U.PORTO
INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

U.PORTO
FMUP FACULDADE DE MEDICINA
UNIVERSIDADE DO PORTO

U.PORTO
FC FACULDADE DE CIÊNCIAS
UNIVERSIDADE DO PORTO

Cuz1, a novel modulator of Cdc48 function in the ubiquitin-proteasome system

Maria Bebiana Vigário da Silva Sá Moura

Tese de doutoramento em Biologia Básica e Aplicada

2014

Maria Bebiana Vigário da Silva Sá Moura

Cuz1, a novel modulator of Cdc48 function in the ubiquitin-proteasome system

Tese de Candidatura ao grau de Doutor em Biologia Básica e Aplicada submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Orientador – Mark Hochstrasser

Categoria – Eugene Higgins Professor

Afiliação – Yale University

Co-orientador – João Henrique Morais-Cabral

Categoria – Professor

Afiliação – Instituto de Biologia Molecular e Celular- Universidade do Porto

“You cannot swim for new horizons until you have courage to lose sight of the shore.”

William Faulkner

ABSTRACT

Regulated protein degradation mediated by the ubiquitin-proteasome system (UPS) is critical to eukaryotic protein homeostasis. Often vital to degradation of protein substrates is their disassembly, unfolding, or extraction from membranes. These processes are catalyzed by the conserved AAA-ATPase Cdc48 (also known as p97). Here, we characterize the Cuz1 (Cdc48-associated UBL/zinc-finger-1) protein, encoded by a previously uncharacterized arsenite-inducible gene in budding yeast. Cuz1, like its human ortholog ZFAND1, has both an AN1-type zinc finger (Zf_AN1) and a divergent ubiquitin-like domain (UBL). We show that Cuz1 modulates Cdc48 function in the UPS. The two proteins directly interact, and the Cuz1 UBL, but not Zf_AN1, is necessary for binding to the Cdc48 N-terminal domain. Cuz1 also associates, albeit more weakly, with the proteasome, and the UBL is dispensable for this interaction. Cuz1-proteasome interaction is strongly enhanced by exposure of cells to the environmental toxin arsenite, and in a proteasome mutant, loss of Cuz1 enhances arsenite sensitivity. Whereas loss of Cuz1 alone causes only minor UPS degradation defects, its combination with mutations in the $Cdc48^{Npl4\text{-}Ufd1}$ complex leads to substantial further impairment. Cuz1 helps limit the accumulation of ubiquitin conjugates on both the proteasome and Cdc48, suggesting a possible role in the transfer of ubiquitinated substrates from Cdc48 to the proteasome or in their release from these complexes.

RESUMO

A degradação regulada de proteínas é mediada pelo sistema ubiquitina-proteassoma que é vital para a homeostasia em células eucariontes. Também crucial para a degradação de proteínas é o seu unfolding, separação e extracção da membrana. Estes processos são catalizados por uma AAA-ATPase, extremamente conservada, chamada Cdc48 (ou p97).

Neste manuscrito procedeu-se à caracterização da proteína de *S. cerevisiae* Cuz1 (Cdc48-associada UBL/Dedo de Zinco), cujo gene é induzido por arsenito. Cuz1, à semelhança do seu ortólogo humano ZFAND1, possui um domínio dedo de zinco do tipo AN1 bem como um domínio semelhante à ubiquitina (UBL).

A proteína Cuz1 modula a atividade da ATPase Cdc48 no sistema ubiquitina-proteassoma. As duas proteínas interagem diretamente e o domínio UBL da proteína Cuz1 é necessário, enquanto o domínio AN1 é dispensável para a interacção com a região N-terminal da proteína Cdc48.

A proteína Cuz1, também se liga, embora de forma mais fraca, com o proteassoma e neste caso o domínio UBL não é necessário. Verifica-se um aumento na interação entre o proteasoma e a proteína Cuz1 após exposição à toxina ambiental arsenito. Em mutantes de proteassoma, a perda de Cuz1 causa um aumento de sensibilidade em relação a este composto. A perda de Cuz1 caracteriza-se também por um pequeno defeito na degradação de substratos do sistema ubiquitina-proteassoma. A combinação da deleção esta proteína com mutações no complexo $Cdc48^{Npl4\text{-}Ufd1}$ agrava o defeito observado.

A proteína Cuz1 limita também a acumulação de substratos ubiquitinados quer no proteassoma quer no complexo Cdc48, sugerindo que a proteína Cuz1 possa estar envolvida na transferência de substratos do Cdc48 para o proteassoma ou na sua libertação destes complexos.

ACKNOWLEDGEMENTS

I owe my appreciation to lots of different people that have been really important for me to get to this point of my life and career.

The first one I have to thank is Mark, my PhD advisor who is a true inspiration not only as a scientist but also as a person. In the last few years he has believed in me and encouraged me to always follow my own ideas. From day one, Mark has always been super supportive not only in science related subjects but also when bureaucracy issues came along.

Past and current members of the Hochstrasser lab were wonderful in the last few years. Many times during labmeetings, I felt I was having breakfast with a bunch of friends and it happens that we were chatting about science, I couldn't have asked for a better and more supportive environment to grow up as a scientist. I have to thank a person from the lab in particular, Robb, who taught me everything about yeast when I first arrived at the lab and with whom I have had the most interesting "stupid" discussions ever. I shall never forget the word prioritize because of him.

This has been a long journey and sometimes a bumpy one, but I had several friends that were there all along and that made it a lot easier. I probably have to apologize to some of them, for just being in the other side of the world and never being there for their big moments. But such is life and still they were there supporting me.

This last few years were not only about science, but about the experiences and the people you meet along the way. So many people I have met both in USA and Germany that made me a better person and with whom I shared unforgettable moments, friendship, laughter and sometimes tears, stories, little things that I will always remember and for which I am so thankful and that made me the person I am today. I also have to give a special shout out to the GABBA crowd. I was fortunate to have shared with them the first 6 months of my PhD and I couldn't have asked for a better group. Even far from each other, in the past 5 years we shared our experiences, our worries, our wins, our losses, our everyday stories.

Most of my friends I consider them to be the family I acquired throughout life, and each one of them somehow has helped me reach this day and I couldn't feel more privileged to know them.

Lastly I need to thank my family, they are the most important people in my life and they have been there all along in my 28 years of life in so many different ways that it is even hard to explain. I owe them everything… My mom is the rock of the family, she is tiny but the toughest person I have ever met. I like to think that my resilience comes from her. My father is the kindest person ever, but I will not miss him calling me on Sundays during my Phd and always asking: "So when are you publishing?".

A big thank you to all the above and all the others that I forgot and that somehow have been there in these last few years.

TABLE OF CONTENTS

CHAPTER 2- MATHERIAL AND METHODS

CHAPTER 3- CUZ1, A NOVEL MODULATOR OF CDC48 FUNCTION IN THE UBIQUITIN-PROTEASOME SYSTEM

LIST OF ABBREVIATIONS

AAA-ATPases- ATPases associated with diverse celular activities

AIRAP- Arsenite-inducible RNA-associated protein

AIRAP-L- Arsenite-inducible RNA-associated protein-like

AIP-1- Arsenite inducible RNA associated protein

ALS- Amyotrophic lateral sclerosis

APF1- ATP-dependent proteolysis factor

APL- acute promyelocytic leukaemia

ATP- adenosine triphosphate

BCA assay- bicinchoninic acid assay

BS1- Binding site 1

CDC48- Cell division cycle 48

CFTR- Cystic fibrosis transmembrane conductance regulator

Co-IP- Coimmunoprecipitation

CORE cassette- Counterselectable Reporter

CP- Core particle

CPY*- mutant carboxypeptidase Y

CRLs- Cullin-RING ubiquitin ligases

CUZ1- Cdc48-associated UBL/zinc-finger-1

DNA- Deoxyribonucleic acid

DUB- Deubiquitinating enzyme

ECL- Enhanced chemiluminescence

EM- Electron microscopy

ER- Endoplasmic-reticulum

ERAD- Endoplasmic-reticulum-associated protein degradation

GAL- Galactosidase

GFP- Green fluorescent protein

GST- Glutathione S-transferase

HECT- Homologous to the E6AP carboxyl terminus

HEPES- 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid

IBMPFD- Inclusion body myopathy associated with Paget's disease of bone and frontotemporal dementia

IgG- Immunoglobulin G

JAMM/MPN- Jab1/MPN domain-associated metalloisopeptidase

LC-MS/MS- Liquid chromatography-tandem mass spectrometry

MJD- Machado-Joseph disease

MWCO- Molecular Weight Cut Off

NER- Nucleotide excision repair

NPL4- Nuclear pore localization protein 4

Ntn hydrolases- N-terminal nucleophile hydrolases

OB fold- oligonucleotide/oligosaccharide-binding fold

ORF- open reading frame

OTU- Otubain

PACE- Proteasome Associated Control Element

PAN- Proteasome-Activating Nucleotidase

PBS- Phosphate buffered saline

PCI domains- Proteasome/CSN/initiation complex

PCR- Polymerase Chain Reaction

PDB- Protein Data Bank

PFU domain- PLAA family ubiquitin binding domain

PGK- Phosphoglycerate kinase

PML- promyelocytic leukaemia

PRU domain- Pleckstrin-like receptor for ubiquitin

RAR- retinoic acid receptor α

RING finger- Really Interesting New Gene

RPN- regulatory particle non-ATPase

RPT- regulatory particle triple A

RMSD- Root-mean-square deviation

RP- Regulatory particle

RVP- Retroviral aspartyl proteases

Sc ratio- Spectral count ratio

SCF- Skp1-Cdc53/CUL1-F-box

SDS-PAGE- sodium dodecyl sulfate polyacrylamide gel electrophoresis

SIM- SUMO-interacting motif

STUBL- SUMO-Targeted Ubiquitin Ligase

TBS-T- Tris-buffered saline with Tween

UB- Ubiquitin

UBA- Ubiquitin-associated

UBC- Ubiquitin-conjugating enzyme

UBL- Ubiquitin-like

UBX- Ubiquitin regulatory X

UCH- Ubiquitin carboxy-terminal hydrolase

UFD1- Ubiquitin-fusion degradation protein 1

UIM- Ubiquitin-interacting motif

UPS- Ubiquitin-Proteasome system

USP- Ubiquitin-specific protease

VWA domain- von Willebrand factor type A domain

VCP-Valosin-containg protein

VIM motif- VCP- interacting motif

XPC- Xeroderma Pigmentosum protein C

WT- Wild-type

Zf- Zinc-finger

LIST OF FIGURES

CHAPTER 1

CHAPTER 3

LIST OF TABLES

CHAPTER 1

CHAPTER 2

CHAPTER 3

Portions of this thesis have been previously published in:

Sá-Moura B., Funakoshi M., Tomko R.J. Jr, Dohmen R.J., Wu Z., Peng J., and Hochstrasser M. (2013) A conserved protein with AN1 zinc finger and ubiquitin-like domains modulates Cdc48 (p97) function in the ubiquitin-proteasome pathway. J Biol Chem. 2013 Nov 22;288(47):33682-96

CHAPTER 1- INTRODUCTION

A. INTRODUCTION TO PROTEIN DEGRADATION

Regulated protein degradation is critical to ensure cellular homeostasis, as nearly every major cellular pathway includes proteins that are specifically targeted for degradation. The half-lives of typical proteins inside cells range from a few minutes to several days (reviewed in (1)), and protein destruction systems can respond to changes in environmental conditions. The most frequently degraded proteins are regulatory proteins, which are often only briefly necessary for a certain process before they undergo destruction. Conversely, structural proteins typically remain stable longer, with examples of proteins that are stable for several years. Damaged and misfolded proteins are also targeted for degradation, and failure to do so has been linked to the etiology of several different diseases (2).

Two major pathways, the ubiquitin-proteasome system (UPS) and lysosomal proteolysis, mediate protein degradation. The UPS is the major route for the regulated degradation of intracellular proteins (3). However, lysosomal proteolysis was discovered first, in the 1950's, and early models (4) assumed that protein degradation would occur exclusively in this organelle. But soon evidence started to accumulate against that possibility. Rabinovitz and Fisher found that rabbit reticulocytes were able to degrade hemoglobin (5). Since rabbit reticulocytes do not contain lysosomes, it was postulated that the degradation of haemoglobin was mediated by non-lysosomal machinery. By the late 1970's, Goldberg and Etlinger were able to isolate a cell-free, ATP-dependent proteolytic preparation from reticulocytes (6). Lysosomal proteases were known to not require ATP, lending more evidence for a novel system of protein degradation.

The reticulocyte extract system was later adopted by Hershko, Ciechanover and colleagues, who fractionated the extract. They were able to isolate two different fractions that were required for ATP-dependent protein degradation. The first fraction contained a small protein called ubiquitin (at the time called APF-1, for ATP-dependent proteolysis factor 1) (7,8). This was the breakthrough that led to the

discovery of protein degradation requiring a two-step mechanism: 1) covalent attachment of ubiquitin to a substrate protein (9) followed by 2) degradation of the ubiquitin-modified substrate by a protease now named the 26S proteasome, which was later found to be in the second fraction along with enzymes that conjugate ubiquitin to the substrate (Fig. 1) (10). As a result of their work on ubiquitin-mediated proteolysis, Hershko, Ciechanover, and their collaborator Irwin Rose were awarded the Nobel Prize in Chemistry in 2004.

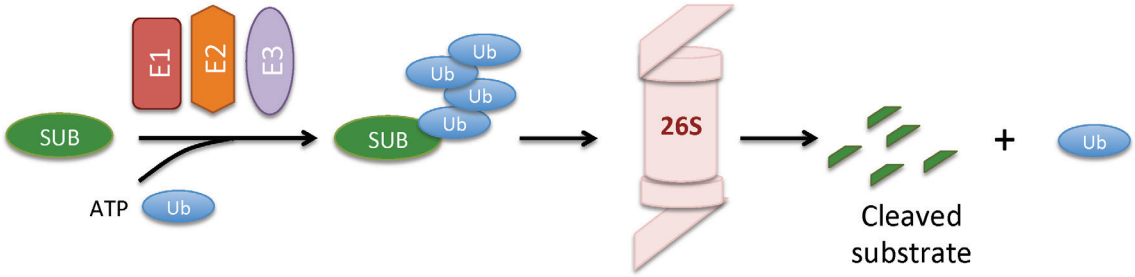


Figure 1- Schematic overview of the mechanism of ubiquitin mediated protein degradation by the 26S proteasome. Ubiquitin (Ub) is attached to the substrate (SUB), in an ATP-dependent manner. The 26S proteasome then recognizes polyubiquitin chains attached to the substrate and then degrade it.

The UPS is now known to be involved in the regulated proteolysis of proteins in almost every cellular process, including the cell cycle, cell differentiation and development, DNA repair, regulation of the immune and inflammatory responses, transcription, cellular signalling, morphogenesis of neuronal networks and the biogenesis of organelles (11-17). Much of what is known about the UPS and its broad utility in cellular physiology was obtained from studies of the pathway and its components in model organisms, most notably *Saccharomyces cerevisiae* (yeast). This thesis focuses on the UPS in yeast, which is indistinguishable from the UPS in humans in its basic organization and molecular mechanisms (18).

The UPS is a highly regulated, strictly controlled system whose disruption has been implicated in many different human diseases including cancer, immunological

and neurodegenerative disorders (19-21). Furthermore, drugs targeting specific components of the UPS are either currently in use or are in development for the treatment of some of these diseases (22,23). An even better understanding of the UPS and each of its components will provide further insight to aid in the treatment of these diseases.

B. UBIQUITINATION OF SUBSTRATES

Ubiquitin

Ubiquitin is a small protein composed of 76 amino acids that is only present in eukaryotes. It is among the most conserved proteins, with the human protein differing by only 3 amino acids compared to the yeast version. Ubiquitin adopts the β-grasp fold, a complex topology consisting of a five-stranded β sheet, an α helix, and a short 3_{10} helix (24). One important feature of ubiquitin is the exposed C-terminal Gly-Gly motif required for ubiquitin conjugation to the lysines of the substrate to be degraded. The prototypical ubiquitin modification to substrate proteins is as ubiquitin chains in which the C-terminus of the distal ubiquitin is attached to the ε-amino group of a lysine of the previous ubiquitin molecule, forming an isopeptide bond. However, monoubiquitination has been shown to be a non-proteolytic signal and is involved in multiple cellular processes including endocytosis of membrane proteins, histone regulation, gene expression and DNA repair (reviewed in (25)). Ubiquitin has 7 different lysines (K6, K11, K27, K29, K33, K48, K63), which allows the formation of chains with different shapes and lengths (26,27) that constitute distinct functional signaling (Fig. 2). The canonical signal for proteasomal degradation is a polyubiquitin chain with at least 4 ubiquitin molecules linked through Lys48 (28), although it has been shown that other kind of linkages can be recognized by the proteasome (29) and that some proteins do not even require ubiquitination to be degraded (30). K63-linked chains create a non-proteolytic signal (31) playing a role in transcriptional regulation, DNA repair, endocytosis and activation of protein kinases. K11 linked

chains have also been linked to proteasomal degradation (29) but the functions of the other type of linkages remain elusive (Fig. 2).

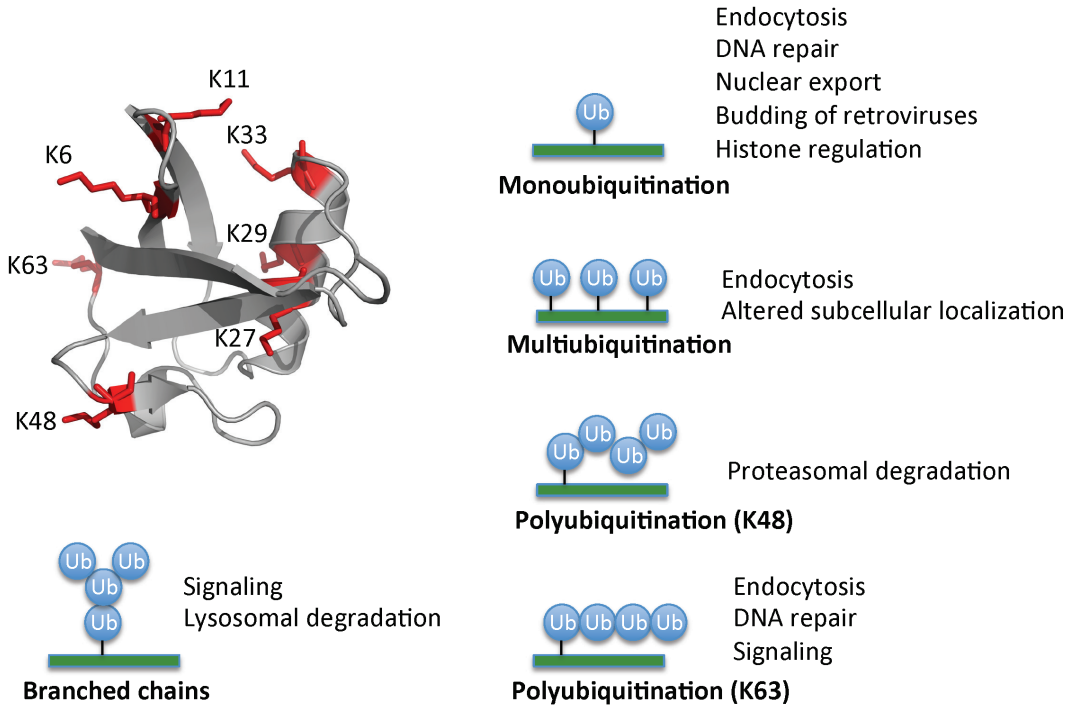


Figure 2- Ribbon diagram of ubiquitin (PDB ID: 1UBQ) showing the typical β-grasp fold. Indicated in red are the ubiquitin lysines that are used for conjugation to the substrates. Ubiquitin can be conjugated to any given substrate as a monomer, as multiple monomers, in chains attached through one of the lysines or in mixed chains. These different types of ubiquitin conjugation have been associated with distinct cellular pathways.

