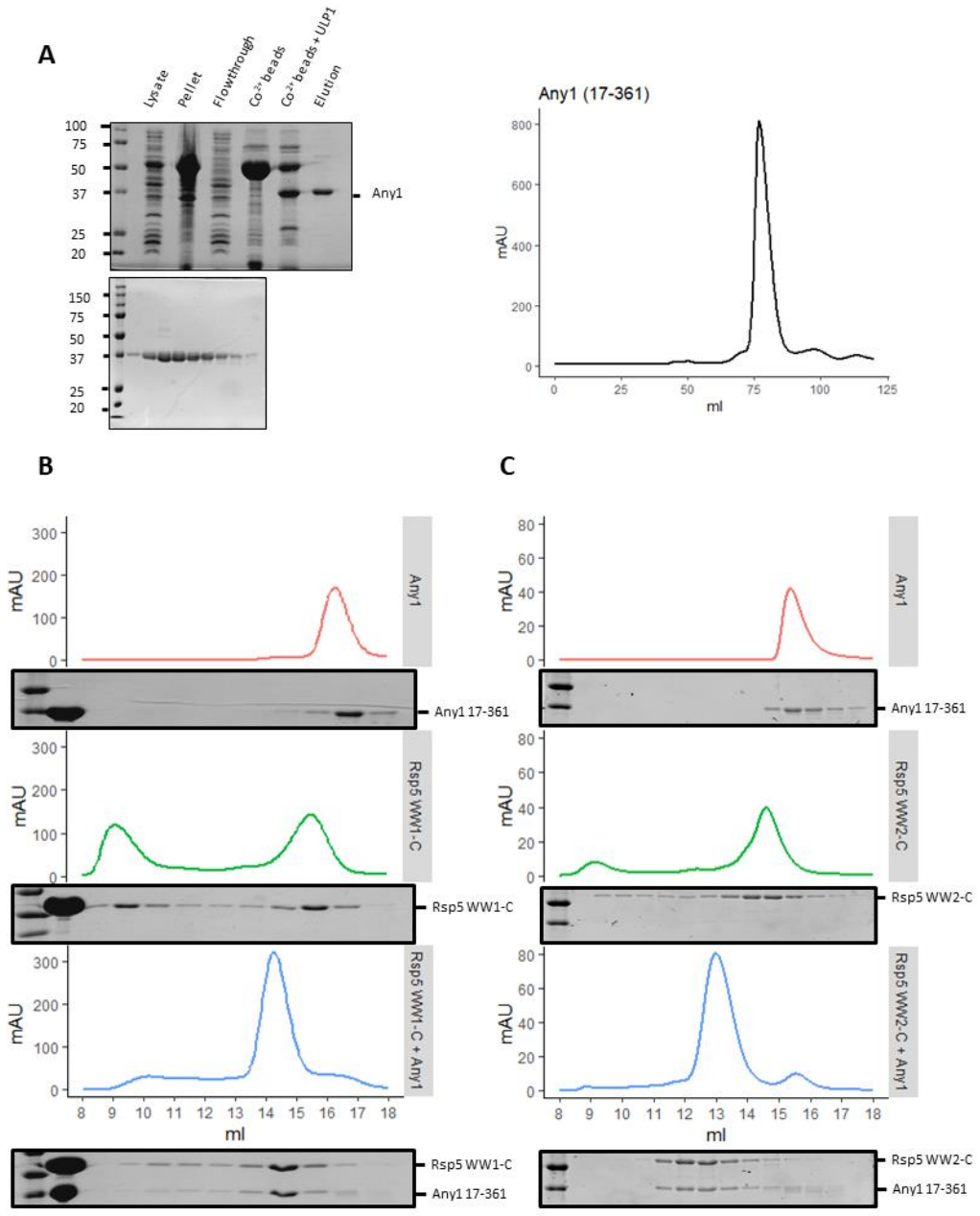
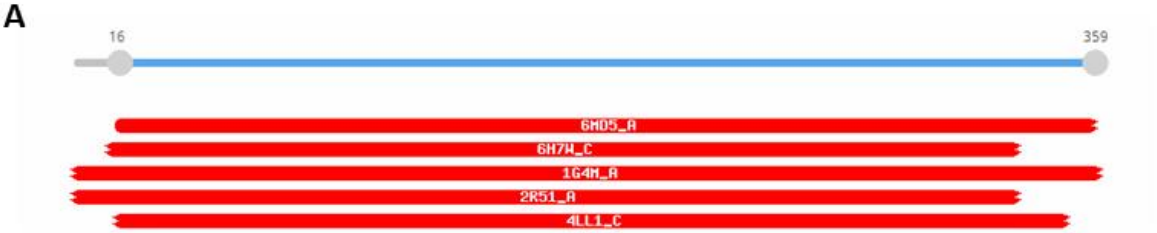