Conjugation of ubiquitin to proteins

The conjugation of ubiquitin to a substrate proceeds in a 3-step process that culminates with the attachment of ubiquitin to the substrate through an isopeptide bond between one lysine from the substrate and the carboxyl terminus of ubiquitin. First ubiquitin is activated in an ATP-dependent manner by an ubiquitin-activating enzyme (E1) (32,33) and then transferred to a specific cysteine residue of an

ubiquitin-conjugating enzyme (E2, also known as ubiquitin carriers). While the yeast genome encodes for a single ubiquitin-activating enzyme, UBA1, the E2 family is larger (13 members), and all of its members have in common a conserved ubiquitin conjugating catalytic fold (UBC) of 150-200 amino acids (34). The last step is catalyzed by an ubiquitin ligase (E3) that attaches ubiquitin to a lysine residue of the target protein (Fig. 3). The E3s confer specificity to this process and therefore they are quite numerous, with humans having several hundred different E3 ligases. The level of specificity is further increased by different pairing of E2s with E3s, which allows the formation of different ubiquitin chains. The E3s belong to 3 different classes according to the domain found in their core protein: U-box, RING finger (Really Interesting New Gene), and HECT (homologous to E6-AP carboxy terminus). The U-box and RING domains are structurally similar (35,36).

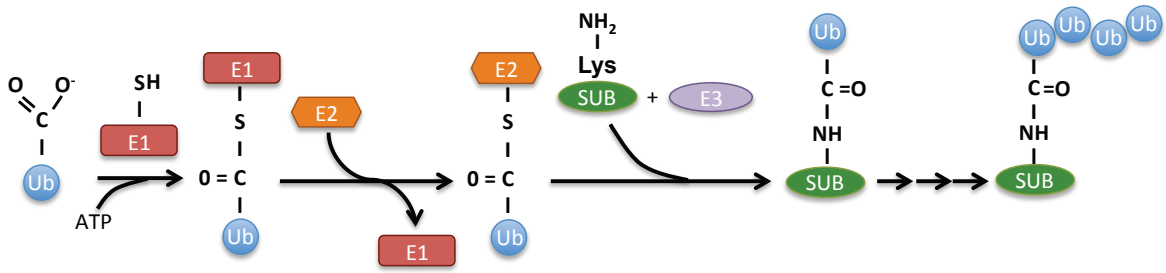


Figure 3- Protein ubiquitination involves the concerted action of the E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzymes and E3 ubiquitin-protein ligases. The E1 enzyme, using the energy from the hydrolysis of ATP, activates the C-terminal carboxylate of ubiquitin, forming a thioester bond with the E1's catalytic cysteine. The ubiquitin is subsequently transferred to the catalytic cysteine of the E2. The mechanism of attachment of ubiquitin depends on the nature of the E3. RING domain and U-box bind both the substrate and the ubiquitin-loaded E2, acting as a scaffold for ubiquitin transfer. HECT domain E3s first form a thiolester intermediate with ubiquitin and then transfer it to the substrate. Further cycles of these reactions will extend the polyubiquitin chain. In some cases, such as in monoubiquitination, the requirement for an E3 might be bypassed, although this has not been shown definitively *in vivo* (37).

One important question is how does the machinery for ubiquitin conjugation, mainly the E3 ligases, specifically recognize the proteins that need to be degraded. Most short-lived proteins possess a degradation signal (or degron), which can be defined as the minimal element within a protein that is necessary for recognition and degradation by the proteolytic apparatus (38). Some well known degrons include the *Deg1* sequence of the *MAT*α2 transcriptional regulator, the exposed N-terminal residue (in some cases modified) of many proteins, the phosphodegrons involved in the destruction of cyclins and cyclin-dependent kinases (CDK) inhibitors (39) and PEST sequences in calmodulin binding proteins (40).

Ubiquitin-like proteins

Several other small proteins adopt a fold similar to ubiquitin, despite not sharing high sequence similarity (Fig. 4). These ubiquitin-like proteins (UBLs) are also covalently attached to substrates (usually proteins, but in one case a lipid) and have biologically distinct functions. The UBLs include RUB1/NEDD8, SUMO, ATG8 and ATG12, UFM1, URM1, ISG15, FAT10 and MNSFβ. Ubiquitin and UBLs share the mechanism for conjugation to proteins and modification by UBLs plays key roles in several cellular regulatory mechanisms including cell cycle progression, gene transcription, transport across the plasma membrane, protein quality control and immune response (41,42). A much large class of proteins contain UBL domains but do not undergo attachment to and removal from other proteins. The best characterized conjugation-competent UBLs are NEDD8 and SUMO and this Introduction will focus on providing more information about them and some of the others UBLs present in *S. cerevisiae* (check also Table 1).

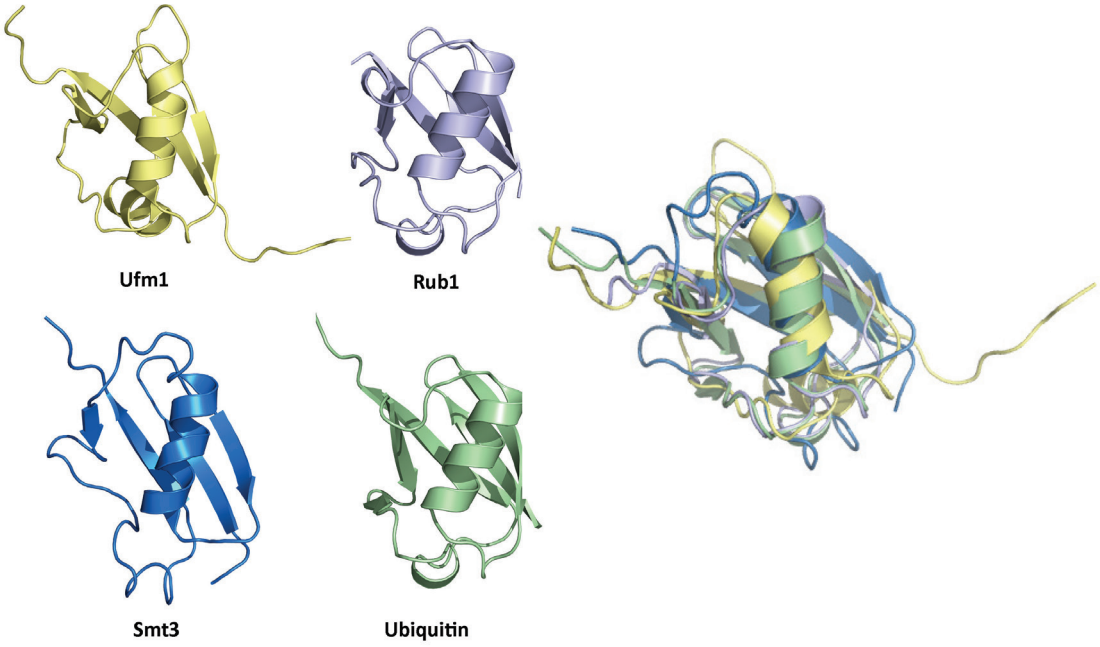


Figure 4- Three-dimensional structures of ubiquitin and ubiquitin like proteins. In common, as shown by their overlay, they possess the ubiquitin fold. (PDB ID: 1UBQ, 1L2N, 1WXS, 1NDD). Adapted from (43).

NEDD8 (neural precursor cell-expressed, developmentally downregulated), known as Rub1 in yeast, is the UBL with the closest sequence to ubiquitin. Similar to ubiquitin, NEDD8 is conjugated to its substrates by an isopeptide linkage between its carboxy-terminal glycine and a lysine of the target protein and a consensus motif for neddylation has been identified: IVRIMKMR. The best-known targets for Neddylation are members of the cullin family. Cullin-RING ubiquitin ligases (CRLs) constitute the largest family of ubiquitin ligases (44). CRLs are multiple-subunit complexes generally consisting of a cullin, a RING-H2 finger protein, a substrate-recognition subunit (SRS) and an adaptor subunit that links the SRS to the complex. Yeast have 3 different cullins: CUL1, CUL3 and CUL8. The neddylation of cullins was first identified for Cdc53/CUL1 (45,46), the cullin component of SCF E3 ligases (Skp1-Cdc53/CUL1-F-box protein) in *Saccharomyces cerevisiae*. Later it was shown that neddylation of CUL1 enhances the ubiquitin-ligating activity of the Rbx1 RING-H2 protein *in vitro*, by potentially facilitating the recruitment of ubiquitin-loaded E2s

(47,48). Additionally neddylation of CUL1 prevents binding of CAND1/p120 that would otherwise dissociate SKP1 from CUL1, therefore inhibiting SCF ligase activity (49).

Small ubiquitin-related modifier (SUMO) is another known UBL. While, in *S. cerevisiae* there is only one form of SUMO, encoded by *SMT3*, in vertebrates there are 4 different isoforms, SUMO-1 to 4, which are encoded by different genes, although it is not clear whether SUMO-4 can actually be conjugated (50). All SUMO genes encode a precursor containing a short C-terminal peptide that needs to be cleaved off to expose the terminal diglycine motif necessary for conjugation. A SUMO conjugation consensus site has been identified and it consists of [I/V/L]-K-X-[D/E], where X is any amino acid (51). Similarly to ubiquitin, SUMO can be conjugated in its monomeric form (monosumoylation) but Smt3 (52), SUMO-2 and SUMO-3 (53) also can form chains (polysumoylation) through a single conserved acceptor lysine that is absent in SUMO-1. Non-covalent interaction between SUMO and other proteins is mediated by SIMs (SUMO-interacting motifs) (54) that consists of a hydrophobic core ([V/I]-x-[V/I]-[V/I]) flanked by a cluster of negatively charged amino acids (55) . This SIM forms a β strand that can be inserted in a parallel or antiparallel direction between the α helix and β strand of SUMO (56). The consequences of SUMOylation are diverse, including alteration of the activity, localization and/or stability of the target protein which in turn will affect different cellular processes including cell signaling, intracellular trafficking, cell cycle, DNA repair and protein degradation (for more details (57)). Interestingly, SUMO can also play a role in ubiquitin conjugation. SUMO-targeted ubiquitin ligases (STUbLs) are a family of E3 enzymes that recognize sumoylated substrates via their SUMO interaction motif and subsequently attach ubiquitin (58,59). In acute promyelocytic leukaemia (APL), the promyelocytic leukemia (PML) protein is fused to the retinoic acid receptor α (RAR). The treatment of this disease involves arsenic, which will induce sumoylation of PML and proteasomal degradation. The molecular basis for this response involves RNF4, a STUbL that recognizes the poly-SUMO chains on PML and targets PML for degradation (60).

Autophagy is a catabolic process by which cells break down their own components by engulfing organelles or portions of the cytosol in double-membrane autophagosomes that will fuse with lysosomes or vacuoles for breakdown by lysosomal hydrolases. In yeast, more than 30 *ATG* (autophagy-related) genes have been identified to play a role in this process. Amongst them there are Atg8 and Atg12, two ubiquitin-like proteins (61,62). Atg12 ends with a glycine so it can be readily conjugated to the substrate, without processing. Atg12 is activated by binding to Atg7 (which acts as an E1) and then transferred to Atg10 (which acts as a E2 enzyme) and is finally conjugated, without the requirement for an E3, to Atg5 via an isopeptide bond (63,64). While most of UBLs are conjugated to proteins, Atg8 is actually the exception, being conjugated to phosphatidylethanolamine (PE) on the autophagic membrane, a process that is crucial for autophagic cargo recruitment and autophagosome biogenesis. The Atg8 precursor is first processed by the cysteine protease Atg4, to expose the terminal glycine. Subsequently, the mature Atg8 is activated by Atg7 (E1), transferred to the E2-like enzyme Atg3 and then conjugated to PE through an amide bond (65). Atg7 has the unique feature of being responsible for the conjugation of two different UBLs and working with two different E2s. Interestingly, it has been shown that Atg12-Atg5, despite not having any of the canonical E3 signature domains, might work as an E3 ligase for the conjugation of Atg8 to PE (66).

The eukaryotic ubiquitin-related modifier Urm1 is the most ancient UBL, and it resembles two prokaryotic proteins MoaD and ThiS, which are required for sulphur incorporation into molybdopterin and thiamine, respectively (67). It has been implicated both in protein modification and sulfur transfer. Urm1 is synthesized already in its mature form and can be covalently conjugated to lysine residues of other proteins. Similar to ubiquitin, Urm1 forms a thioester with an E1-like protein, and forms isopeptide bonds between Urm1 and the substrate. Ahp1p, in budding yeast, was the first urmylation substrate to be identified and for long was thought to be the only one (67), but recently it was discovered that in response to oxidative stress several human proteins are modified by Urm1, including proteins involved in nuclear transport, tRNA modification, RNA binding and processing, nuclear transport

and Urm1 pathway (68). Urm1 is also a sulphur carrier and it is involved in the 2-thiolation of 5-methoxycarbonylmethyl-2-thiouridine (mcm5s2U) at the wobble positions of certain tRNAs. Urm1 activation is similar to the prokaryotic sulfur carrier proteins. Urm1 forms an acyl disulfide bond with the E1, leading to the formation of a thiocarboxylated Urm1. This dual role for Urm1 suggests that it is the evolutionary link between eukaryotic UBLs and prokaryotic sulfur carrier proteins (69).

Table 1- Ubiquitin and ubiquitin-like proteins share the same overall fold, but have very distinct conjugation machinery. Their substrates as well as the pathways they affect are also highly variable.

UBL	E1	E2	E3	Protease	Substrates	Function
UB	UBA1	UBC1-8, 10, 11 and 13	Hundreds	Multiple	Multiple eg: short lived proteins	Several (check Figure 2)
SUMO	UBA2, AOS1	UBC9	Hundreds eg: SIZ/PIAS	ULP1, ULP2, SENPs (mammals), DESI1,2, USPL1	Multiple eg: sentrins	Protein degradation DNA repair Cell cycle Others
NEDD8	UBA3, ULA1	UBC12		CSN5, DEN1	Cullins/RBX1, p53	Activation of cullin based E3s
ATG8	ATG7	ATG3	ATG12-ATG5	ATG4	Phosphatidylethanolamine	Autophagy
ATG12	ATG7	ATG10			ATG5	Autophagy
URM1	UBA4				AHP1	Response to oxidative stress tRNA uracyl thiolation
FAT10	UBA6				Hundreds	Ubiquitin-independent proteasomal degradation Immune response
ISG15	UBE1L	UbcH8	HERC5	UBP43	Hundreds	Resistance to viral infection

UFM1	UBA5	UFC1	UFL1	UfSP1 and UfSP2	UFBP1 C20orf116	ER-stress induced apoptosis

C. THE PROTEASOME

Protein degradation by the proteasome: overview

Ubiquitin-dependent degradation is carried out by the 26S proteasome, a 2.6 MDa complex that is found in the cytosol and the nucleus of eukaryotic cells (70). This multisubunit complex consists of a proteolytically active 20S core particle capped on one or both ends by the 19S regulatory particle. Ubiquitin chains conjugated to a substrate protein are specifically recognized by the 19S regulatory particle. Ubiquitin is then removed by deubiquitinating enzymes and the substrate is unfolded by a ring of ATPases present at the base of the 19S. This ATPase ring is also responsible for opening the gate to the 20S proteolytic chamber and translocating the substrate into the chamber, where it is degraded (71,72) (Fig. 5).

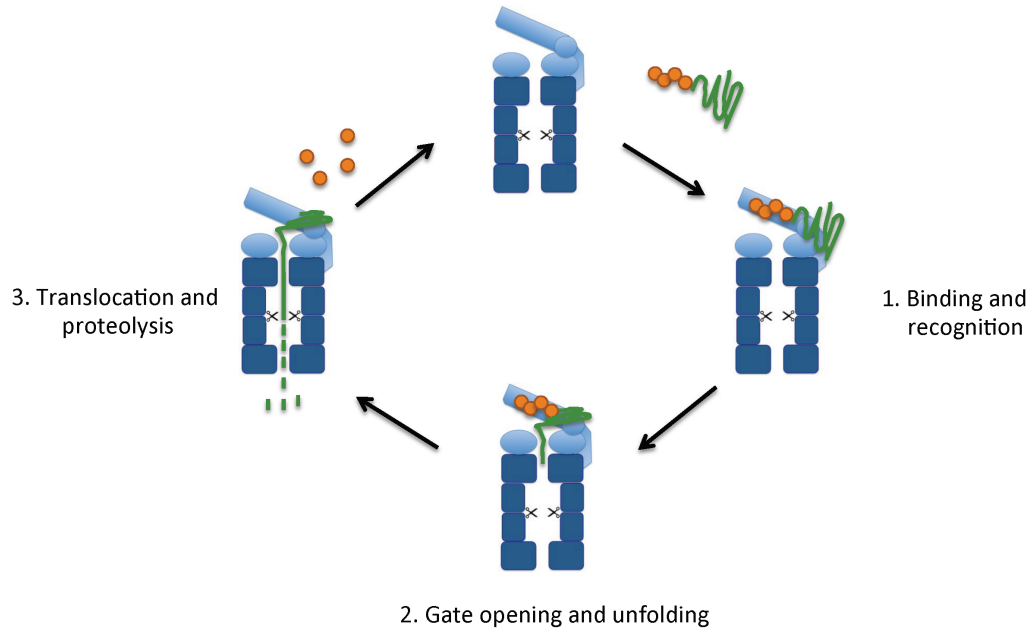


Figure 5- Schematic overview of the mechanism of degradation at the proteasome. The ubiquitinated substrate is recognized and bound by proteasome directly through Rpn10 or Rpn13. The substrate is unfolded by the ring of ATPases at the base of the 19S, which is also responsible for the opening of the gate to the proteolytic chamber as well as the translocation of the protein into the chamber. Deubiquitinating enzymes are responsible for the removal of ubiquitin prior to degradation. Once inside the proteolytic chamber, the substrate is degraded.

26S proteasome constitution: 20S core particle and 19S regulatory particle

The *S. cerevisiae* 20S proteasome is well characterized structurally, consisting of 14 different subunits: seven α subunits (α1-α7) and seven β subunits (β1-β7) (73). They are organized in a barrel shaped complex consisting of four stacked rings: 2 inner rings consisting of β subunits and two outer rings consisting of the α subunits (Fig. 6). The α subunit N-terminal residues form a gate at the center of the ring that impedes substrate translocation into the degradation chamber in the absence of an activator (74,75). All of the α subunits are genetically related (~30% average identity between the seven α subunits in *S. cerevisiae*) but the sequence of their N-terminus is highly variable (74). β-rings form a central proteolytic chamber where the β1, β2 and β5 subunits contain the proteolytic active sites, and each site has different substrate specificities, cleaving preferentially after acidic, basic, or hydrophobic residues, respectively (76). Proteasome β-subunits belong to the Ntn-hydrolases group (N-terminal nucleophile) and are synthesized as inactive precursors that upon autocatalytic processing expose the N-terminal amino acid residue, thus rendering them proteolytically active (77). In mammals, upon immune stress, an alternative subset of β subunits is induced. β1i, β2i and β5i are incorporated into “immunoproteasomes”, which increase the generation of specific peptides that are important for immunosurveillance (78).

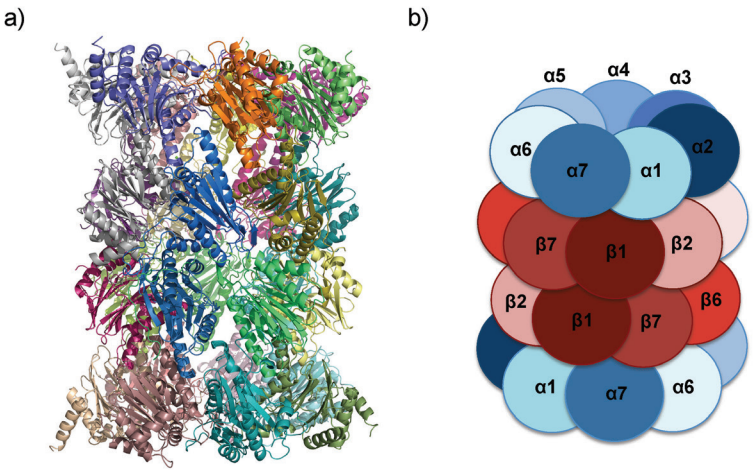


Figure 6- The 20S proteasome. a) Side view of the crystal structure of the 20S *S. cerevisiae* proteasome (PDB ID: 1RYP). The core particle adopts the shape of a barrel with large central cavities and narrow constrictions (13 Å) at the ends. It is approximately 150 Å in length and has a diameter of 115 Å. b) Scheme of the overall arrangement of the 20S subunits. The β subunits form the inner rings while the α subunits form the outer rings.

Although the 26S proteasome, with the 19S RP as activator, is the prototypical proteasome, several other regulators of the 20S have been identified (79). These include PA26/PA28 and PA200/Blm10, which are capable of opening the gate to the 20S, and thereby activating it (80). More recently, the AAA ATPase Cdc48, which is known to play central roles in the UPS, has also been suggested to serve as a 20S activator in some cases (81). The roles of Cdc48 in the UPS will be addressed in greater detail later in this thesis. The proteasome associated protein Ecm29 also deserves mention, as it functions as a scaffold protein stabilizing the 20S-19S interaction and suppressing gate opening (82). Finally, it should be noted that archaea and certain eubacteria have 20S-like proteasomes and these are capped by PAN or ARC/Mpa AAA+ ATPases, respectively.

Free purified 20S is able to degrade peptides and some unfolded proteins (18), but the degradation of polyubiquitinated proteins only occurs after association

with the 19S complex. As described above, the 19S is responsible for the recognition, deubiquitination, unfolding and translocation of the substrate, ultimately leading to activation of the 20S. The 19S regulatory particle is a complex of at least 19 subunits that can be subdivided into base and lid. Obtaining high-resolution crystal structures of the 19S regulatory particle has been difficult given the large size and extensive conformational dynamics of the complex. However, several recent studies using mainly electron microscopy have revealed the molecular architecture as well as some mechanistic information about of the regulatory particle (Fig. 7) (83,84).

The base of the RP consists of a heterohexameric ring of six different AAA+ ATPases (ATPases associated with various cellular activities) Rpt1-Rpt6 (for Regulatory particle triple A protein) and three non-ATPase subunits, Rpn1, Rpn2 and Rpn13. The C-terminal tails of the Rpt proteins mediate the attachment of the base to the α-ring of the 20S particle by establishing contacts with the α ring surface (85). Interestingly the ATPase ring and the α-ring are not axially aligned until substrate is engaged. The order of the ATPase subunits Rpt1/Rpt2/Rpt6/Rpt3/Rpt4/Rpt5 was determined by engineered disulfide cross-linking experiments (86). In addition to the AAA+ domain that is thought to directly interact with substrates, each ATPase also includes an oligonucleotide/oligosaccharide binding (OB) fold as well as an N-terminal helix. The OB folds form an extra N-ring above the AAA+ domains with the N-terminal helices protruding from that ring to form a trio of coiled coils (87). The coil regions of Rpt1/Rpt2 and Rpt6/Rpt3 are responsible for the interaction with Rpn1 and Rpn2. Both Rpn1 and Rpn2 adopt a toroidal α-solenoid fold composed of eleven tandem helix-turn-helix pseudo-repeats consisting of copies of a 35–40 amino acid repeat motif called proteasome/cyclosome (PC) repeats (88). Rpn1 and Rpn2 are the largest subunits of the proteasome and act as scaffolds for docking of several proteasome-interacting proteins through recognition of their UBL domains (89,90). This group includes Ubp6, a deubiquitinating enzyme, as well as the shuttle factors Rad23, Dsk2 and Ddi1 (91-94), which will be discussed in a later section.

The lid is composed of 9 different subunits, Rpn3, Rpn5-Rpn9, Rpn11, Rpn12 and Sem1/Rpn15. Six of the nine lid subunits possess proteasome/CSN/initiation

complex (PCI) domains, and interact with one another through these domains in a horseshoe shape arrangement, while their N-terminal α-helical domains project out like fingers. The order of the subunits of the lid is Rpn9, 5, 6, 7, 3, and 12 (83,84). Rpn12 is the last subunit to be incorporated into the lid, appearing to act as a sensor for proper assembly of the other eight subunits. It is thought that upon correct association of all lid subunits, the Rpn12 C-terminal helix completes formation of a helical bundle that then licenses lid-base joining (95,96). The other two lid subunits Rpn8 and Rpn11 both possess Mpr1/Pad1/N-terminal (MPN) domains. Rpn11 possesses deubiquitinating (DUB) activity. Rpn8 and Rpn11 form a heterodimer that is located above the AAA-ATPase ring and establishs several contacts with the PCI domains of the lid subunits. Sem1 is a very small acidic protein that has been found in a complex with Rpn3 and Rpn7. The Sem1 C-terminal helix contacts the PCI domain of Rpn7 while the N-terminus is likely responsible for keeping Rpn3 and Rpn7 together (97).

The proteasome possesses 2 intrinsic ubiquitin receptors: Rpn10 and Rpn13. Their exact locations have been determined by analyzing the cryo EM structure of the proteasome in *rpn10Δ*, *rpn13Δ* and *rpn10Δ rpn13Δ* yeast strains. Rpn10 and Rpn13 localize in the apical region of the 26S, above the coiled coils of Rpt4/Rpt5 and Rpt1/Rpt2, respectively (98).

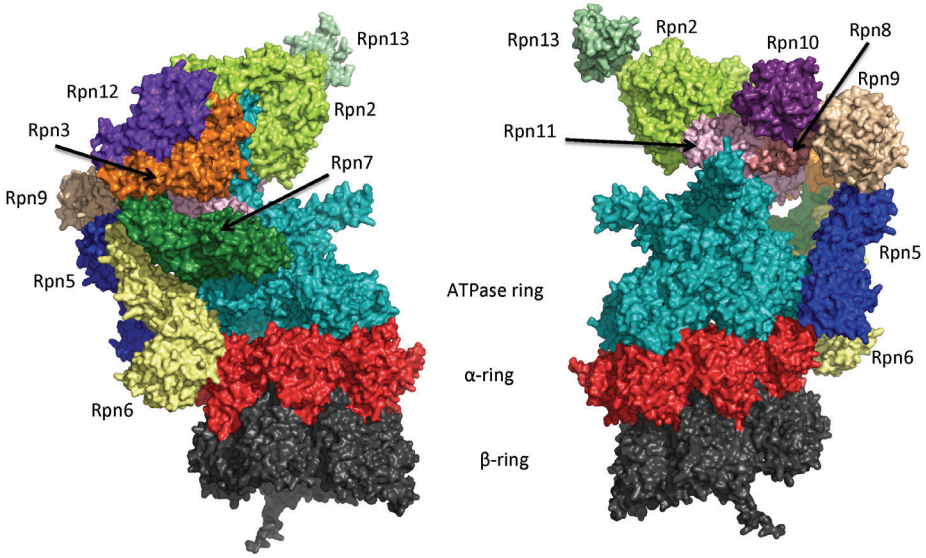


Figure 7- Near atomic reconstruction of the 26S proteasome structure obtained by cryo EM with 7.4Å resolution (PDB ID: 4B4T). Half of the core particle is displayed in

complex with the regulatory particle. In gray the β-ring and in red the α-ring, with the ATPase ring (in cyan) sitting on top. Several RP subunits are highlighted. Rpn10 is positioned to bind subunits from both the lid and base complex, as well as Rpn5 and Rpn6, which establish contacts with the 20S. Rpn1 is missing from this model because its density was poorly resolved in the structure.

Substrate delivery and recognition

The primary intrinsic proteasome receptors for polyubiquitinated substrates are Rpn10 and Rpn13 (99,100). Rpt5 has also been suggested to be an additional receptor as it can be cross-linked to polyubiquitin chains (101) but there is no further evidence for this function.

Rpn10 is composed of an N-terminal von Willebrand factor A (VWA) domain and a C-terminal ubiquitin interacting motif (UIM) that, as the name indicates, binds ubiquitin (91,102). The UIM is 20 amino acids long and includes the highly conserved LAL(M)AL motif that is essential for the recognition and binding of K48 linked polyubiquitin chains (99). While yeast Rpn10 possesses only one UIM, the human and *Drosophila* counterparts have two and three UIMs, respectively. The VWA domain of Rpn10 is involved in contacting other proteasome subunits, namely Rpn1, Rpn2, Rpn9 and Rpn12, which are subunits from both the base and lid, thereby placing Rpn10 at the interface of these two subcomplexes (98). Consequently, mutations in the Rpn10 VWA domain result in dissociation of the 19S into base and lid subcomplexes (103). Interestingly, a substantial fraction of the Rpn10 pool is not associated with the proteasome (104). This extraproteasomal Rpn10 fraction is involved in regulating the access of Dsk2, a known shuttle factor, to the proteasome (105), and also in affecting ubiquitin chain formation (106).

Rpn13 is another ubiquitin receptor of the proteasome. Its human homolog interacts with Uch37 (107), enhancing its isopeptidase activity (108). This enzyme is thought to exert a proofreading activity, rescuing poorly ubiquitinated substrates (109). There is no Uch37 ortholog in yeast, and *S. cerevisiae* Rpn13 even lacks the

C-terminal part that is known to bind the DUB. The N-terminal PRU domain (pleckstrin-like receptor of ubiquitin) of Rpn13 binds to both ubiquitin and Rpn2 (100,110).

Intriguingly, *S. cerevisiae* lacking both Rpn10 and Rpn13 are still viable, suggesting alternative mechanisms for the delivery of polybiquitinated substrates to the proteasome (100). The three proteins Rad23, Dsk2, Ddi1 are extraproteasomal proteins involved in delivering polyubiquitinated substrates to the proteasome (111-113). These proteins, collectively called shuttle factors, possess a UBL domain that is recognized by Rpn1 at the proteasome. In addition to the UBL domain, Rad23, Dsk2 and Ddi1 also possess UBA domains (ubiquitin associated) that bind polyubiquitinated substrates (114). Consistent with this shuttling function, proteasomes purified from a *rad23Δ dsk2Δ* strain are defective in binding ubiquitinated conjugates (115). Moreover strains deficient in any of these shuttle factors display defects in protein degradation by the proteasome (116-118). Notably, yeast cells simultaneously lacking Rpn10, Rpn13, and the three UBL-UBA shuttle factors remain viable. Another protein that may be involved in the delivery of substrates to the proteasome is Cdc48, a AAA ATPase. Cdc48 binds ubiquitin directly and also indirectly through adaptors (119). These mechanisms of substrate delivery will be discussed in more detail the next chapter.

Deubiquitination of substrates

Another crucial step in proteasome-mediated substrate degradation is deubiquitination. In order to maintain cellular homeostasis, ubiquitin is not degraded at the proteasome but is instead cleaved off from the conjugates prior to their degradation by deubiquitinating enzymes (DUBs). In *S. Cerevisiae,* the proteasome possesses one intrinsic DUB, Rpn11, which is located at the lid (120). In addition to ubiquitin recycling, proteasome-localized DUBs are also responsible for proofreading and assuring that only the correct substrates are degraded, as described for Uch37 (109).

DUBs are also crucial apart from the proteasome as they process ubiquitin precursors as well as trim ubiquitin chains throughout the cell (reviewed extensively in (121)) (Fig. 8). Yeast has 20 DUBs, while mammals have about 100 DUBs encoded in their genomes. Most DUBs specifically cleave after the carboxyl terminus of the last residue of ubiquitin (Gly76). DUBs can be divided into five different subfamilies based on sequence and catalytic motifs. Four of the subfamilies, namely ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs/UBPs), ovarian tumor (OTU) related proteases and the Josephin or Machado-Joseph disease (MJD) proteases are all cysteine proteases, while the fifth group, the Jab1/MPN domain-associated metalloisopeptidase (JAMM/MPN+) proteases, belong to the metalloprotease family. In addition to the catalytic domain, most DUBs also contain domains that mediate protein-protein interactions, including ubiquitin binding domains (UBDs) (122).

Rpn11, the most conserved of all lid subunits, belongs to the JAMM/MPN+ protease family, and its activity is essential for viability in *S. cerevisiae* (120,123). This DUB has been shown to cleave at the proximal ubiquitin molecule, therefore removing the entire ubiquitin chain from the substrate (123). Rpn11 deubiquitinating activity is ATP-dependent. This dependence suggests that Rpn11 activity is coupled to the unfolding and translocation by the Rpt ring in the base of the 19S, and indeed this mechanism was explained in more detail a recent report (87). Rpn11 normally localizes to the side of the central pore, allowing the substrate access. Upon substrate engagement, Rpn11 shifts towards the center of the α-ring, locating itself right above the pore and the catalytic groove in a position that allows Rpn11 to remove the polyubiquitin tag of the substrate.

Ubp6 is a proteasome-interacting protein that has been shown to have enzymatic activity against ubiquitin. Ubp6, know as USP14 in humans, associates with the 19S base, particularly with Rpn1 through its ubiquitin-like domain at the N-terminus (92,124). Unlike Rpn11, Ubp6 cleaves polyubiquitin chains starting at the distal end, thus shortening them in a stepwise manner. Interestingly, Ubp6 is a potent inhibitor of the proteasome (124), delaying substrate degradation (125). Ubp6 is involved in opening the 20S gate, where the binding of polyubiquitinated substrates

to the DUB rather than its catalytic activity is responsible for regulating gate opening (126). Conversely Ubp6 catalytic activity has been shown to be required for the 19S assembly and presumably deubiquitinates proteasome precursors loaded with ubiquitinated substrates (127).

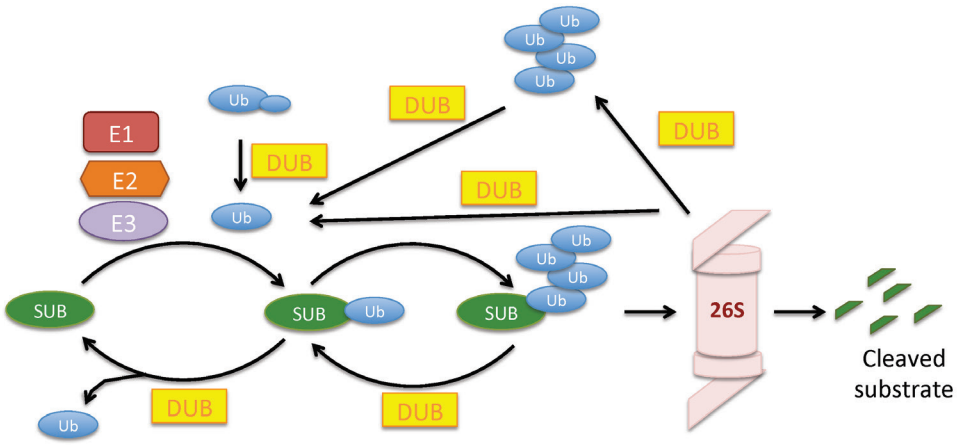


Figure 8- Catalytic functions of deubiquitinating enzymes. Ubiquitin is synthesized as a fusion protein precursor, fused either to itself, or to ribosomal proteins. Deubiquitinating enzymes are involved in generating mature monomeric ubiquitin. Deubiquitinating enzymes also reverse the conjugation of ubiquitin as well as edit ubiquitin chains. One of the most important role of DUBs is in the recycling of ubiquitin at the proteasome. The substrate is degraded but the ubiquitin tag is cleaved off either by Rpn11 (the one intrinsic DUB at the proteasome) or by associated DUBs prior to degradation.

Gate opening and substrate unfolding and translocation

As mentioned before, the 20S contains the proteolytic active sites sequestered in a central chamber (73,76,128). Most intracellular protein degradation occurs in gated chambers in which the proteolytic active sites are protected from the surrounding environment. The gated narrow pore (around 15 Å) allows only the entry of small peptides or unfolded or partially unfolded polypeptides. In eukaryotes, the ATPase ring at the base of the 19S particle is responsible for several different tasks:

1) binding to the 20S particle, 2) interacting with the protein substrate, 3) opening the gate to the proteolytic chamber, 4) unfolding the substrate, 5) translocating the substrate into the 20S chamber. The substrate interacts directly with the ATPase ring which uses the energy from binding and hydrolysis of ATP and converts it into the mechanical force that is responsible for substrate unfolding. The ATPase ring can only bind a total of four ATP molecules, and the ATP hydrolysis likely happens in pairs of para-located subunits of the ring (129).

Insight into this opening mechanism came from work in archaea in which the 20S proteasome associates with an ATPase called PAN (proteasome-activating nucleotidase) (130-132), which is the closest homolog of the eukaryotic ATPase ring. PAN forms a homohexameric ring that has a conserved three amino acid C-terminal motif, called the HbYX (hydrophobic-tyrosine-X) motif that is required for PAN-mediated opening of the entry gate to the proteolytic chamber (85). This HbYX motif is also present in Rpt2, Rpt3 and Rpt5 and these Rpt subunits contact conserved α-subunit residues and insert into shallow pockets formed by the α-ring. This interaction induces conformational changes that result in the opening of the 20S pore (85,133).

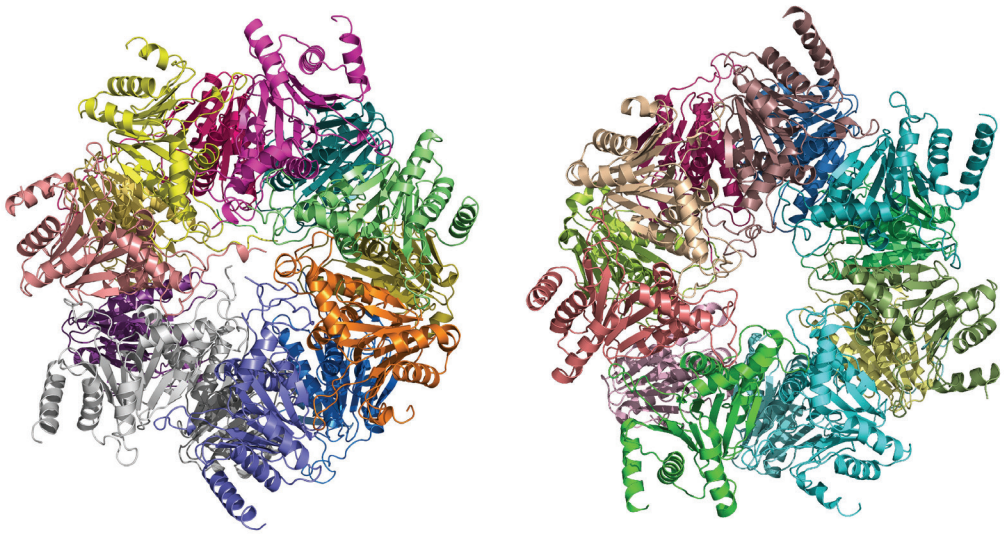


Figure 9- The 20S proteasome α-annulus. Top views of the crystal structure of the proteasome. In the closed conformation the N-terminal residues of the α-subunits block entry to the 20S proteasome (PDB ID: 1RYP). In the open conformation the

Trypanosome brucei PA26 carboxy-terminal tails (not displayed in the figure) help open the yeast 20S annulus (PDB ID: 1FNT).

Recently, work by Matyskiela et al (87) revealed in more detail the mechanism of substrate processing by the proteasome during substrate degradation. During protein degradation the regulatory particle assumes two different conformations: a pre-engaged conformation and a translocation-competent conformation. Upon substrate binding, several major conformational alterations occur that culminate in a coaxial alignment of the N-ring, the AAA ring and the 20S entry pore and repositioning of the Rpn11 active site over the N-ring, thus optimizing the structure for substrate entry, deubiquitination, unfolding and translocation (87).

D. CDC48

Often vital to degradation of protein substrates is their disassembly from protein complexes, unfolding, or extraction from membranes. These processes are catalyzed by the conserved AAA-ATPase Cdc48, also called valosin containing protein (VCP) or p97 in mammals (134). This abundant protein (about 1% of the cellular protein pool) has a variety of functions in numerous pathways. It is well-conserved and consists of a globular N-terminal domain, two type II AAA ATPase domains that share 40% sequence identity (called D1 and D2) followed by a disordered C-terminal tail (Fig. 10). Cdc48 assembles into a homohexameric ring with a central pore, thus allowing this protein to work as a segregase and/or unfoldase in an ATP-dependent manner.