Figure 3: Purification of the *S. pombe* α-arrestin Any1. (A) Any1 17-361 was cloned into a pET28a vector containing an N-terminal His-SUMO tag and transformed into Rosetta *E. coli*. Expressed protein was purified using Cobalt affinity chromatography followed by cleavage of the His-SUMO tag by His-ULP1 (Top Left). Proteins recovered from the column were subjected to size exclusion chromatography (Right), and the protein contents of the fractions were analyzed by SDS-PAGE (Bottom Left). Any1 and Rsp5 (B) WW1-C or (C) WW2-C were mixed at a concentration of 50µM. A volume of 250µL was loaded onto a Superdex 200 Increase 10/300 GL sizing column. Complex formation between Rsp5 and Any1 was assessed by SDS-PAGE after SEC separation.

A




| Nr | Hit | Name | Probability | E-value | SS | Cols | Target Length |
|---|---|---|---|---|---|---|---|
| 1 | 6MD5_A | Vacuolar protein sorting-associated protein 26A; retromer, vps26, endoscope, ENDOCYTOSIS; 1.7A {Danio rerio} | 99.95 | 5.4e-28 | 38.3 | 291 | 319 |
| 2 | 6H7W_C | Vacuolar protein sorting-associated protein 26-like; retromer, endosome, sorting nexin, BAR; 11.4A {Chaetomium thermophilum (strain DSM 1495 / CBS 144.50 / IMI 039719)} | 99.95 | 2e-28 | 34.9 | 267 | 292 |
| 3 | 1G4M_A | BETA-ARRESTIN 1; Sensory transduction, alternative splicing, SIGNALING; 1.9A {Bos taurus} SCOP: b.1.18.11 | 99.95 | 1.2e-27 | 34.9 | 315 | 393 |
| 4 | 2R51_A | Vacuolar protein sorting-associated protein 26B; retromer, fibronectin III, arrestin domain; HET: MSE; 2.1A {Mus musculus} | 99.94 | 5.2e-27 | 36.5 | 278 | 340 |
| 5 | 4LL1_C | Thioredoxin-interacting protein; Arrestin-like domain, ANTITUMOR PROTEIN-PROTEIN BINDING; 2.0A {Homo sapiens} | 99.94 | 6.1e-27 | 35.6 | 286 | 315 |

B

Figure 4: Any1 structural prediction and crystallization. (A) Sample of HHpred results from alignment of Any1 amino acid sequence to PDB protein structures. (B) Any1 crystals were found in Hampton Screen condition 36: 0.1 TRIS hydrochloride pH 8.5, 8% w/v Polyethylene glycol 8000 (Right). The formation of larger, bunched crystals was observed after further screening in 33mM Ammonium Sulfate, Tris hydrochloride pH 7.9, 3.5% PEG 8000 (Middle), and in 50mM Ammonium Sulfate, Tris hydrochloride pH 7.7, 3% PEG 8000 (Right).

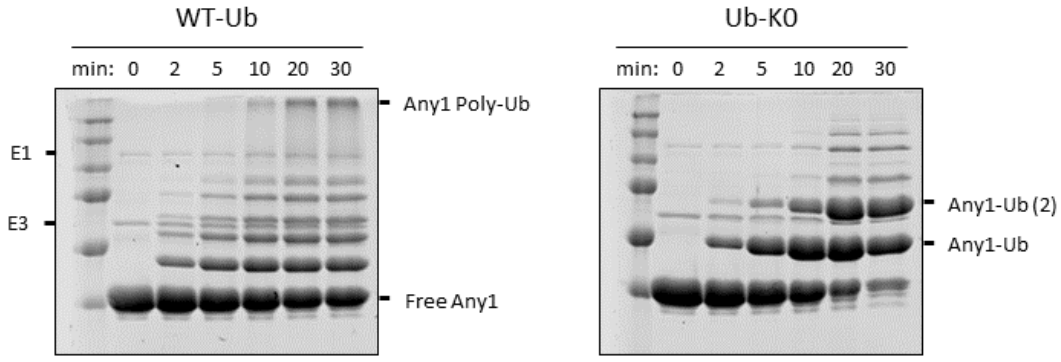



Figure 5: *In vitro* ubiquitination of Any1 by Rsp5 WW2-C. Reactions mixed as described in Methods were initiated by addition of either Wild-type Ubiquitin (Left) or Ub-K0 (Right). Samples were isolated from the pooled reaction at time points from 2-30 minutes and denatured by addition of SDS-PAGE loading buffer.