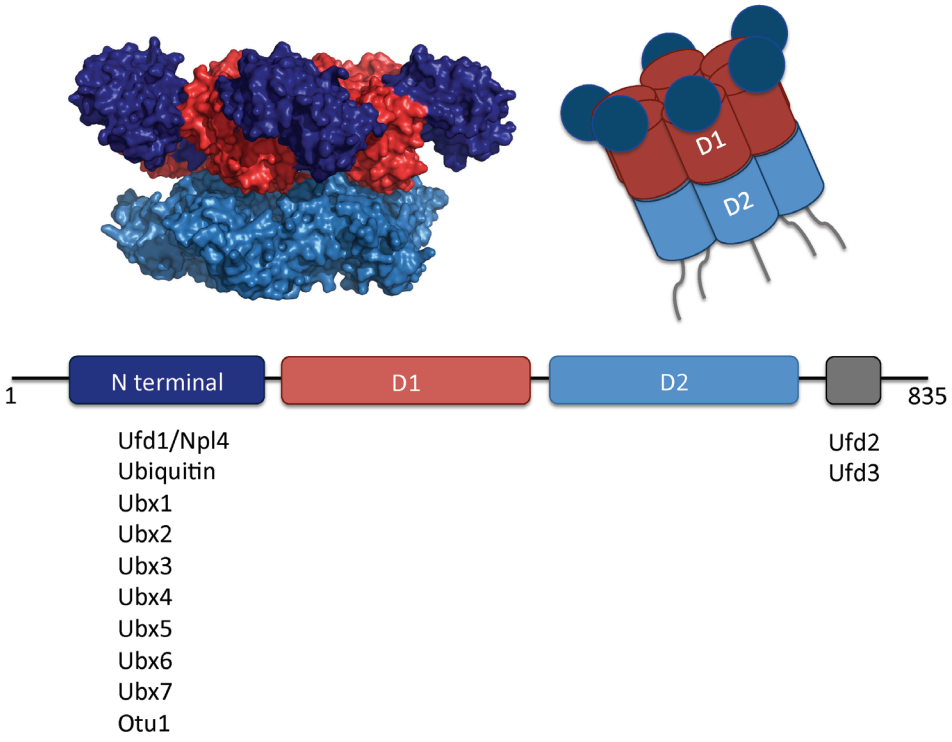


Figure 10- Structure of p97/Cdc48 and its domain architecture (PDB ID: 3CF1). Cdc48 N- and C-terminal domains are responsible for binding to cofactors. The D1 and the D2 domains are not equivalent. D1 has been shown to play a role in the hexamerization while D2 is necessary for the ATPase activity of Cdc48 (135-137).

Cdc48 was first found in a genetic study in which conditional mutations affecting the cell division cycle (and hence the name cdc) were identified (138). Sequencing of that gene unveiled a novel protein of 92 kDa (134). The link to the UPS came some years later, when it was found that Cdc48 interacts with Doa1 and that like Doa1, Cdc48 is required for the ubiquitin-dependent degradation of UFD (Ub fusion degradation) model substrates (139).

Cdc48 cofactors

Cdc48 interacts with a plethora of cofactors and this is key to explaining the wide range of functions and different pathways in which this protein plays a role. Cdc48 cofactors are involved in substrate binding, substrate processing and recruitment of Cdc48 to specific locations within the cell (Table 2). Cdc48 exerts its functions in various subcellular locations: the endoplasmic reticulum (ER), lysosome, Golgi, nucleus, cytosol, mitochondria, peroxisome, and plasma membrane (140). Substrate processing Cdc48 cofactors include those that can extend or trim Ub chains, called an E4 ligase and a deubiquitinating enzyme (DUB), respectively.

Members of the largest family of Cdc48 cofactors are related by the UBX (ubiquitin regulatory X) domain; there are seven UBX proteins in *S. cerevisiae*. Structural studies of the UBX domain, which binds specifically to the Cdc48 N-terminal domain (141), reveal a β-grasp fold similar to that of ubiquitin (142). The UBX domain is the most prominent and widespread Cdc48-interacting module, and yeast expresses seven UBX-containing proteins (Table 1) (143-145). The UBX domain consists of about 80 amino acid residues that assume a conformation that structurally resembles the ubiquitin fold. The side chains of the conserved R…FPR motif of the UBX domain are required for the association with Cdc48/p97 (142). This motif is exclusive to UBX domains as it does not exist in either ubiquitin or ubiquitin-like proteins. The first characterized UBX domain-containing Cdc48 cofactor was p47 (called Ubx1 or Shp1 in yeast), which is required for homotypic membrane fusion in the nuclear envelope, Golgi and ER (146,147). Ubx2 is a transmembrane protein of the ER that helps recruit Cdc48 to ubiquitin ligase complexes in the ER membrane (148). Ubx4 is involved in the release of polyubiquitinated substrates to downstream players (149), while Ubx5 appears to recruit Cdc48 complexes to sites of DNA repair (150). Precise functions for the other UBX proteins in yeast have not yet been clearly defined.

The most well known Cdc48 cofactor is the heterodimeric cofactor Ufd1/Npl4, which plays a role in the ERAD and UFD pathways (151,152). One Cdc48 hexamer interacts with one Npl4-Ufd1 heterodimer via a short binding site (BS1) in the C-

terminal region of Ufd1 and a region in Npl4 with a similar fold to UBX and ubiquitin (the ubiquitin-D or UBD domain) (153). Both Npl4 and Otu1 possess a ubiquitin-like domain (called UBD) that is responsible for binding the Cdc48 N-terminal region (153). Rather than having a R…FPR motif as in a UBX domain, the Npl4 UBD possesses a surface that is reminiscent of the hydrophobic patch on ubiquitin (154).

Another Cdc48 cofactor, Vms1, has recently been shown to function in both ERAD and in mitochondrial protein degradation. Vms1 deletion leads to an increase in Cdc48-associated ubiquitinated proteins at the ER membrane, thus pointing to a possible role for this cofactor in the release of substrates from Cdc48. In mitochondrial degradation, Vms1 is necessary to recruit Cdc48 to the mitochondrial membrane (155,156). Vms1 binds to the Cdc48 N-terminus through a VIM motif (VCP-interacting motif). The VIM sequence motif has a minimal consensus sequence of RX_5AAX_2R (157).

In recent years Cdc48 (VCP/p97) has received considerable attention due to its involvement in various cellular processes including cell cycle progression, protein degradation, DNA repair, gene transcription, homotypic membrane fusion, apoptosis and membrane trafficking. In each of these processes, Cdc48 is thought to function as a ubiquitin-selective protein segregase, using its ATPase activity to cause conformational changes to ubiquitin-modified proteins, thereby removing these proteins from their interaction partner(s) or specific cellular environment. How does an enzyme with a single activity (ATPase) operate in such a diverse set of processes and with the required specificity? A number of ancillary proteins, referred to as Cdc48 cofactors (Table 2), are crucial to the specificity and versatility of Cdc48 (140). These Cdc48 cofactors regulate Cdc48 activity towards specific substrates in a timely and spatially controlled manner (158).

Table 2- Cdc48's cofactors in yeast: function, pathways they are involved in complex with Cdc48, and motifs they use for binding the ATPase.

Cofactor	Function	Pathway	Interaction Motif
Npl4-Ufd1	Recruitment of Ub conjugates to Cdc48	ERAD, cell cycle control, nuclear transport, DNA repair, transcription, ribosome-associated degradation, clearance of damaged mitochondria	BS1, UBD domain
Ubx1/Shp1	Recruitment of Ub conjugates to Cdc48	Autophagy, nuclear envelop formation, ER biogenesis, Golgi assembly	Ubx domain
Ubx2	Recruitment of Cdc48 to ubiquitin-ligase complexes in the ER membrane , localization of phospholipid:diacylglycerol acyltransferase (Lro1)	ERAD, lipid droplet maintenance	
Ubx3	Unknown	Unknown	
Ubx4	Release of Ub-conjugated substrates from Cdc48	ERAD, DNA repair	
Ubx5	Recruit Cdc48 complexes to chromatin sites of DNA repair	ERAD, DNA repair	
Ubx6	Unknown	Unknown	
Ubx7	Unknown	Unknown	
Otu1	DUB, counteracts Ufd2	ERAD	UBD domain
Vms1	Recruit Cdc48 to the mitochondrial membrane, release of Ub-conjugated substrates from Cdc48	ERAD, mitochondria stress response	VIM (VCP interacting motif)
Ufd2	E4 ligase	Cell cycle control, ERAD, cardiac development, and nervous system maintenance	Ufd2 mutations G274D and C385Ydisrupt binding to Cdc48
Doa1	Not determined, competes with Ufd2	Ribophagy, tRNA import to mitochondria, DNA repair, multivesicular body sorting	PUL (PLAA, Ufd3, Lub1)

Cdc48 function involves ubiquitin binding by either Cdc48 itself (119), its cofactors, or a combination of these interactions (159-161), and ubiquitin interacting domains have been defined in Cdc48 and its cofactors. Shp1 and Ubx2 both possess UBA domains (145,162), while Ubx5 possesses a UBA domain and a UIM motif (163). Doa1, a Cdc48 cofactor that binds Cdc48 through a PUL domain, also possesses a PLAA-family ubiquitin-binding (PFU) domain (164).

VCP/p97 has been implicated in the ethiology of several neurodegenerative disorders (165). Missense mutations in p97 have been found in patients with IBMPFD (Inclusion body myopathy associated with Paget's disease of bone and frontotemporal dementia) (166,167). In *Drosophila*, *ter94* (p97 homolog) has been identified as a modulator of expanded polyglutamine-induced eye degeneration (168). p97 is also a well know binding partner of ataxin-3, the protein whose expansion causes Machado-Joseph disease (169). Moreover p97 has been found in inclusions from neurons of patients suffering from several different neurodegenerative diseases including Machado-Joseph disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (ALS) (165). A full understanding of how Cdc48 and cofactors work can provide invaluable tools that can be used in the treatment of these diseases.

Cdc48 in ERAD

One of the best understood roles of Cdc48 is its involvement in ERAD (endoplasmic reticulum-associated degradation). Proteins destined for the secretory pathway are translocated into the ER lumen where, with the help of several chaperones they fold into their native conformation. In addition to chaperones, ER also contains several other proteins like lectins, N-glycan processing enzymes, protein disulphide isomerases and prolyl cis–trans isomerases (reviewed in (170)). These diferente components of the ER not only aid in folding but also in maturation and post-translocational modifications like the formation of disulfide bridges and well as glycosilation (171). In the ER, proteins undergo a quality control mechanism that assesses protein's folding state. Only proteins that are properly folded and modified

are allowed to leave the ER and follow the secretory pathway (172). But protein folding is error-prone and therefore the resulting misfolded or damaged proteins must be destroyed. Proteins in the ER lumen or associated with the ER membrane must be retrotranslocated to the cytosol or extracted from the ER membrane so that they are accessible for degradation by the UPS (173-175). In *S. cerevisiae* there are two integral ER membrane E3 ligases: Doa10 and Hrd1. Both are RING finger ligases required for the polyubiquitination of ER substrates. There are three different types of ERAD substrates, categorized based on the location of the lesion within the protein substrate: ERAD-L (lumen of the ER), ERAD-M (membrane) and ERAD-C (cytosol). Doa10 works predominantly on ERAD-C substrates while Hrd1 ubiquitinates ERAD-L and ERAD-M substrates, although some flexibility exists (176). Ubiquitination by one of these ubiquitin ligases occurs at the cytosolic side of the ER membrane prior to Cdc48 action (28). It has been shown that Cdc48 together with Ufd1/Npl4 are required for the extraction and subsequent degradation of mutant carboxypeptidase (CPY*), a misfolded ER lumen protein (177). It has also been found that myc-Hmg2p, a membrane protein, interacts with Cdc48 and is stabilized in *cdc48* mutants (178). Moreover, Cdc48 stimulates both the extraction and degradation of CFTR transmembrane domains (179).

In summary, Cdc48 helps in the extraction of both ER lumenal and transmembranar proteins and also aids in segregating proteins from binding partners. Following retrotranslocation, Cdc48 may help substrate degradation by editing the ubiquitin chains.

CONCLUSION

The rapid and selective degradation of proteins inside a cell is key to ensure homeostasis. The ubiquitin-proteasome is the major route for the majority of regulated degradation inside the cell. And although ubiquitin-proteasome system has been the target of several studies, many of the componentes of this system still

remain uncharacterized. Understanding in depth this system might provide unvaluable tools that can be used in the treatment of several UPS-related diseases.

Herein we characterize Cuz1 (previously called Ynl155w and renamed Cdc48-associated Ubl/zinc-finger protein-1, Cuz1), a novel arsenite-inducible protein in yeast that was otherwise uncharacterized, and that we have identified to be a modulator of Cdc48 role in the ubiquitin-proteasome pathway.

Cuz1 interacts directly with Cdc48, an ATPase notably involved in endoplasmic reticulum associated degradation (ERAD). We have also shown that Cuz1 interacts with polyubiquitinated conjugates affecting Cdc48's interaction with them. Furthermore we have identified a ubiquitin related domain that is required for both interactions to occur. Our data suggests that Cuz1 is a novel Cdc48 cofactor. Deletion of Cuz1 lead to defective degradation of misfolded proteins and and combination of Cuz1 deletion with $Cdc48^{Npl4\text{-}Ufd1}$ mutants shows a further impairement in the degradation of certain model substrates.

Interestingly, Cuz1 also interacts with the proteasome and this interaction is enhanced by arsenite. Recent studies have revealed the role of several proteins induced by arsenite that play a crucial role in cell defense against this toxin. In fact, upon proteasome impairment this protein plays a role in helping cells deal with toxicity associated with this compound.

CHAPTER 2- MATERIAL AND METHODS

Yeast strain and plasmid construction- Yeast rich (YPD) and minimal (SD) media were prepared as described previously, and all yeast manipulations were carried out according to standard procedures (180).

The *YNL155W* (*CUZ1*) gene was isolated by PCR amplification from genomic yeast DNA, and inserted into various plasmids. The correct sequence of CUZ1 was verified by DNA sequencing of the entire CUZ1 insert. Plasmid pRS314-FLAG-Cuz1 was derived from pRS314-Cuz1 using Site-directed, Ligase-Independent Mutagenesis (SLIM) (181).

To obtain the FLAG-CUZ1 strain, we used the *delitto perfetto* methodology (182) which allowed us to insert a FLAG epitope sequence upstream of the CUZ1 gene and maintain the endogenous CUZ1 promoter sequences. After insertion of the CORE-I-*SceI* cassette from pGSKU, the FLAG tagged CUZ1 coding sequence was amplified from pRS314-FLAG-Cuz1 using primers whose 5' segments had 40 nucleotides of identity to sequences upstream and downstream, respectively, of the CORE cassette insertion. This PCR product was then transformed into yeast to replace the CORE cassette in *CUZ1* by homologous recombination. Correct recombination was verified by DNA sequencing and anti-FLAG immunoblotting.

Yeast strains were made by mating pre-existing strains or by standard PCR-based homologous recombination techniques. Yeast chromosomal gene deletions and tagging were made by PCR-mediated marker amplification and gene replacement in diploid cells; the resulting diploid heterozygotes were dissected to verify 2:2 marker segregation and to isolate haploid strains. Complete lists of *Saccharomyces cerevisiae* strains and plasmids used in this study are presented in Table 1 and Table 2, respectively.

The DNA sequence encoding a V5 tag was introduced at the C-terminus of chromosomal *CDC48* in wt diploid (MHY606) to obtain MHY7900. The V5 tag sequence was generated by PCR amplification using the pFA6a-6xGly-V5-His3MX6 plasmid as a template.

DNA sequences encoding 6His-Cdc48 or 6His-Cdc48(1-220) were PCR-

amplified from genomic yeast DNA using an oligonucleotide that introduced a 6xHis tag and then cloned into pET42b using *Nde*I and *Xho*I restriction sites, which removed the sequence for the GST tag from the plasmid. DNA sequencing confirmed that the ORF contained no mutations.

Plasmid pGEX-KT was used to express full-length Cuz1 and different Cuz1 deletion variants as GST fusions in *E. coli*. *CUZ1* sequences were obtained by either amplifying the desired DNA fragments from yeast genomic DNA and inserting them downstream of the GST coding sequence in pGEX-KT or by site directed mutagenesis to insert a stop codon at the desired sites. To make pGEX-KT-Cuz1-4S, Cys-to-Ser codon mutations were introduced into the *CUZ1* sequence using two sequential Quikchange (Stratagene) site-directed mutagenesis reactions.

Table 1- List of yeast strains used in this study.

Strain	Genotype	Source
MHY500	*MATa his3-∆200 leu2-3,112 ura3-52 lys2-801 trp1-1*	(183)
MHY606	*his3-∆200 /his3-∆200 leu2-3,112/ leu2-3,112 ura3-52/ ura3-52 lys2-801/lys2-801 trp1-1/trp1-1 gal2/gal2*	(184)
MHY2483	*MATalpha his3-∆200 leu2-3,112 ura3-52 lys2-801 trp1-1 npl4-1*	(185)
MHY2836	*MATa his3-Δ200 leu2-Δ1 ura3-52 lys2-801 trp1-Δ63 ade2-101*	(186)
MHY3566	*MATa his3-∆200 leu2-3,112 ura3-52 lys2-801 trp1-1 ufd1-2*	(185)
MHY4464	*MATa his3-Δ200 leu2-Δ1 ura3-52 lys2-801 trp1-Δ63 ade2-101 cim3-1*	(187)
MHY4466	*MATa his3-Δ200 leu2-Δ1 ura3-52 lys2-801 trp1-Δ63 ade2-101 cim5-1*	(187)
MHY5532	*MATa his3-Δ200 leu2-Δ1 ura3-52 lys2-801 trp1-Δ63 ade2-101 cuz1Δ::kanMX6*	This study

MHY5533	*MATalpha his3-Δ200 leu2-Δ1 ura3-52 lys2-801 trp1-Δ63 ade2-101 cuz1Δ::kanMX6*	This study
MHY5550	*MATa his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 cuz1Δ::kanMX6*	This study
MHY5551	*MATalpha his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 cuz1Δ::kanMX6*	This study
MHY5635	*MATa his3-Δ200 leu2-Δ1 ura3-52 lys2-801 trp1-Δ63 ade2-101 cim3-1 cuz1Δ::kanMX6*	This study
MHY5637	*MATa his3-Δ200 leu2-Δ1 ura3-52 lys2-801 trp1-Δ63 ade2-101 cim5-1 cuz1Δ::kanMX6*	This study
MHY5840	*MATalpha his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 RPN5-6xGly-3xFLAG::hphMX4*	This study
MHY5841	*MATalpha his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 RPN11-6xGly-3xFLAG::hphMX4*	This study
MHY6952	*MATa his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 PRE1-6xGly-3xFLAG::hphMX4*	This study
MHY7812	*MATa his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 FLAG-CUZ1*	This study
MHY7900	*MATalpha his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 CDC48-6xGly-V5::HIS3MX6*	This study
MHY7969	*MATa his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 CDC48-6xGly-V5::HIS3MX6 RPN5-6xGly-3xFLAG::hphMX4*	This study
MHY8185	*MATa his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 npl4-1 cuz1Δ::kanMX6*	This study
MHY8196	*MATalpha his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 npl4-1 cuz1Δ::kanMX6*	This study
MHY8210	*MATa his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 cdc48-6*	This study
MHY8218	*MATalpha his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 CDC48-6xGly-V5::HIS3MX6 FLAG-CUZ1*	This study

MHY8223	*MATa his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 ufd1-2 cuz1Δ::kanMX6*	This study
MHY8224	*MATalpha his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 ufd1-2 cuz1Δ::kanMX6*	This study
MHY8228	*MATa his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 cdc48-6 cuz1Δ::kanMX6*	This study
MHY5616	*MATa ura3-52 leu2Δ1 his3Δ-200 trp1Δ63 lys2-801 ade2-101 cuz1Δ::kanMX6 rpn4Δ::KanMX4*	This study
MHY5617	*MATalpha ura3-52 leu2Δ1 his3Δ-200 trp1Δ63 lys2-801 ade2-101 cuz1Δ::kanMX6 rpn4Δ::KanMX4*	This study
MHY6612	*MATa ura3-52 leu2Δ1 his3Δ-200 trp1Δ63 lys2-801 ade2-101 rad23Δ::TRP1 cuz1Δ::kanMX6*	This study
MHY6613	*MATalpha ura3-52 leu2Δ1 his3Δ-200 trp1Δ63 lys2-801 ade2-101 rad23Δ::TRP1 cuz1Δ::kanMX6*	This study
MHY3808	*MATalpha ura3-52 leu2Δ1 his3Δ-200 trp1Δ63 lys2-801 ade2-101 rad23Δ::TRP1*	This study
MHY6064	*MATa ura3-52 leu2Δ1 his3Δ-200 trp1Δ63 lys2-801 ade2-101 dsk2Δ::HIS3 cuz1Δ::kanMX6*	This study
MHY3607	*MATa ura3-52 leu2Δ1 his3Δ-200 trp1Δ63 lys2-801 ade2-101 dsk2Δ::HIS3*	This study
MHY6604	*ura3-52 leu2-3,112 his3-D200 trp1-1 lys2-801 gal2 pdr5Δ::KanMX6*	This study

Table 2 - List of plasmids used in this study.