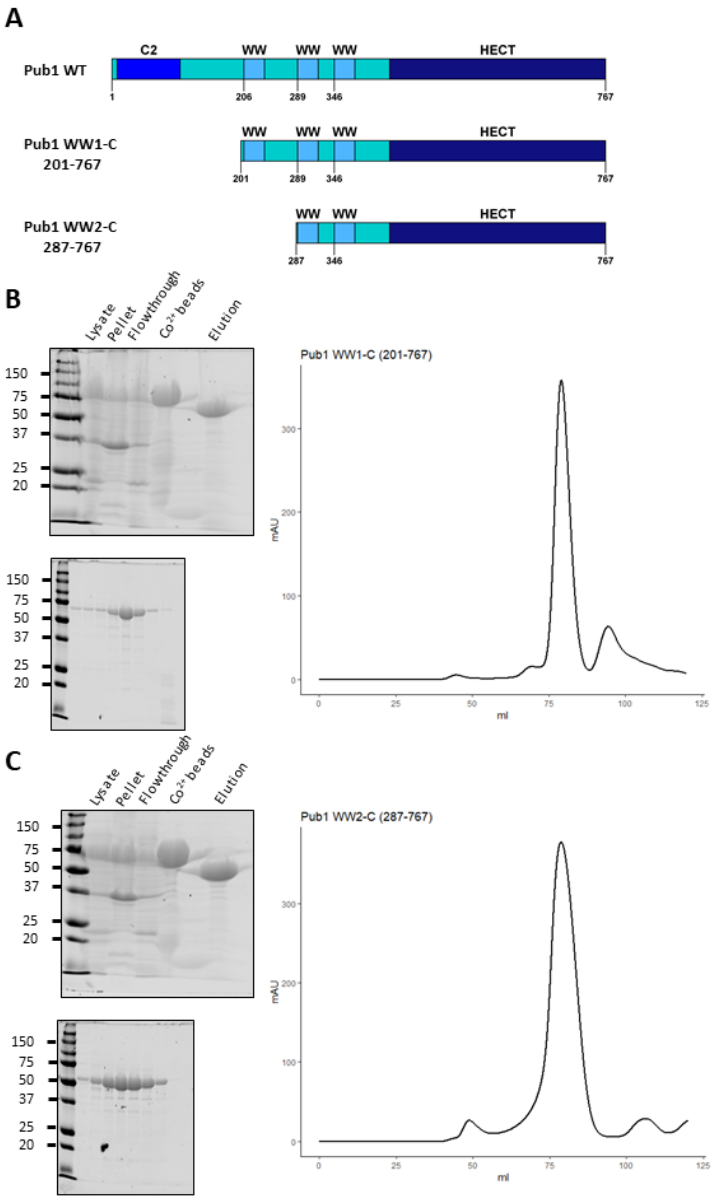



Figure 6: Generation and purification of Pub1 truncations. (A) Domain map of full-length Pub1 from *S. pombe*. Truncations were made to the N-terminal region of the protein to generate Pub1 WW1-C (201-767) and Pub1 WW2-C (287-767). Both truncations were cloned into a pET28a vector containing an N-terminal His-SUMO tag for expression and purification of protein from *E. coli.* Purification of (B) Pub1 WW1-C and (C) WW2-C was conducted using Cobalt affinity chromatography followed by removal of the His-SUMO tag by His-ULP1 (Top Left). Proteins recovered from the column were subjected to size exclusion chromatography (Right) and fractions were analyzed by SDS-PAGE (Bottom Left).