Plasmid	Source
Yep112 HA-Ub	(188)
pGEX-KT	(189)
pGEX-KT-Cuz1	This study
pGEX-KT-Cuz1-4C	This study
pGEX-KT-Cuz1-1-59	This study
pGEX-KT-Cuz1-1-161	This study
pGEX-KT-Cuz1-59-161	This study
pGEX-KT-Cuz1-60-274	This study
pGEX-KT-Cuz1-168-274	This study
pET42b 6His-Cdc48	This study
pET42b 6His-Cdc48-1-220	This study
pGEX-KG-Rad23	(190)
pDN431	(191)
pUb^{V76}-lacZ	(192)
pRS314-Cuz1	This study
pRS314-FLAG-Cuz1	This study

Identification of Cuz1-binding proteins by LC-MS/MS- Late log-phase 2-liter cultures of yeast cells were harvested by centrifugation. Cell pellets were washed with ice-cold water, centrifuged, flash frozen in liquid nitrogen, and stored at -80^{o}C. Cell lysis was achieved by grinding cells to a fine powder in liquid nitrogen (193). The powder was resuspended in a buffer containing 50 mM HEPES pH 7.5, 200 mM NaCl, 10% glycerol, 0.5% Triton X-100 and Complete Protease Inhibitor tablets (Roche). The extract was centrifuged for 25 min at 30,000 x *g* to pellet cell debris. Protein concentration was determined using the BCA assay (Pierce), and 96 mg of protein extract (~40 ml) were mixed with 0.4 ml of FLAG-M2 antibody resin (50% slurry; Sigma-Aldrich). After 2 h rotating at 4°C, the beads were washed four times with 10 ml of the resuspension buffer. Beads were resuspended in 0.6 ml of buffer

and then transferred to a new tube to which 3xFLAG peptide was added to a final concentration of 0.2 mg/ml. After incubation for 45 min at 4°C, the batch eluate was concentrated using a Vivaspin 500 concentrator (MWCO 10,000 kDa; GE Healthcare). SDS-PAGE followed by silver staining was used to evaluate 10% of the concentrated eluate. The remainder was frozen with liquid nitrogen and used for liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis.

The mass spectrometry analysis was performed according to an optimized procedure for LC-MS/MS (194). Briefly, the immunoprecipitated proteins were resolved and excised from a Coomassie blue–stained SDS gel and digested with trypsin. The extracted peptides were loaded on a C_{18} capillary column (75 mm inner diameter, 10 cm length, 2.7 mm HALO C18 resin, tip size 15 mm; New Objective, MA), and then eluted during a 60-min gradient of 10-40% solvent B (solvent A: 0.1% formic acid; solvent B: 70% acetonitrile, 0.1% formic acid, flow rate of 300 nl/min). The eluted peptides were analyzed on a hybrid LTQ Orbitrap Velos MS (ThermoFisher Scientific) with one MS survey scan and up to 10 data-dependent MS/MS scans. Acquired MS/MS spectra were searched against yeast Uniprot database using the SEQUEST algorithm. Searching parameters included mass tolerance of precursor ions (±20 ppm) and product ion (±0.5 Da), tryptic restriction, dynamic mass shifts for oxidized Met (+15.9949), two maximal modification sites, two maximal missed cleavages, as well as only b and y ions counted. To evaluate the false discovery rate during spectrum-peptide matching, all original protein sequences were reversed to generate a decoy database that was concatenated to the original database (195). Assigned peptides were grouped by charge state and then filtered by matching scores (XCorr and DCn) to reduce the protein false discovery rate to 1%.

Dilution series- Cells were grown overnight in 5 ml YPD or selective media. Cultures were diluted to an $O.D._{600}$ of 0.2 and further diluted in 6- or 10-fold series. A fraction of each dilution (4 μL) was then spotted on the indicated plates using a multichannel pipette. Growth was followed over several days and plates were photographed using

a G:Box BioImaging System (Syngene).

Western blot analysis- Proteins were separated by electrophoresis and then transferred to a PVDF membrane. Membranes were blocked with 5% nonfat dry milk in TBS-T (10 mM Tris-HCl pH 8.0, 150mM NaCl and 0.1 % Tween-20) for 45 min and then incubated with the appropriate primary antibody, diluted in 5% nonfat dry milk in TBS-T, for 1 hour at room temperature.

The primary antibodies used in this study were anti-HA (Covance), anti-GST (Abcam), anti-FLAG (Sigma-Aldrich), anti-V5 (Invitrogen), anti-PGK (Invitrogen), anti-β-galactosidase (Millipore), anti-Tetra His (Qiagen), anti-Ub (Covance), anti-Pre6 (a gift from Dr. Dieter Wolf) and anti-Cdc48 (a gift from Dr. Thomas Sommer). An anti-Cuz1 polyclonal antiserum was raised in rabbits and subsequently purified.

Membranes were washed 3 times with TBST for 5 min and then incubated for 1 hour with secondary antibody either anti-rabbit IgG linked to horseradish peroxidase (GE Healthcare) or anti-mouse IgG linked to horseradish peroxidase (GE Healthcare) diluted in 5% nonfat dry milk in TBST. Membranes were again washed 3 times in TBST for 5 min each. ECL detection reagents were added as indicated and the membrane was exposed to the film.

Co-immunoprecipitation and immunoblot analyses- For co-immunoprecipitation (co-IP) experiments, cultures were grown at 30°C to mid-logarithmic phase (A_{600} ~1); where indicated, As_2O_3 was added to a final concentration of 0.2 mM, and the cultures were then incubated with shaking for 2 h, except where indicated otherwise. Cells were harvested by centrifugation. To test the interaction of Cdc48 with Cuz1, lysates were prepared by resuspending cell pellets in co-IP buffer A (25 mM Tris-HCl pH 8.0, 200 mM NaCl, 2 mM $MgCl_2$, 5% glycerol, 1% Triton X-100 and protease inhibitors) and, when indicated, 2 mM ATP. The resuspended cells were disrupted using glass beads in an MP Biomedicals FastPrep bead-beater followed by centrifugation at 21,000 x *g* for 10 min to remove cell debris. After protein quantification, 2.5 mg of protein extract were incubated with 50 µl of FLAG-M2 slurry for 2 h at 4˚C. The beads were washed three times with 1 ml of co-IP buffer and then

resuspended in 25 µl of gel loading buffer. Proteins were resolved by SDS-PAGE and analyzed by immunoblotting.

To test the interaction of polyubiquitinated substrates with Cdc48, cells expressing V5-tagged Cdc48 were transformed with a plasmid expressing a HA-tagged ubiquitin gene under the control of the *CUP1* promoter (188). $CuSO_4$ was added to a final concentration of 0.1 mM when the cultures were diluted. Cells were resuspended in co-IP buffer B without ATP, lysed, and after protein quantification, 1.5 mg of protein was incubated with 40 µl of anti-V5 agarose (Sigma). After 2 h, the beads were washed 3 times with 1 ml of PBS containing 0.2% Tween 20. To test the interaction of the proteasome with polyubiquitinated substrates, cells were grown in the presence of $CuSO_4$ to logarithmic phase and exposed to As_2O_3 for 2 h. Extracts were prepared with co-IP buffer B without ATP; 1 mg of protein was incubated with anti-FLAG resin, which was then washed with PBS containing 0.2% Tween 20. For testing the interaction of Cdc48 with the proteasome, co-IP buffer C was used: 50 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 5 mM $MgCl_2$, 0.5% Triton X-100.

To test the interaction of Cdc48 with proteasomes *in vivo*, cultures were grown at 30°C to mid-logarithmic phase (A_{600} ~1), and where indicated, As_2O_3 was added to a final concentration of 0.2 mM, and the cultures were then incubated with shaking for 30 min. Cells were harvested by centrifugation. Lysates were prepared by resuspending cell pellets in co-IP buffer C containing 50 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 5 mM $MgCl_2$, 0.5% Triton X-100 and protease inhibitors and using glass bead lysis. After protein binding to FLAG-M2 slurry, the resin was washed three times with 1 ml of PBS containing 0.2% Tween 20. The bound material was eluted by boiling with gel loading buffer and analyzed by western blot.

To test the interaction of Cuz1 with proteasomes *in vivo*, cultures were grown at 30°C to mid-logarithmic phase (A_{600} ~1); where indicated, As_2O_3 was added to a final concentration of 0.2 mM and the cultures were then incubated with shaking for 2 h. Cells were harvested by centrifugation. Lysates were prepared by resuspending cell pellets in co-IP buffer B containing 50 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 5 mM $MgCl_2$, 0.1% Triton X-100, 5 mM ATP and protease inhibitors and using glass bead lysis. After protein binding to FLAG-M2 slurry, the resin was

washed three times with 1 ml of PBS containing 0.2% Tween 20. The bound material was eluted by boiling with gel loading buffer and analyzed by western blot.

To test the interaction of the proteasome with polyubiquitinated substrates, cells with ubiquitin overexpressed from a copper-stimulated *CUP1* promoter on a high-copy plasmid were grown in the presence of $CuSO_4$ to logarithmic phase and exposed to As_2O_3 for 2 h. When indicated, MG132 was added to the cultures to a final concentration of 40 μM and the culture incubated with shaking at 30°C for 1.5 hours. Extracts were prepared with co-IP buffer B without ATP; 1 mg of protein was incubated with anti-FLAG resin, which was then washed with PBS containing 0.1% Tween 20. Bound material was eluted by boiling in SDS gel-loading buffer.

Recombinant protein purification and in vitro binding assays – Expression of GST and GST fusion proteins was induced in *E. coli* BL21 (DE3) transformants by addition of 1 mM IPTG and overnight growth at 30°C. The GST fusion proteins were purified with glutathione-agarose (Thermo Scientific) and eluted with reduced glutathione according to manufacturer instructions. Expression of 6His-Cdc48 in *E. coli* Rosetta2 (DE3) pLysS cells was induced by adding 1 mM IPTG for 4 h at 30°C. The recombinant protein was purified using HisPur Cobalt Resin (Thermo Scientific) and eluted using a buffer containing 150 mM imidazole. All the purified recombinant proteins were dialyzed against 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM $MgCl_2$, and 10% glycerol.

For testing the interaction of Cdc48 with GST-Cuz1 and Cuz1 deletion derivatives, the purified proteins were used in a 1:1 molar ratio of Cuz1 monomer to Cdc48 monomer in GST pulldown assays. Binding reactions were incubated for 2 h at 4°C in a final volume of 0.4 ml of co-IP buffer A. For measuring interaction of ubiquitinated substrates with GST-Cuz1 and its derivatives, recombinant proteins (3 μg) were incubated with 800 mg of yeast extract from cells overexpressing HA-tagged ubiquitin. Extract preparation as well as GST pulldowns were performed with co-IP buffer D: 50 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 5 mM $MgCl_2$, 1% Triton X-100. After incubation for 2 h at 4°C, beads were washed with the same buffer and proteins were eluted by boiling in gel loading buffer.

To test the direct interaction between GST-Cuz1 and K48 linked 2,7 molecules ubiquitin chains (Boston Biochem) we used 3 µg of GST fusion proteins and mixed it with purified chains in PBS 1X with 0.1% Triton X-100. After incubation for 2 h at 4°C, beads were washed with the same buffer and proteins were eluted by boiling in gel loading buffer.

To test the interaction between 26S proteasome and Cuz1 or Cuz1 truncations *in vitro*, 26S or 19S proteasomes were purified as described in (92) and then mixed with recombinant GST-Cuz1 and truncations in co IP buffer B. The proteins were bound to 40 µL of glutathione-agarose for 2 h and washed three times with 1 ml of PBS containing 0.2% Tween 20. The bound material was eluted by boiling with gel loading buffer followed by western blotting analysis.

To test interaction between the shuttle factors and the proteasome, strains expressing FLAG-tagged proteasome were transformed with either p424GAL or p424GAL-Cuz1. Cells were grown in SD-Trp and then switched to SD-Raf. Cuz1 was expressed by adding galactose to the media to a final concentration of 2%. Extracts were prepared in a buffer containing 50 mM Hepes pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 5 mM ATP and one tablet of EDTA-free protease inhibitor (from Roche). The extract (800 mg) was mixed with 20 µg of shuttle factor and 40 µL of glutathione-agarose and incubated for 2 hours at 4°C. Beads were washed 3 times with PBS 1X, 0.1% Tween-20 and 5 mM ATP. Beads were resuspended in gel loading buffer, and heated to 100°C for 5 min; the lysates were centrifuged for two min at 21,000 x *g* to remove cell debris. Levels of bound proteasome were analyzed by western blot analysis using anti-FLAG antibody. Bands were quantified using the G:Box BioImaging System

Cycloheximide-chase/immunoblot and pulse-chase analyses – For analysis of UFD and ERAD model substrates degradation by cycloheximide-chase/immunoblot assays, cultures were grown at room temperature (~23°C) to logarithmic phase and switched for one hour to 37°C. Cycloheximide was added to a final concentration of 0.25 mg/ml, and 2.5 OD_{600} equivalents of cells were harvested at each time point. The chase was performed at 37°C. Cell pellets were resuspended in 0.1 ml of water

plus 0.1 ml of 0.2 M NaOH and incubated for 5 min at room temperature. Cells were pelleted by centrifugation, resuspended in gel loading buffer, and heated to 100°C for 5 min; the lysates were centrifuged for two min at 21,000 x *g* to remove cell debris. Pulse-chase analysis was performed essentially as described previously (183). Cultures were grown at 23°C to exponential phase; after washing, cells (~10 OD_{600} equiv.) were incubated for 4 min at 28˚C and labeled with ~0.2 mCi [35S]-Translabel (MP Biomedicals) for 10 min and chased with excess cold methionine and cysteine at 28°C. Immunoprecipitation was performed using anti-β-galactosidase antibody and protein-A-agarose (Repligen). Immunoprecipitated proteins were separated by SDS-PAGE and analyzed by autoradiography using a Storm 860 Phosphorimager system and ImageQuant 5.2 software (Molecular Dynamics).

Analysis of Cuz1 degradation was performed by cycloheximide-chase/immunoblot assays. Cultures were grown at 30°C to logarithmic phase. Cycloheximide was added to a final concentration of 0.25 mg/ml, and 2.5 OD_{600} equivalents of cells were harvested at each time point. The chase was performed at 30°C. Cell pellets were resuspended in 0.1 ml of water plus 0.1 ml of 0.2 M NaOH and incubated for 5 min at room temperature. Cells were pelleted by centrifugation, resuspended in gel loading buffer, and heated to 100°C for 5 min; the lysates were centrifuged for two min at 21,000 x *g* to remove cell debris. Degradation was followed by western blot with anti-Cuz1 antibody.

In-gel substrate-overlay assay- For these assays, yeast whole extracts derived from liquid nitrogen-frozen cells that were ground to a fine powder were resolved by nondenaturing PAGE, and the gels were incubated with the fluorogenic Suc-LLVY-AMC peptide (as described in (196)). Proteasome bands were visualized by exposure to UV light.

CHAPTER 3- CUZ1, A NOVEL MODULATOR OF CDC48 FUNCTION IN THE UBIQUITIN-PROTEASOME SYSTEM

INTRODUCTION

Proteasome-associated proteins

Proteasome composition has been thoroughly analyzed (92,94) and there are many proteins that reversibly associate with the proteasome and are important for regulatory functions. These proteasome-interacting proteins (PIPs) include amongst others ubiquitin ligases, deubiquitinating enzymes and UBA-UBL proteins (197,198). In response to various conditions, proteasome function and composition can be subject to regulation in order to help the cell cope with the new challenges.

Arsenic is the 20th most abundant element on earth. It is found in both organic and inorganic states. It is most often found in its inorganic state, as arsenite trioxide (As_2O_3, white arsenic), realgar (As_4S_4, red arsenic) and orpiment (As_2S_3, yellow arsenic). The trivalent species is more toxic.

The metalloid arsenic is a natural environmental contaminant to which we are exposed in food, water, air, and soil but has been used throughout times with different purposes. It has been identified as a human carcinogen (199) and has also been implicated in vascular disorders, neuropathy and diabetes (200). Interestingly arsenic is an effective treatment of acute promyelocytic leukemia (APL) (201).

The classical view is that arsenic toxicity derives from its ability to react with vicinal sulfhydryl groups in proteins, substitute essential metal ions in metal-dependent proteins, or catalyze formation of reactive oxygen and nitrogen species (202). Arsenite specifically interacts with actin and tubulin, disrupting the cytoskeleton in *S. cerevisiae* (203). Moreover, arsenite affects protein folding by acting on unfolded polypeptides and directly inhibiting chaperone activity, triggering the formation of protein aggregates (204). Exposure of yeast cells to arsenite induces a complex transcriptional program that upregulates several genes involved in the

ubiquitin-proteasome system, including chaperones, proteasomal genes, and ubiquitination genes, amongst others. Rpn4 is a key transcription factor involved in this process (203,205).

Several arsenite-inducible proteins containing AN1 zinc-finger domains interact with the proteasome. AIRAP, (arsenite-inducible RNA associated protein) is a specific mammalian proteasome subunit induced by arsenite and electrophiles that binds to the 19S base. Upon arsenite exposure there is an accumulation of misfolded proteins. AIRAP is thought to change the biochemical properties of the proteasome, facilitating substrate degradation and helping the cells cope with the stress (206). AIP-1, AIRAP-L's *C.elegans* homologue, and it has also been shown to interact with the proteasome. This protein has been linked to resistance to proteotoxicity and maintenance of animal life span (207). AIRAP-L (AIRAP-like) is another mammalian protein that binds to the proteasome, and like AIRAP-containing proteasomes, proteasomes containing AIRAP-L show enhanced activity towards specific substrates. In addition AIRAP-L shares some specific properties with AIP-1, namely its importance for animal lifespan (207).

Substrate delivery to the proteasome is a complex process and the exact details are still not well understood (reviewed in (208)). Proteasomes possess intrinsic ubiquitin receptors, namely Rpn10 and Rpn13 (check introduction for further details). Another group of proteins, collectively called shuttle factors are involved in the delivery of polyubiquitinated substrates to the proteasome. This group includes Rad23, Dsk2 and Ddi1.

Rad23 was first identified to be involved in DNA repair pathway and the nuclear excision repair complex. Rad23 binds several DNA repair factors that are involved in nucleotide excision repair (NER) and specifically binds and stimulates Rad4/XPC activity, in addition to promoting the assembly of DNA repair complexes (209-212). The link to the ubiquitin-proteasome system came afterwards, when it was discovered that Rad23 could interact with the proteasome through its UBL (ubiquitin-like) domain (89,213), in addition to binding ubiquitin (111,112) and K48-linked ubiquitin chains (116) through its UBA domains (ubiquitin- associated). Moreover, mutations in *RAD23* displayed a pleiotropic effect *RPN10* deletion, including slow

growth, an increase in the level of Ub conjugates and a stabilization of specific UPS targets (117).