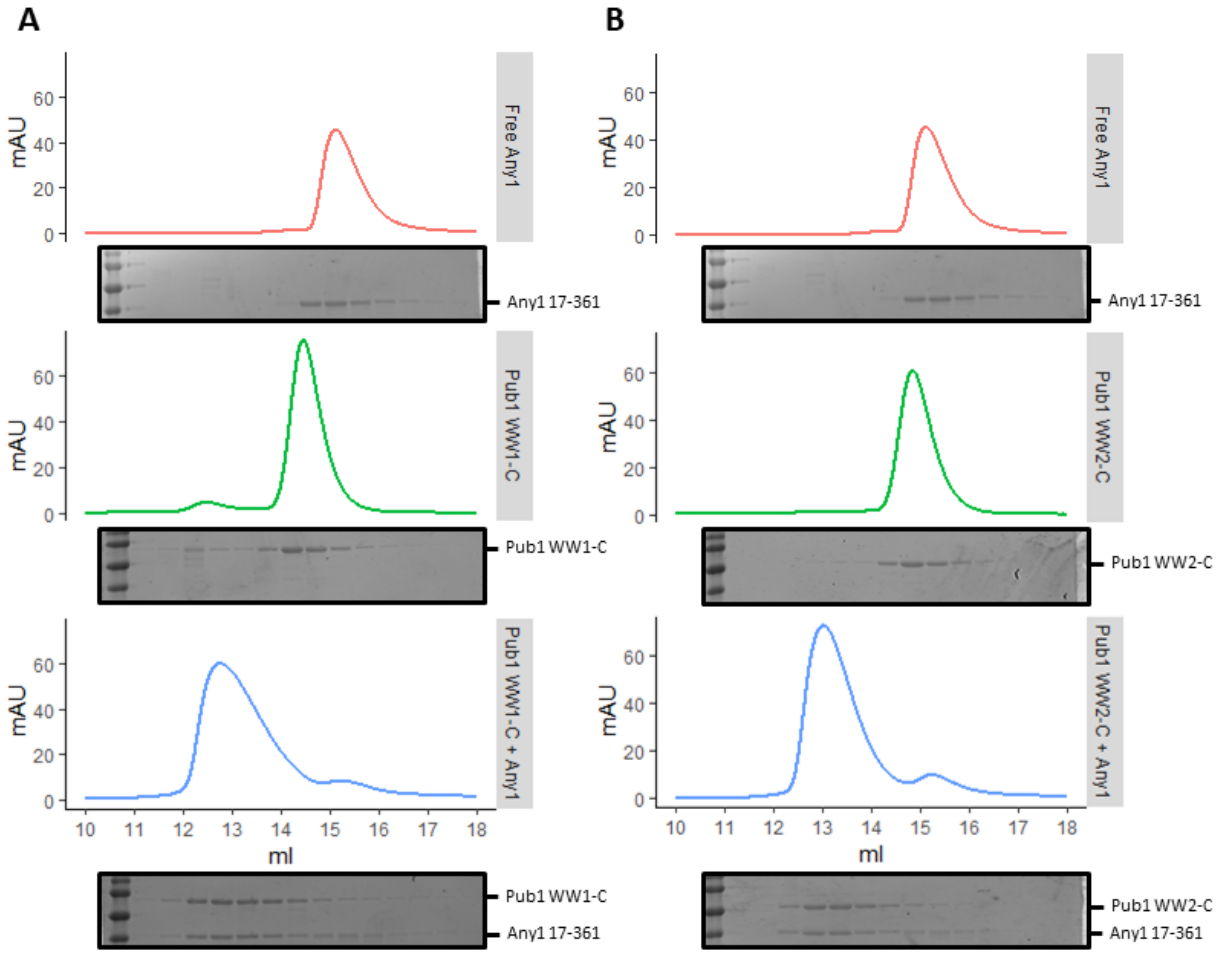



Figure 7: Complex formation between purified Pub1 and Any1 17-361. Any1 and Pub1 (A) WW1-C or (B) WW2-C were mixed at a concentration of 50µM. A volume of 250µL was loaded onto a Superdex 200 Increase 10/300 GL sizing column. Complex formation between Rsp5 and Any1 was assessed by SDS-PAGE after SEC separation.

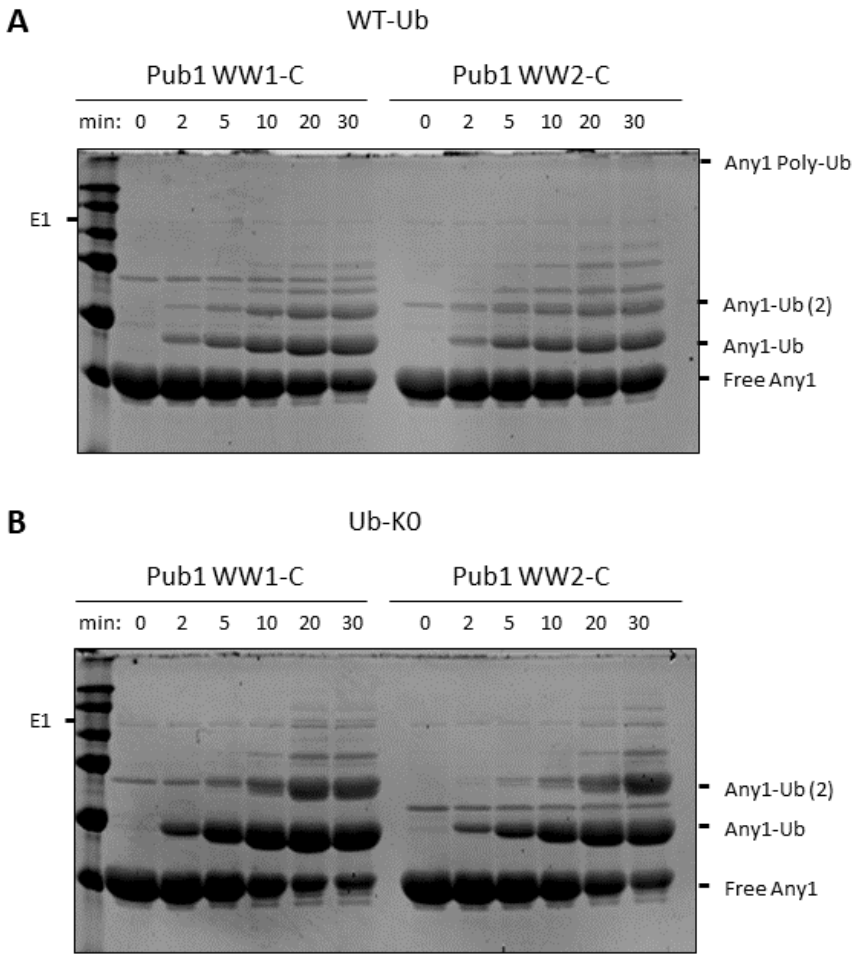



Figure 8: *In vitro* ubiquitination of Any1 by Pub1. Reactions mixed as described in Methods were initiated by addition of either Wild-type Ubiquitin (A) or Ub-K0 (B). Samples were isolated from the pooled reaction at time points from 2-30 minutes and denatured by addition of SDS-PAGE loading buffer. Both Pub1 WW1-C (Left) and WW2-C (Right) were screened for their catalytic capabilities.