Dsk2 (dominant supressor of *kar1*) was first identified as a supressor of *kar1*, which is defective in spindle pole duplication (214). Later it was discovered that Dsk2 through its C-terminal UBA domain, interacted with polyubiquitin chains, displaying special preference for K48-linked chains (113). In addition, Dsk2 also interacts with the proteasome specifically with the Rpn1 subunit and similarly to Rad23, Dsk2 also used its UBL domain for binding the proteasome (89,113). Overexpression of Rpn10 supressed the growth defect caused by Dsk2 overexpression, which could be explained by the fact that extra-proteasomal Rpn10 controls the access of Dsk2 to the proteasome (105). This hypothesis was further supported since Dsk2 could be found in proteasomes purified from *rpn10Δ* yeast but not from wild type (105). Interestingly, Rpn10 and Dsk2 function together as ubiquitin chain-length sensor, by forming a ternary complex between Rpn10, Dsk2 and a polyubiquitin chain. Dsk2 UBA domain outcompetes Rpn10 UIM for binding to mono or di-ubiquitin and consequently only chains with at least 4 ubiquitin molecules will actually bind Rpn10 (215).

Ddi1 (DNA damage inducible 1), also known as Vms1, is involved in multiple cellular processes including ubiquitin-proteasome system, cell-cycle control and suppression of protein secretion from the cell (216-219). Ddi1 contains a N-terminal ubiquitin-like domain (UBL), followed by an aspartyl protease domain conserved from retroviruses (RVP) and a C-terminal ubiquitin associated domain (UBA). Similarly to the other known shuttle factors, Ddi1 interacts with the proteasome through its UBL domain (219) and to ubiquitin through its UBA domain (112). Ddi1 is involved in the degradation of several substrates including the Ho endonuclease and Ufo1. Ddi1 UBA domain interacts with ubiquitinated Ho and Ufo and transfers it to the proteasome in a Ddi UBL dependent manner (219,220).

In summary, shuttle factors, although not being integral proteasome subunits, are able to recruit polyubiquitinated substrates to the proteasome by binding these proteins via UBA and UBL domains, respectively. These characteristics fit with the

possibility that shuttle factors might be involved in the transfer of conjugates on their way to the proteasome.

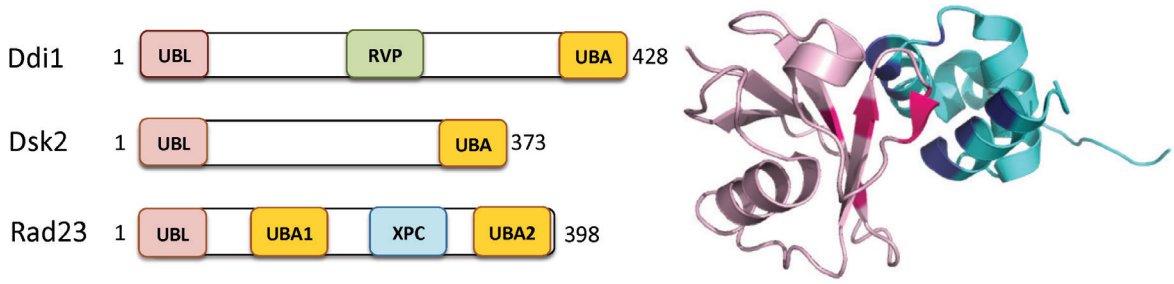


Figure 1- Schematic view of the domain organization of the known shuttle factors. UBA- Ubiquitin associated domain; UBL- Ubiquitin like domain; RVP- retroviral aspartyl-protease domain; XPC- Xeroderma Pigmentosum protein C domain. Interaction between Dsk2 UBA (in cyan) and ubiquitin (in light pink). Important residues displayed in darker shades (PDB ID: 1WR1).

Cdc48 may also been involved in the delivery of polyubiquitinated substrates to the proteasome. Previous work from several groups shows that Cdc48 interacts with the proteasome, although the details are far from clear. Reports point for a possible direct interaction between the mammalian versions of Cdc48 and 20S core particle in vitro. Interestingly, the ATPase domains from Cdc48 are distantly related to the ATPase domains of the 19S regulatory particle, and both complexes share the C-terminal HbYX (hydrophobic residue, tyrosine, any residue) motifs that dock into pockets formed at the interfaces of the alpha subunits on the open end of the CP (at least in the case of the proteasomal ATPases) (79). In *T. acidophilum*, deletion of the N-terminal domain of Cdc48 strengthened 20S binding and increased its proteolytic activity, which suggests that binding of cofactors or/and substrates could potentially play a role in the dissociation of the Cdc48-20S proteasome or prevention of their association (81). Other reports point to a possible interaction between the 26S and Cdc48, either for specific substrates (221) (such as those that are hard to unfold) or in situations in which there is an accumulation of polyubiquitinated proteins, such as with arsenite stress (222). Cdc48 is also involved in promoting the disassembly of the

Ufd2-Rad23 complex which will facilitate the delivery of polyubiquitinated substrates from Rad23 to the proteasome (223).

This chapter adresses the functional characterization of Cuz1. First we discovered a new interaction between Cdc48 and Cuz1 and provided evidence for Cuz1 functioning as a novel Cdc48 cofactor. Cdc48-Cuz1 interaction requires the binding of a ubiquitin related domain in Cuz1 to the Cdc48 N-terminal region. We have shown that Cuz1, likely in complex with Cdc48, plays a role in ubiquitin-dependent protein degradation by showing that *CUZ1* deletion, both alone or in combination with $Cdc48^{Npl4\text{-}Ufd1}$ mutants, affects the degradation rates of some model UPS substrates. I propose a role for Cuz1 in the release of polyubiquitinated conjugates from Cdc48 to downstream components of the ubiquitin-proteasome pathway.

Several peptides from proteasome subunits were found in the mass spectrometry analysis of proteins bound to Cuz1. This prompted us to investigate the interaction between the 26S proteasome and Cuz1. In contrast to its interaction with Cdc48, Cuz1 interaction with the proteasome involves the Cuz1 N-terminal region. Interestingly, exposure to arsenite enhances the interaction between Cuz1 and the proteasome. The growth defect displayed by *cim5-1/rpt1-1 cuz1Δ* yeast exposed to arsenite reveals a functional link between the proteasome and Cuz1 in the resistance to the metalloid. Furthermore, when *RPN4* is absent, deletion of *CUZ1* is deleterious to the cells, further supporting a role for this protein in proteasome-dependent resistance to arsenite stress.

We also explored the influence of Cuz1 in the delivery of polyubiquitinated proteins to the proteasome. Overexpression of Cuz1 affects the interaction of the shuttle factors with the proteasome with a concomitant effect in the amount of polyubiquitinated conjugates bound to the proteasome. Furthermore deletion of *RPN10* and *RPN13*, which are proteasome intrinsic receptors, leads to an accumulation of Cuz1 at the proteasome, consistent with the involvement of Cuz1 in the delivery of conjugates to the proteasome.

As observed for Cuz1, exposure to arsenite causes an accumulation, albeit less dramatic, of Cdc48 at the proteasome. Therefore, we analyzed the influence of Cuz1 in the interaction of Cdc48 with the proteasome and we showed that deleting *CUZ1* causes an increase in the levels of Cdc48 interacting with the proteasome indicating that Cuz1 might also be involved in cell's defense against arsenite.

RESULTS

Yeast Cuz1/YNL155w as potential UPS factors – An initial bioinformatic search for uncharacterized *S. cerevisiae* genes that might function in the ubiquitin-proteasome system (UPS) led us to two genes, *YNL155W* and *YOR052C*. These genes are preceded by one or two PACE (*p*roteasome-*a*ssociated *c*ontrol *e*lement) sequences. PACE elements are 9bp motifs (5′-GGTGGCAAA-3′) that bind Rpn4 (224), a 60 kDa protein that contains a C_2H_2 zinc finger domain as well as two acidic regions. Rpn4 interacts with the proteasome and was originally thought to be a proteasome subunit, hence the name regulatory particle non ATPase subunit (4). Interestingly, transcriptional activation by Rpn4 is a feature common to nearly all proteasome subunit genes and also many other genes involved in the ubiquitin-proteasome system including genes involved in ubiquitination and genes involved in stress response, amongst others. Rpn4 is a short-lived protein ($t_{1/2} \leq$ 2min) degraded by the proteasome (225) via ubiquitin-dependent and independent pathways (226). Therefore levels of proteasomes are elegantly regulated by a negative feedback loop controlled by Rpn4: Rpn4 upregulates transcription of proteasome genes and quickly gets degraded by the assembled active proteasome. Interestingly, expression of the RPN4 gene is also regulated by a variety of signals. The promoter region of RPN4 is recognized by the transcription factors Yap1, Pdr1 and Pdr3 as well as the heat shock factor Hsf1. Cellular stresses known to induce RPN4 transcription include arsenic.

The presence of upstream PACEs was our original search criterion. Both *YNL155W* and *YOR052C* encode predicted proteins with AN1-type zinc finger (Zf_AN1) domains, which coordinate a pair of zinc ions and are part of a widespread

structural motif known as the treble-clef domain (227). Treble-clef domains include the RING and IBR domains, both sequence signatures of ubiquitin ligases. The treble-clef fold includes 3 different structural elements: an N-terminal “lateral flap”, a central β-hairpin and a C-terminal α-helix (227). The AN1 zinc finger domain was first identified in the C-terminus of a protein, called An1 from *Xenopus Laevis*. In addition this protein also contains a ubiquitin-like domain at its N-terminal region (228).

Similarity between YNL155w and YOR052c is limited to the Zf_AN1 domains (50% sequence identity over 28 residues, using LALIGN). Two mammalian Zf_AN1 proteins, ZFAND2A/AIRAP (Zn finger-AN1 domain/arsenite-inducible RNA-associated protein) and ZFAND2B/AIRAPL (AIRAP-like), were reported to bind to the 26S proteasome and may modulate its activity (further information on these proteins will be provided in Chapter 4) (206,207). AIRAPL has also been reported to bind p97/Cdc48 (229) and its *C. elegans* ortholog, AIP-1 has been genetically linked to resistance to proteotoxic stress resistance and increased longevity (207).

Both YNL155w and YOR052c belong to the set of proteins in Cluster of *Orthologous* Groups 3582 (*COG3582*). More detailed sequence comparisons show that the human ZFAND family member closest to YNL155w is ZFAND1 (although the latter protein has an additional Zf_AN1 motif at residues 64-105 (Fig. 2)). The biochemical function of ZFAND1 has not yet been elucidated, but ZFAND1 mutations have been linked to several cancers including ovarian carcinoma (230). YOR052c is a much more divergent protein, with only a low level of similarity to mammalian proteins. Human ZFAND1 has diverged substantially from AIRAP (less than 20% identity) and AIRAPL (29% identity over 139 residues).

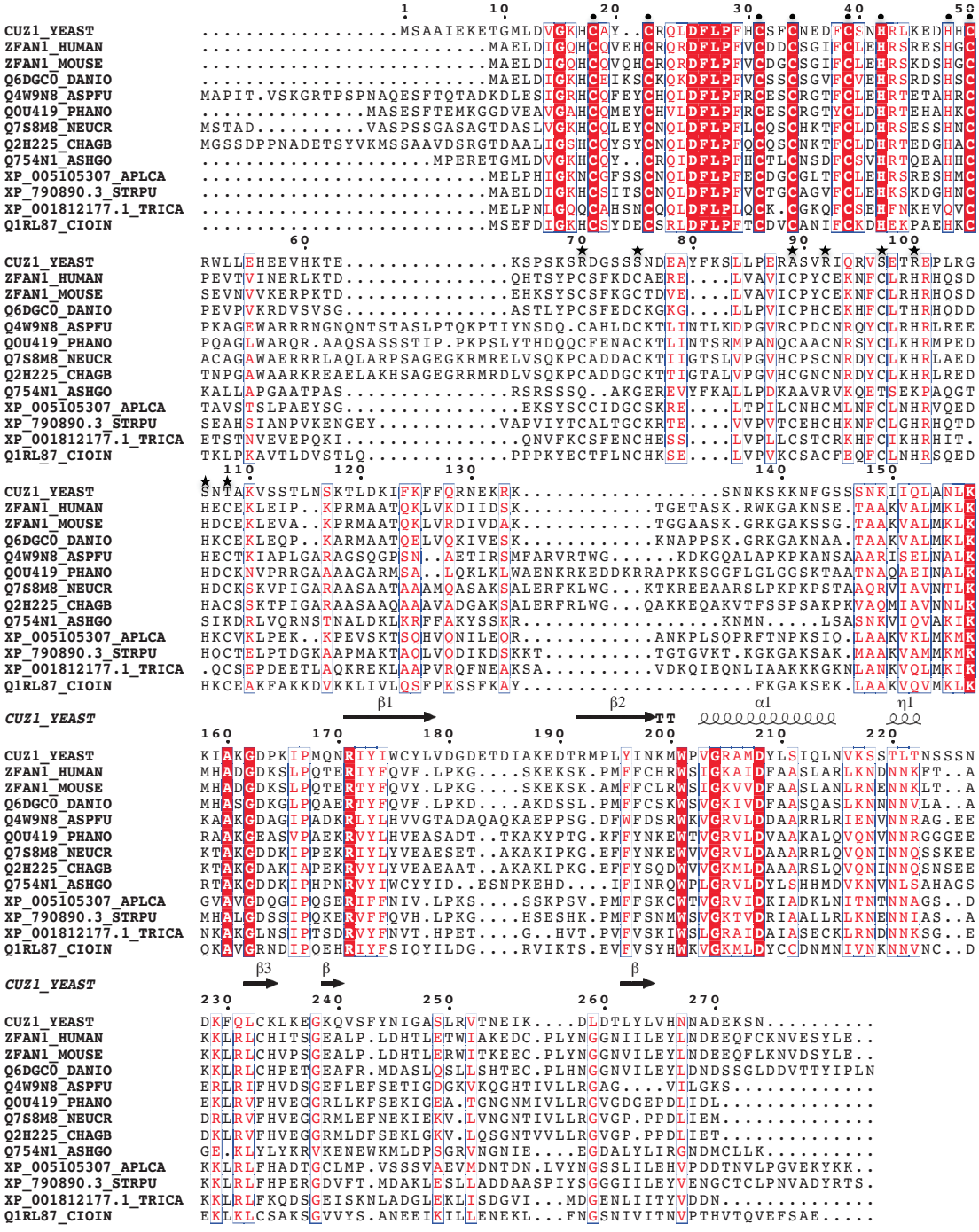


Figure 2- Cuz1 is evolutionarily conserved, containing an AN1-type zinc-finger (Zf_AN1) domain in its N-terminal region. Aligned proteins were selected based on the phylogenetic tree of Cuz1 orthologs from Phylome DB. Alignments were performed with ClustalOmega and edited with ESPript. The expected zinc-coordinating residues of the N-terminal Zf_AN1 domain are indicated with a "•". With

the exception of Cuz1 and Q754N1, all other proteins possess a second Zf_AN1 domain; the putative metal-coordinating residues of this second domain are marked with a "★". Proteins are from the following species: YEAST, *Saccharomyces cerevisiae*; HUMAN, *Homo sapiens;* MOUSE, *Mus musculus;* DANIO, *Danio rerio*; ASPFU, *Aspergillus fumigatus;* PHANO, *Phaeosphaeria nodorum SN15;* NEUCR, *Neurospora crassa*; CHAGB, *Chaetomium globosum*; ASHGO, *Ashbya gossypii*; APLCA, *Aplysia californica*; STRPU, *Strongylocentrotus purpuratus*; TRICA, *Tribolium castaneum*; CIOIN, *Ciona intestinalis.* Secondary structure elements were added using ESPript based on the PDB file of the obtained model for the ubiquitin-like domain.

In addition to the AN1-type Zn finger, sequence and structural homology searches revealed that both YNL155w and ZFAND1 contain an ubiquitin-like domain (UBL) near the C-terminus (Fig. 3 and 4). Conversely, YOR052c, AIRAP, or AIRAP-L lack this C-terminal UBL. Using the Phyre2 structural modeling program (231), the YNL155w sequence between residues 161-265 is readily fit to the structure of ubiquitin (90% confidence score over 79 residues). The two structures were aligned with an RMSD of 2.15 Å over 64 core residues (Fig. 3). Ramachandran plot analysis (MolProbity) of the modeled polypeptide shows 88% of the backbone conformations fall in allowed regions. Similar results were obtained with human ZFAND1 residues 158-250 (Fig. 3).

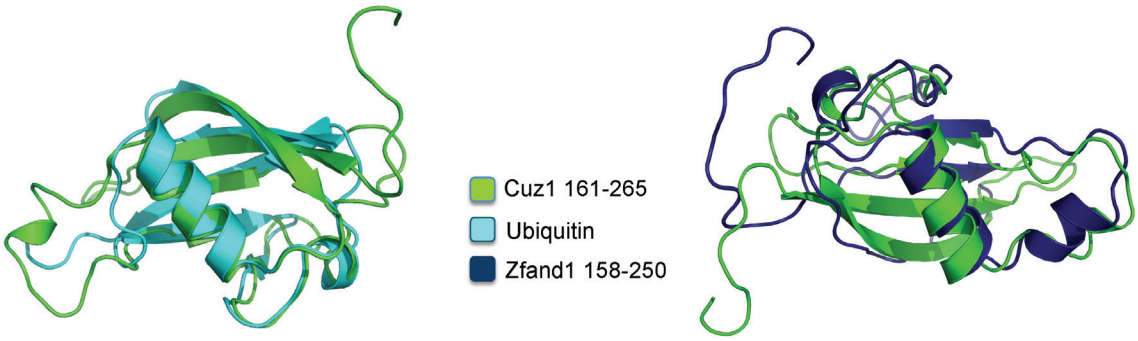


Figure 3- Cuz1 and ZFAND1 contain a C-terminal ubiquitin-like domain (UBL). Protein fold and 3D-structure predictions were obtained using Phyre2 (231). Cuz1 C-terminal model includes fragment 161-265 in green and the alignment with ubiquitin (1UBQ) is shown in cyan (obtained using Pymol). Phyre2 output model was predicted based on human ubiquillin 3 (d1yqba1). The ZFAND1 model includes fragment 158-250 (shown in dark blue) and is based on the structure of the N-terminal DUSP-UBL domain of human Usp15 (with a confidence of 86.2%)

Because of these structural features and the association of YNL155w with Cdc48 (next section), we named the YNL155w protein Cuz1 for Cdc48-associated UBL/Zn-finger protein-1.

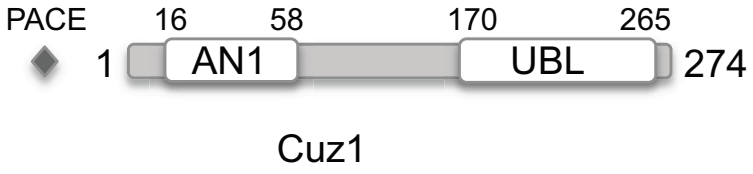


Figure 4- Sequence features of yeast Cuz1/YNL155w. Cartoon depicting domain organization of Cuz1. Proteasome-associated control elements (PACE) are found upstream of the corresponding gene.

A proteomic screen for Cuz1-binding proteins– As a first step to determining Cuz1 potential functions in the UPS, we searched for this protein interaction partners. Toward this end, the chromosomal *CUZ1* locus was modified to encode an N-terminally FLAG-tagged Cuz1 protein. FLAG-Cuz1 was purified under nondenaturing conditions on an anti-FLAG affinity resin. FLAG-Cuz1 and interacting proteins were eluted from the resin with excess 3xFLAG peptide. As a negative control, a parallel purification from yeast cells expressing untagged Cuz1 was performed. A fraction of each eluate was first evaluated by SDS-PAGE and silver staining (Fig. 5). The band corresponding to FLAG-Cuz1 was the most prominent species, and a second protein close to 100 kDa in mass was also seen in the FLAG-Cuz1 eluate and not in the

untagged control.

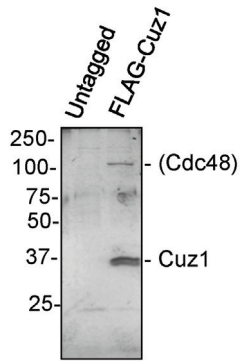


Figure 5- Identification of Cuz1-binding proteins *in vivo*. A) Proteins from a yeast strain expressing FLAG-Cuz1 from the chromosomal *CUZ1* locus, or an untagged control strain, were affinity-purified using anti-FLAG resin; 10% of the purified sample was resolved on a 10% SDS-PAGE gel followed by silver staining.

The remaining fraction of each purified sample was analyzed by LC-MS/MS. Peptides from over 370 different proteins were identified in both the control (untagged Cuz1) and FLAG-Cuz1 preparations. Only those proteins represented by at least five times as many spectral counts in the tagged sample as compared to the untagged control are shown in Table 1. From this analysis, Cdc48 appeared to be the major Cuz1-interacting protein *in vivo*. Cdc48, with a predicted molecular mass of 92 kDa, is likely to be the protein migrating near the 100 kDa size standard in Fig. 5. Notably, we also detected several proteins known to interact with Cdc48: Npl4, Ubx1/Shp1, and more weakly, Ubx2. Npl4 is a Cdc48 cofactor that is bound to the Cdc48 hexameric ring as part of an Npl4-Ufd1 heterodimer (151,232). Ufd1 peptides were not detected in this sample but were detected in two subsequent purifications (see Table 2).

The other proteins identified in the FLAG-Cuz1 purification are also likely to be significant. Ubiquitin was represented by more than eight times the number of spectral counts seen in the control purification (Table 1). Ubiquitin-conjugate binding by Cuz1 (likely indirect) was verified by co-immunoprecipitation analysis (see Fig.

11). Rpn3, Rpn7 and Rpt3, all subunits of the proteasome RP, were also identified (interaction between Cuz1 and the proteasome was subsequently validated; Fig.13). The remaining proteins in Table 1 were not pursued further.

In summary, the mass spectrometry data suggest that Cuz1 functions primarily with the Cdc48 ATPase *in vivo*, possibly with multiple distinct Cdc48-cofactor complexes. The apparent association of Cuz1 with proteasomes and ubiquitin, together with its binding to Cdc48, indicates that Cuz1 may indeed act as a component of the UPS, as was originally suggested by the UBL and Zf_AN1 domains in its polypeptide sequence. Interestingly, a mass spectrometry analysis similar to the one described above was performed using Cuz1 with a C-terminal 3xFLAG tag, and one striking difference was the absence of Cdc48 and cofactors from the list of possible binding partners (M. Funakoshi and M. Hochstrasser, unpublished data). This result is consistent with the Cuz1 C-terminal region playing a role in Cdc48 interaction.

Table 1- The remaining sample from the immunopurification was subjected to LC-MS/MS analysis. Using spectral counts as a semi-quantitative index, the majority of proteins show similar abundance in both samples (untagged versus FLAG-Cuz1). This table shows the proteins from the FLAG-Cuz1 purification that had a spectral count (SC) ratio ≥5-fold above the untagged control.

	Spectral counts		Peptide counts				
Protein	**Control**	**Tag**	**Control**	**Tag**	**Description.**	**MW (kDa)**	**SC ratio**
CDC48	11	2932	5	55	Cell division control protein 48	92	255
CUZ1	11	945	4	22	AN1-type zinc finger protein YNL155W	31	82.2
NPL4	0	20	0	5	Nuclear protein localization protein 4	66	40

UBX1	0	17	0	6	UBX domain-containing protein 1 (SHP1)	47	34
QNS1	4	98	3	17	Glutamine-dependent NAD(+) synthetase	81	21.8
RPN3	0	8	0	7	26S proteasome regulatory subunit RPN3	60	16
RPT3	0	8	0	4	26S protease regulatory subunit RPT3	48	16
RGI2	0	7	0	2	Respiratory growth induced protein 2	19	14
ERG9	0	6	0	2	Farnesyl pyrophosphate synthase	40	12
HXT6/7	0	5	0	3	High-affinity hexose transporters HXT6, HXT7	63	10
SPT5	0	5	0	2	Transcription elongation factor SPT5	116	10
RPL40B	12	102	3	4	Ubiquitin-60S ribosomal protein L40	15	8.2
RPS31	12	102	3	4	Ubiquitin-40S ribosomal protein S31	17	8.2
RPL40A	12	102	3	4	Ubiquitin-60S ribosomal proteinL40	15	8.2
UBI4	12	102	3	4	Polyubiquitin	43	8.2
PCK1	5	44	3	8	Phosphoenolpyruvate carboxykinase [ATP]	61	8
YGR250C	0	4	0	3	Uncharacterized RNA-binding protein YGR250C	89	8
UBX2	0	4	0	2	UBX domain-containing protein 2	67	8
VAR1	0	4	0	1	Ribosomal protein VAR1, mitochondrial	47	8
RPB2	2	18	2	9	RNA polymerase II second largest subunit B150	139	7.2
ARO1	36	224	17	44	Pentafunctional AROM polypeptide	175	6.1
RPB5	0	3	0	2	DNA-directed RNA polymerases I, II, and III subunit	25	6
FAS1	0	3	0	2	Fatty acid synthase subunit beta	229	6

RPN7	0	3	0	2	26S proteasome regulatory subunit RPN7	49	6
FPS1	0	3	0	2	Glycerol uptake/efflux facilitator protein	74	6
FBP1	0	3	0	2	Fructose-1,6-bisphosphatase	38	6
FRD1	0	3	0	2	Fumarate reductase	51	6
GAD1	2	14	2	6	Glutamate decarboxylase	66	5.6
RPB1	8	45	6	16	RNA polymerase II largest subunit B220	191	5.3

Cuz1 associates directly with the Cdc48 AAA-ATPase– To validate the *in vivo* interaction of Cuz1 and Cdc48 that was suggested by the LC-MS/MS analysis, we fused the chromosomal copy of *CDC48* with a sequence encoding a V5 epitope tag. The tag on the essential Cdc48 protein caused no detectable growth defect. Using a strain that had both the chromosomal *CDC48-V5* and *FLAG-CUZ1* alleles, we generated whole-cell extracts under nondenaturing conditions and immuno-precipitated FLAG-Cuz1 and any associated proteins with anti-FLAG antibody beads. As seen in Fig. 6A, FLAG-Cuz1 efficiently co-precipitated the Cdc48-V5 protein. Interestingly, addition of 2 mM ATP to the extraction buffer consistently reduced the amount of co-precipitated Cdc48 protein (Fig. 6A, lanes 3 and 5 vs. lanes 2 and 4). This effect of ATP was confirmed by LC-MS/MS analysis; by comparing Cdc48 spectral counts in FLAG-Cuz1 purifications in buffers with or without added ATP, a greater than two-fold reduction in counts was seen when ATP was added compared to extracts without the added nucleotide (Table 2).

To determine whether the association of Cuz1 with Cdc48 was direct or mediated by other proteins, *in vitro* binding assays were carried out using recombinant GST-Cuz1 and 6His-Cdc48 proteins both purified from *E. coli*. GST or GST-Cuz1 was immobilized on glutathione-agarose beads and incubated with 6His-Cdc48. GST-Cuz1, but not GST, was able to pull down 6His-Cdc48, indicating that their interaction did not require any other yeast proteins (Fig. 6B). Cdc48 interacts

with its cofactors through either the Cdc48 N-terminal domain or its C-terminal tail, although the N-terminal domain is the predominant interaction site (161). Therefore, we asked whether a fragment encompassing the Cdc48 N-terminal domain (residues 1-220) would be sufficient for Cuz1 binding. As shown in Fig. 6C, this was in fact the case.

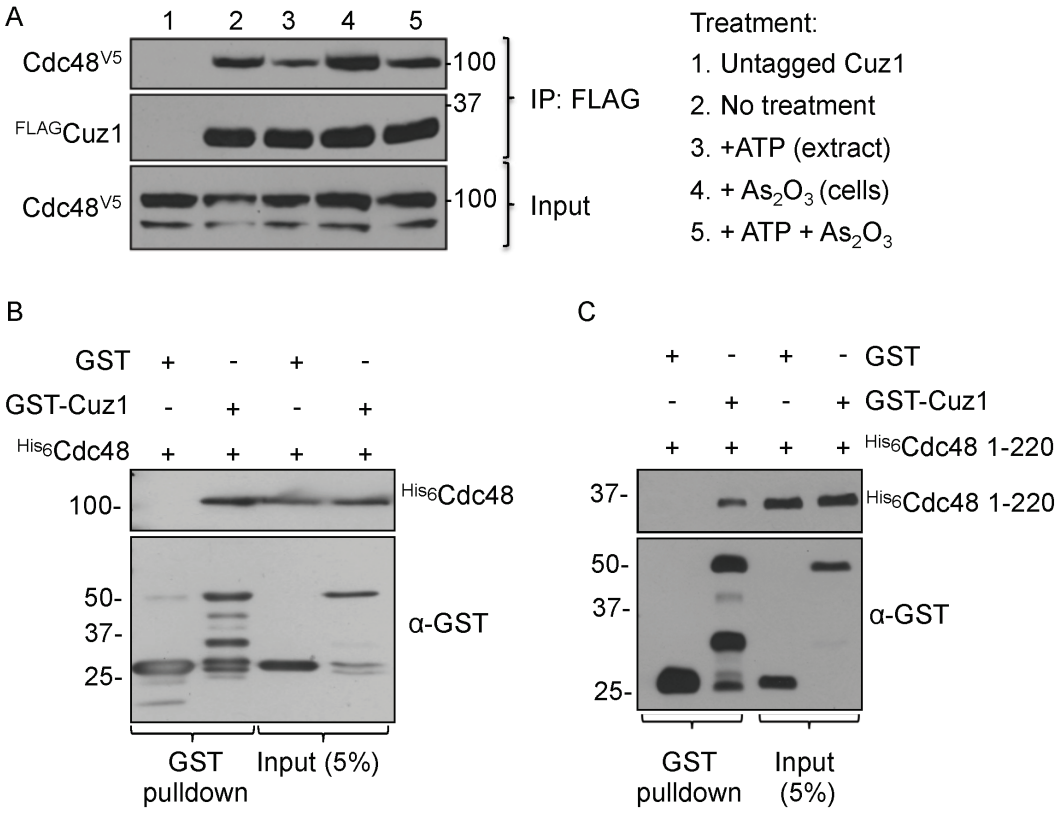


Figure 6- Cuz1 interacts directly with Cdc48. A) Cuz1 and Cdc48 associate *in vivo*, and ATP reduces their interaction. Cells expressed FLAG-tagged Cuz1 and Cdc48-V5 or, as a control, untagged Cuz1 and Cdc48-V5. Cultures were treated with 0.2 mM As_2O_3 for 2 h prior to lysis where indicated. ATP (2 mM) was added to the extracts where indicated. Following immunoprecipitation of FLAG-Cuz1, anti-V5 and anti-FLAG immunoblot analysis was performed. B) Cdc48 interaction with Cuz1 is direct. 6His-Cdc48, GST and GST-Cuz1 were expressed in and purified from *E. coli*. GST pulldowns were followed by anti-His-tag and anti-GST immunoblotting. C) Cuz1 interacts with N-terminal domain of Cdc48. Binding of GST-Cuz1 to 6His-Cdc48 1-

220 was tested as described for the full-length construct in (B).

Table 2- Effect of ATP and arsenite on the Cuz1 interaction network. Late log phase 2-liter cultures expressing FLAG-tagged Cuz1, were treated for 2 h prior to lysis with 0.2 mM As_2O_3, and 2 mM ATP was added to the extracts where indicated. FLAG-Cuz1 was purified as described in the Experimental Procedures. Eluates from these purifications were analyzed by LC-MS/MS. Control binding assays (untagged Cuz1) and assays with FLAG-Cuz1 with no treatment or treated with ATP plus As_2O_3 were done in duplicate.

	Spectral counts								
	Control		No treatment		ATP	As_2O_3	ATP and As_2O_3		
Protein	**1**	**2**	**1**	**2**			**1**	**2**	**Description**
CUZ1	61	56	2071	2071	1969	1447	1255	2151	AN1-type zinc finger protein YNL155W
CDC48	92	167	7649	7337	3415	3918	1142	2113	Cell division control protein 48
ARO1	723	474	402	643	698	406	445	486	Pentafunctional AROM polypeptide
RL402	21	7	66	58	38	94	35	60	Ubiquitin-60S ribosomal protein L40
QNS1	2	0	0	13	0	217	0	0	Glutamine-dependent NAD(+) synthetase
GAD1	0	0	0	15	0	124	24	28	Glutamate decarboxylase
RPB1	45	24	7	7	0	17	0	0	RNA polymerase II subunit RPB1
RGI2	2	0	9	11	7	30	19	18	Respiratory growth induced protein 2
RPN3	0	0	2	0	0	49	10	20	26S proteasome regulatory subunit RPN3
FAS1	0	0	0	0	0	72	3	4	Fatty acid synthase subunit beta
RPN1	4	3	7	0	0	40	3	17	26S proteasome regulatory subunit RPN1
RPN2	12	0	6	0	2	32	6	14	26S proteasome regulatory subunit RPN2
RPB2	25	12	0	0	0	15	11	3	RNA polymerase II subunit RPB2
UBX1	0	0	10	0	0	51	0	3	UBX domain-containing protein 1
NPL4	0	0	16	10	0	20	0	0	Nuclear protein localization protein 4
FBP1	4	0	0	0	10	2	10	14	Fructose-1,6-bisphosphatase
RPN5	0	0	3	0	0	26	0	8	26S proteasome regulatory subunit RPN5
RPT3	0	0	0	2	0	17	0	17	26S protease regulatory subunit RPT3
RPT4	0	0	0	0	0	17	4	5	26S protease subunit RPT4
SPT5	12	7	0	2	0	0	0	4	Transcription elongation factor SPT5
UBX2	0	0	6	5	0	9	0	0	UBX domain-containing protein 2
UFD1	0	0	9	4	0	2	0	0	Ubiquitin fusion degradation protein 1

RPN6	0	0	0	0	0	11	0	9	26S proteasome regulatory subunit RPN6
RPT6	0	0	0	0	0	18	0	0	26S proteasome regulatory subunit RPT6
RPN8	0	0	0	0	0	9	0	8	26S proteasome regulatory subunit RPN8
HXT7	0	0	0	0	0	6	10	0	High-affinity hexose transporter HXT7
RPN7	0	0	0	0	0	15	0	0	26S proteasome regulatory subunit RPN7
RPN9	0	0	0	0	0	9	0	5	26S proteasome regulatory subunit RPN9
ERG9	5	0	0	0	0	0	2	6	Farnesyl pyrophosphate synthase
FRD1	0	0	0	0	0	0	0	12	Fumarate reductase
RPT5	0	0	0	0	0	7	0	4	26S protease regulatory subunit RPT5
RPN11	0	0	0	0	0	7	0	4	26S proteasome regulatory subunit RPN11
RPT2	0	0	0	0	0	10	0	0	26S protease regulatory subunit RPT2
RPT1	0	0	0	0	0	10	0	0	26S protease regulatory subunit RPT1

To identify which region(s) of Cuz1 was involved in Cdc48 binding, several GST-Cuz1 deletion variants were created (Fig. 7). Neither the Cuz1 zinc-finger domain by itself (GST-Cuz1$_{1-59}$) nor a fragment consisting of the less conserved central region between the Zf_AN1 and UBL domains (GST-Cuz1$_{59-161}$) showed detectable binding to Cdc48. Moreover, a fragment including both of these regions (GST-Cuz1$_{1-161}$) also failed to pull down Cdc48. Conversely, mutation of four cysteines in Zf_AN1 to serines, which should eliminate zinc binding, did not impair binding to Cdc48 (4C to S, construct 2). Together, these data indicate that the Cuz1 zinc finger is neither necessary nor sufficient for Cdc48 interaction.

Most of the known Cdc48 adaptors bind Cdc48 via a UBX domain, which has a ubiquitin-like (β-grasp) fold. The Cdc48 cofactor Npl4 binds to Cdc48 through a UBX-related domain (UBD) (153). Cuz1 possesses a domain predicted to have a ubiquitin-like fold (UBL; check Fig. 2, 3 and 4), and a C-terminal deletion that removed the UBL and little else prevented Cdc48 binding (Fig. 7, GST-Cuz1$_{1-161}$). This indicates that the Cuz1 UBL is necessary for Cdc48 binding.

We tested two N-terminal Cuz1 truncations to help narrow down the region that is sufficient for Cdc48 interaction. GST-Cuz1$_{60-274}$ and GST-Cuz1$_{168-274}$ were expressed at similar levels, but only the longer construct was able to bind Cdc48 (Fig. 7, constructs 6, 7). This confirmed the lack of a requirement for the Zf_AN1 domain for Cdc48 binding and suggested either that the UBL is not sufficient for binding or that additional sequences N-terminal to residue 168 are necessary for a fully

functional UBL.

Considered together, the deletion data indicate that the Cuz1 UBL is necessary for Cdc48 binding but may not be sufficient, while the zinc finger domain is neither necessary nor sufficient for this interaction. Moreover as referred before a mass spec analysis performed with a C-terminal version of Cuz1 didn't show Cdc48 as a binding partner, which is consistent with the UBL requirement (data not shown).

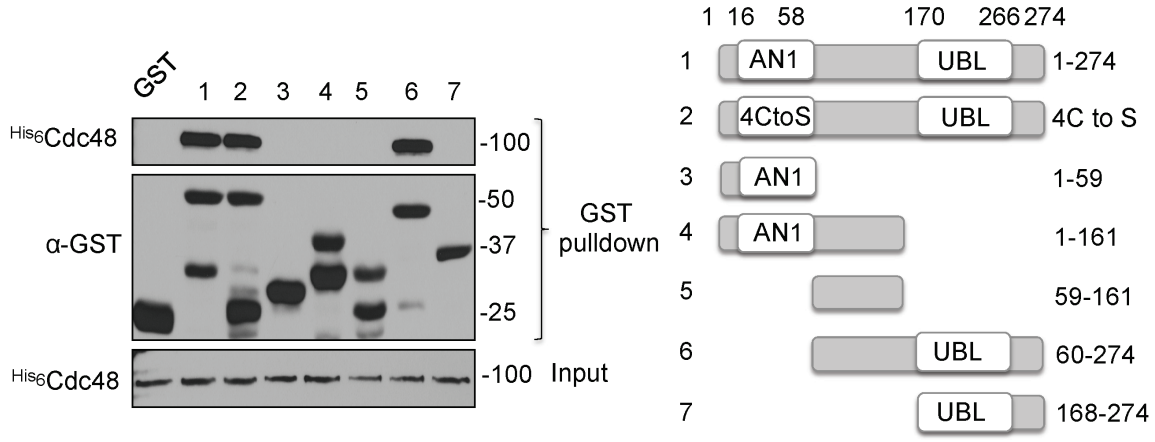


Figure 7- The Cuz1 Zf_AN1 domain is neither necessary nor sufficient for Cdc48 interaction, whereas the UBL domain is required. All proteins used in the GST pulldown analysis were purified from *E. coli*.

Cuz1 functions with Cdc48 in the ubiquitin-proteasome system– Cdc48 has a broad array of functions, but one of its best-characterized roles is in protein degradation by the UPS (233). We used pulse-chase analyses to determine whether Cuz1 contributes to the degradation of the UFD substrate Ub^{V76}-β-galactosidase (Ub^{V76}-βgal) (234). Deletion of *CUZ1* caused a very mild but reproducible slowdown in Ub^{V76}-βgal degradation kinetics (Fig. 8). We then combined the *cuz1Δ* allele with temperature-sensitive mutations in $Cdc48^{Npl4\text{-}Ufd1}$ components. The defects in Ub^{V76}-βgal degradation were already sufficiently severe in these single mutants at the semi-permissive temperature of 28°C that we could not detect any additional defect when the mutations were combined with *cuz1Δ* (not shown).