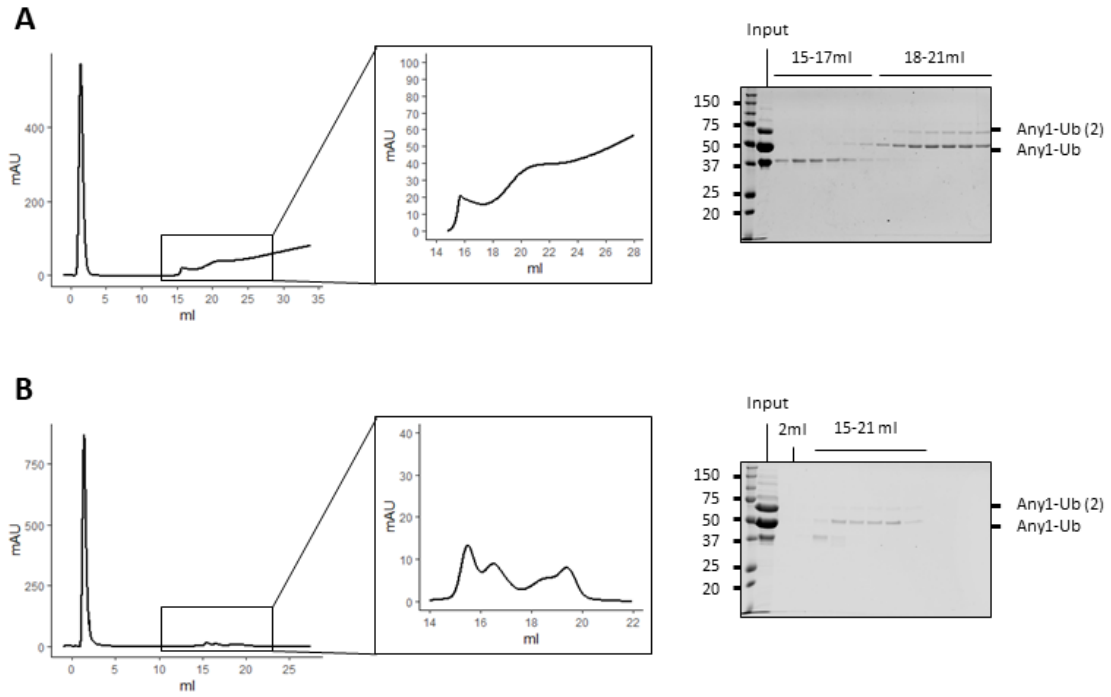



Figure 9: Experimental purification of Any1 ubiquitination reaction products. Ubiquitination of Any1 by Pub1 was initiated by the addition of 6xHis-Ub-K0. Reactions proceeded at room temperature for 30 minutes before loading the entire reaction onto columns. (A) Nickel affinity chromatography was conducted by passing ubiquitination reaction contents through a HisTrap FF 1mL nickel column. Proteins were recovered from the column by gradient elution ranging from 20mM-500mM imidazole over 20 column volumes (Left, Center). Fraction contents (taken from expanded regions of the UV plot) were analyzed by SDS-PAGE (Right). (B) Cation exchange chromatography (CIEX) was conducted by passing ubiquitination reaction contents buffered in 50mM HEPES, pH 7.0, through a HiTrap SP HP 1mL column. Proteins were recovered from the column by gradient elution ranging from 50-500mM NaCl over 20 column volumes.

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