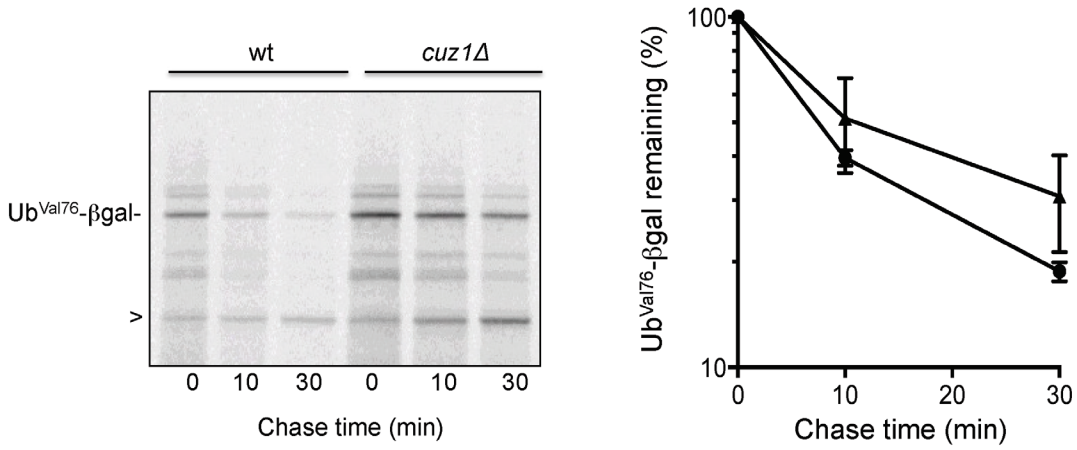


Figure 8- Deletion of Cuz1 causes cellular protein degradation defects. A) Pulse-chase analysis of Ub^{V76}-β-gal in the indicated yeast strains. Representative autoradiograph of a gel is shown at left. Arrowhead indicates a 90 kDa degradation product observed with Ub^{V76}-β-gal degradation in yeast. Bands above the primary Ub^{V76}-β-gal are polyubiquitinated species. Graph at right shows the mean degradation rates observed from three independent experiments. Error bars represent standard errors (Mark Hochstrasser data).

We also examined degradation of the classical ERAD substrate CPY*, a mutant derivative of the vacuolar carboxypeptidase Y enzyme that is retrotranslocated from the ER lumen for degradation by the cytoplasmic proteasome (235). Degradation of an HA-tagged CPY* protein at 37^{o}C appeared to be weakly impaired by loss of Cuz1 (Fig. 9). Notably, when *cuz1Δ* was combined with mutations in Npl4 or Ufd1, CPY*-HA degradation was further impeded (the *cdc48-6* single mutant was already strongly defective; not shown). These results indicate that Cuz1 has an auxiliary or partially redundant role in $Cdc48^{Npl4\text{-}Ufd1}$-dependent protein degradation by the UPS.

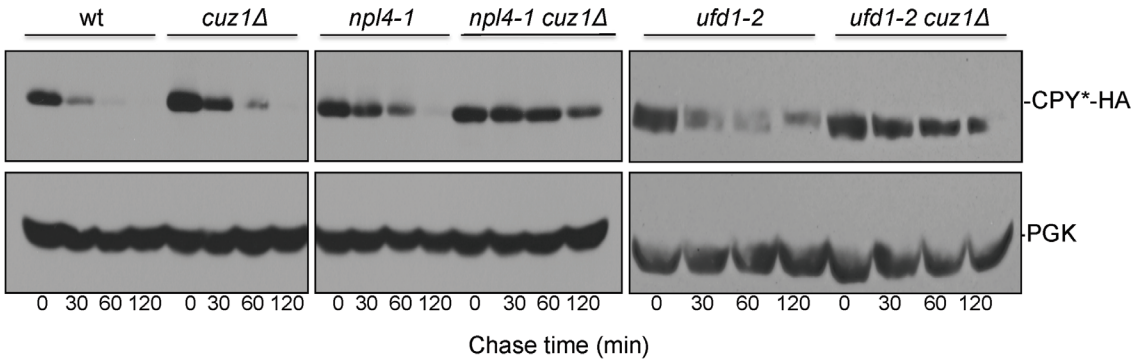


Figure 9- Degradation of CPY*-HA was analyzed by cycloheximide-chase/immunoblot analysis. CPY*-HA was detected by anti-HA-immunoblotting. As a loading control, the membrane was subsequently probed with anti-PGK antibodies (bottom panels).

When growth of these same strains was examined under various conditions, distinct genetic interactions between *cuz1Δ* and different $Cdc48^{Npl4\text{-}Ufd1}$ mutations were observed. The *ufd1-2 cuz1Δ* double mutant grew more slowly than the *ufd1-2* single mutant in the presence of tunicamycin, an ER stress inducer (Fig. 10A). We did not see clear differences in growth when *cuz1Δ* was combined with *cdc48-6*, and in combination with *npl4-1*, an apparent increase in growth rate was seen relative to the *npl4-1* single mutant. This was seen with cells derived from two different *npl4-1 cuz1Δ* spores, but when *CUZ1* was reintroduced into these cells on a plasmid, no change in growth was seen, suggesting that the double mutants carried a cryptic suppressor of *npl4-1* (not shown). Collectively, the growth data suggest an overlap in Cuz1 and Ufd1 function, whereas the results with the *cdc48-6* and *npl4-1* mutants are not readily interpreted.

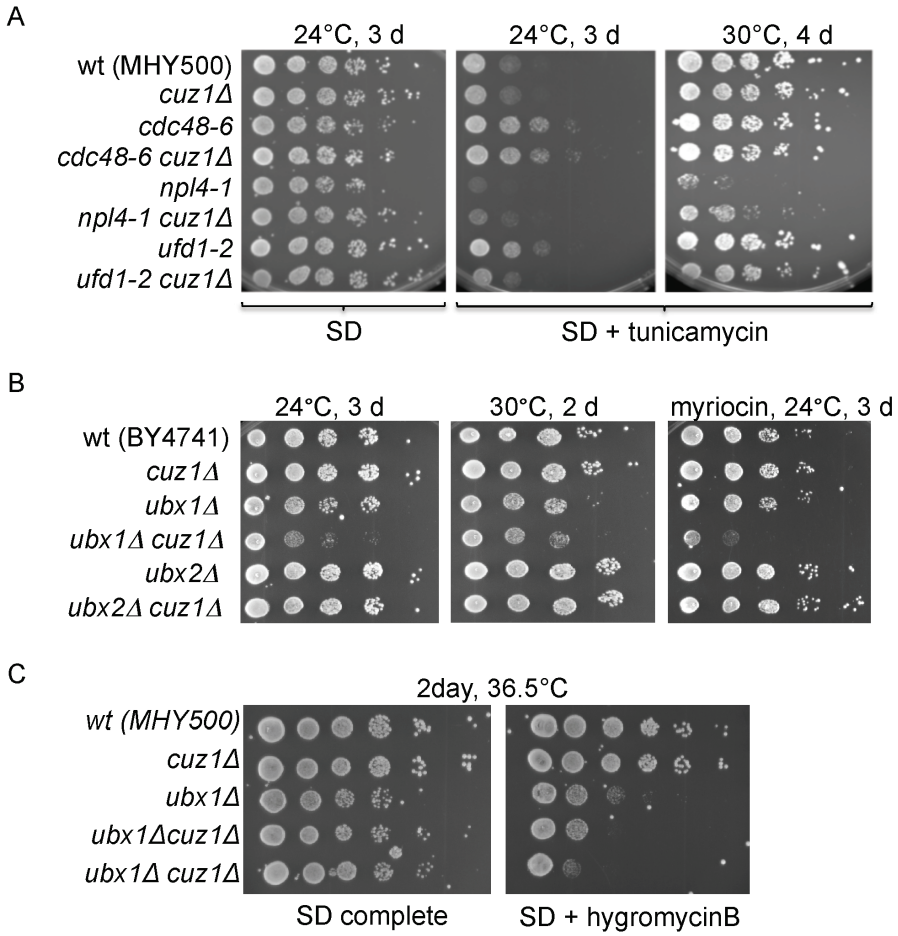


Figure 10- A) Growth assays reveal genetic interactions of *cuz1Δ* with mutations in $Cdc48^{Npl4\text{-}Ufd1}$. Six-fold serial dilutions of cultures were spotted onto plates (SD minimal medium or SD with 0.5 mg/ml tunicamycin). The apparent growth advantage of *cdc48-6* and *cdc48-6 cuz1Δ* in medium containing tunicamycin could in principle be due to a low constitutive induction of the ER unfolded-protein response in these cells. D) Double mutant analysis of *cuz1Δ* with different *UBX* gene deletions. Myriocin was used at 0.2 mg/ml. Ten-fold serial dilutions of cultures were spotted onto the plates. C) Negative genetic interactions between *cuz1Δ* and *ubx1Δ* were also observed in a different strain background. Six-fold serial dilutions of cultures were spotted onto SD plates or SD supplemented with 0.075 mg/ml hygromycin B.

Because the Cdc48-binding Ubx1/Shp1 and Ubx2 proteins were found to copurify with Cuz1 based on LC-MS/MS, we tested for genetic interactions between *cuz1Δ* and deletions of all seven yeast *UBX* genes as well as the gene for the Cdc48 cofactor Vms1. For *ubx2Δ–ubx7Δ* and *vms1Δ*, no obvious differences in growth rate were seen under a variety of conditions when these alleles were combined with *cuz1Δ* (Fig. 10B and not shown). In contrast, a *ubx1Δ cuz1Δ* double mutant grew markedly slower than either single mutant at lower temperatures (24^{o}C), but this effect was attenuated at 30^{o}C and no longer detected at 36.5^{o}C except in the presence of protein synthesis inhibitor hygromycin (Fig. 10C and not shown). Membrane stressors such tunicamycin or the sphingolipid synthesis inhibitor myriocin, also exacerbated the synthetic defect. The *ubx1Δ cuz1Δ* genetic interaction, together with the analogous defects seen in the *ufd1-2 cuz1Δ* mutant, is consistent with our LC-MS/MS results and supports a function for Cuz1 in multiple Cdc48-adaptor complexes.

Cuz1 interacts with ubiquitinated proteins in vivo – Many Cdc48 cofactors help recruit or process polyubiquitinated protein substrates (236). This prompted us to investigate a possible interaction between Cuz1 and ubiquitinated proteins. GST-Cuz1 was immobilized on glutathione-agarose beads and incubated with whole cell extracts from yeast that expressed HA-tagged ubiquitin. GST and GST-Rad23 were used as negative and positive controls, respectively. GST-Cuz1 bound to high molecular mass ubiquitinated species that were visualized near the top of the resolving gel and in the stacking gel (Fig. 11A). The pattern of ubiquitin conjugates that bound was similar to those bound by the well-characterized ubiquitin chain-binding GST-Rad23 protein, although the level of bound conjugates was lower than found with Rad23. No conjugates were detected when GST was used (Fig. 11A).

We tested whether the ubiquitinated protein-Cuz1 interaction was likely to be direct by performing the pulldown assay with purified polyubiquitin chains. Lys48-linked chains (chain length of 2 to 7 ubiquitins) didn't interact with GST-Cuz1, in contrast to GST-Rad23 (Fig. 11B). While it remains possible that Cuz1 directly interacts with ubiquitin chains of distinct linkages, it is more likely that the interactions seen in yeast lysates were indirect (or only occur in the context of a protein complex, such as one bearing additional ubiquitin-binding sites).

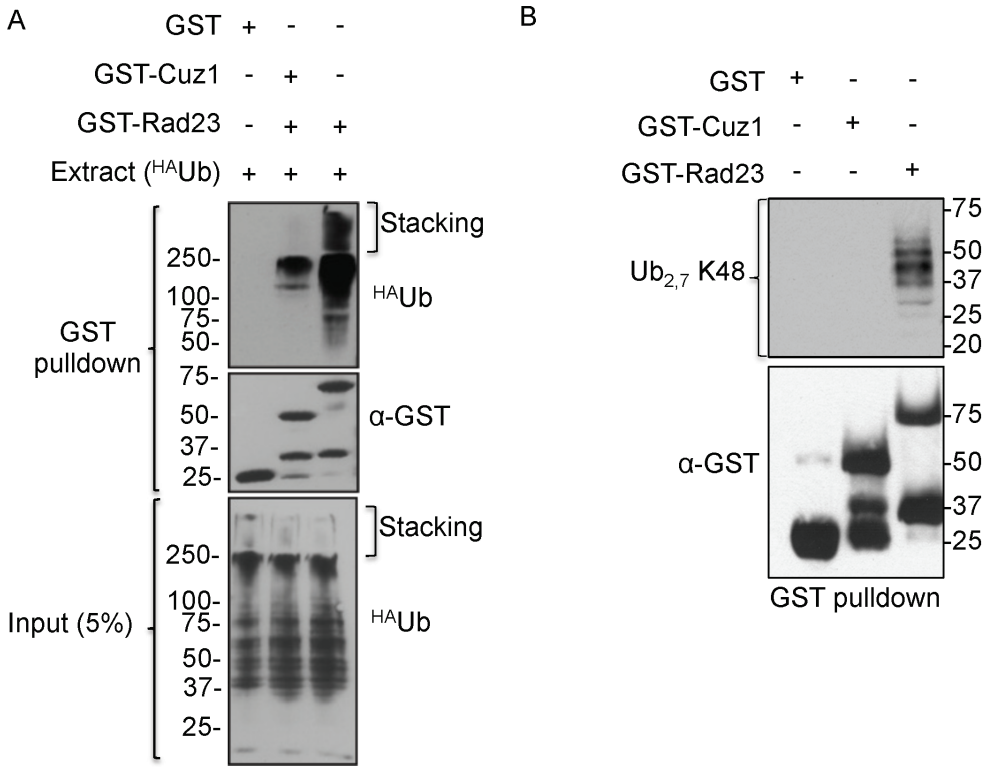


Figure 11- Cuz1 associates with ubiquitin-protein conjugates *in vivo*. A) Recombinant GST-Cuz1 was incubated with extracts from yeast overexpressing HA-tagged ubiquitin, and protein eluted from the glutathione resin was analyzed by anti-HA and anti-GST immunoblotting. B) Similar experiment to the previous was performed but to test the interaction of GST-Cuz1 with purified K48 chains of 2 to 7 molecules of ubiquitin. GST-Rad23 and GST were again used as positive and negative control, respectively.

Using the same Cuz1 deletion constructs described above for examining Cdc48 interactions, we mapped the region(s) in Cuz1 responsible for interaction with ubiquitinated proteins in yeast extracts (Fig. 12A). We observed the exact same binding behaviors as seen when Cdc48 interaction was evaluated (Fig. 7). Specifically, binding did not require the Zf_AN1 domain, but association was observed with a C-terminal Cuz1 fragment including the UBL (GST-Cuz1$_{60-274}$) but with no other truncations. A parsimonious explanation of these results is that Cdc48 mediates the interaction of ubiquitinated proteins with Cuz1, presumably in the context of Cdc48 complexes with one or more of its known cofactors.

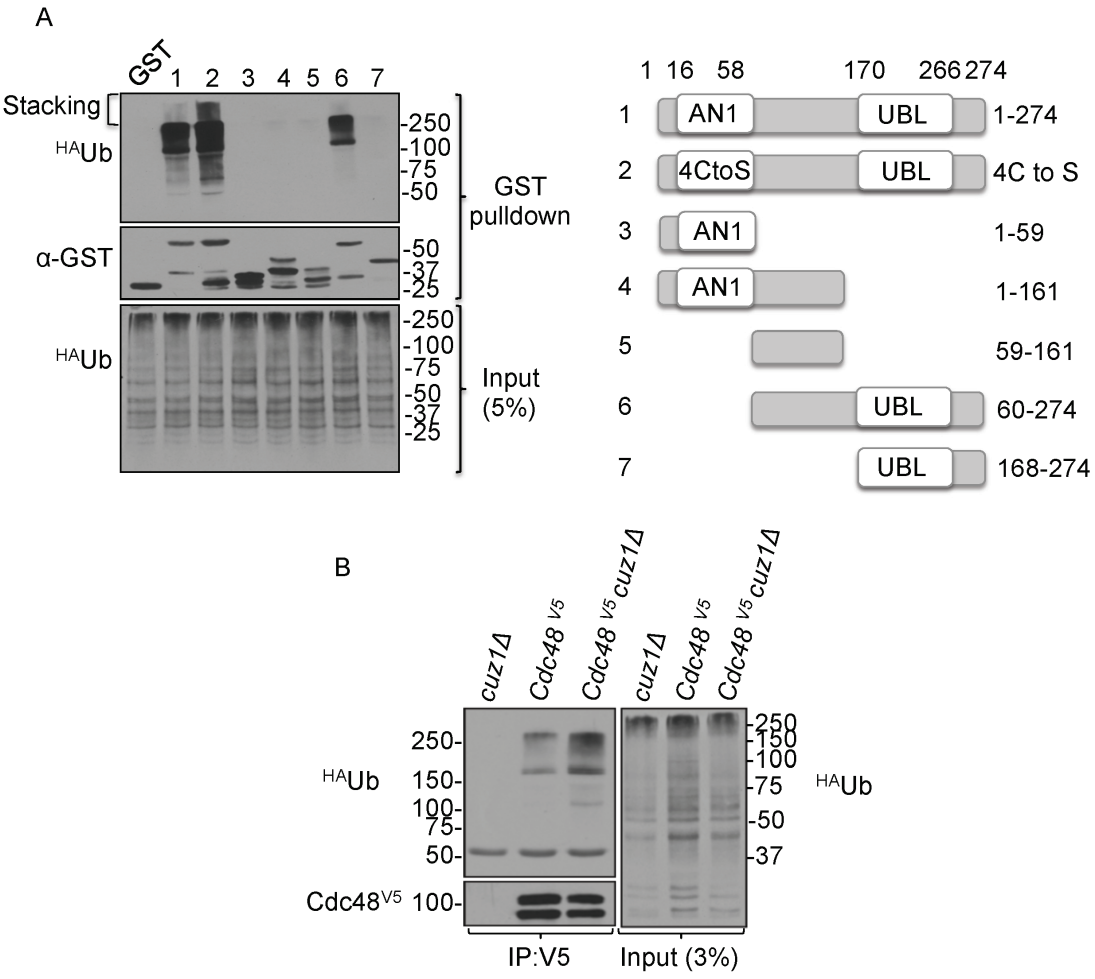


Figure 12- A) The Cuz1 UBL domain is required for ubiquitin-conjugate interaction, but the Zf_AN1 domain is neither necessary nor sufficient. The indicated GST tagged constructs were used in GST pulldown assays performed as in (A). B) Loss of Cuz1

enhances association of polyubiquitinated conjugates with Cdc48 *in vivo.* Analysis was done with cultures of *CUZ1 or cuz1Δ* cells expressing Cdc48-V5 from the endogenous *CDC48* locus and expressing HA-tagged ubiquitin from a plasmid.

These results raise the question of whether Cuz1 influences the interaction of Cdc48 with ubiquitinated substrates *in vivo*. We transformed a plasmid overexpressing HA-tagged ubiquitin into *cuz1*Δ and *CUZ1* strains carrying the chromosomal *CDC48-V5* allele. If Cuz1 were involved in the recruitment of substrates to Cdc48, loss of Cuz1 might reduce ubiquitin-conjugate interaction with the ATPase complex. Conversely, if Cuz1 were affecting substrate release from Cdc48, an increase in the level of ubiquitinated substrates on Cdc48 may be observed. In fact, when Cdc48-V5 was immunoprecipitated from extracts derived from *cuz1*Δ cells, there was a reproducible increase in the levels of high molecular mass ubiquitinated species that were co-precipitated (Fig. 12B). This suggests a potential role for Cuz1 in releasing ubiquitinated substrates from Cdc48 or in transferring them from Cdc48 to the proteasome or proteasome shuttle factors.

Cuz1 binds to the proteasome– The preceding data raised the possibility that Cuz1 is involved in transferring ubiquitinated substrates from Cdc48 to the proteasome. This idea is supported by our mass spectrometry data, which revealed low levels of proteasome subunits co-purifying with Cuz1 (Table 1 and 2). Moreover, previous studies had shown that AIRAP and AIRAP-L, mammalian proteins related to Cuz1, can interact with the 26S proteasome (206,207). To determine whether Cuz1 interacts with the yeast 26S proteasome, we affinity purified 26S proteasomes from yeast cells that expressed proteasomes with a 3XFLAG epitope tag on either the Pre1 (β4) subunit of the core particle (CP) or the Rpn5 subunit of the regulatory particle (RP). Anti-Cuz1 immunoblot analysis of the purified complexes revealed association of Cuz1 in both cases (Fig. 13A). Additionally, GST-tagged Cuz1 purified from *E. coli* associated with isolated 26S proteasome particles, suggesting that the Cuz1-proteasome interaction is direct (Fig. 13B). We noted that Cuz1 is a relatively

long-lived protein, so its association with the proteasome is unlikely to be as a substrate (Fig. 13C)

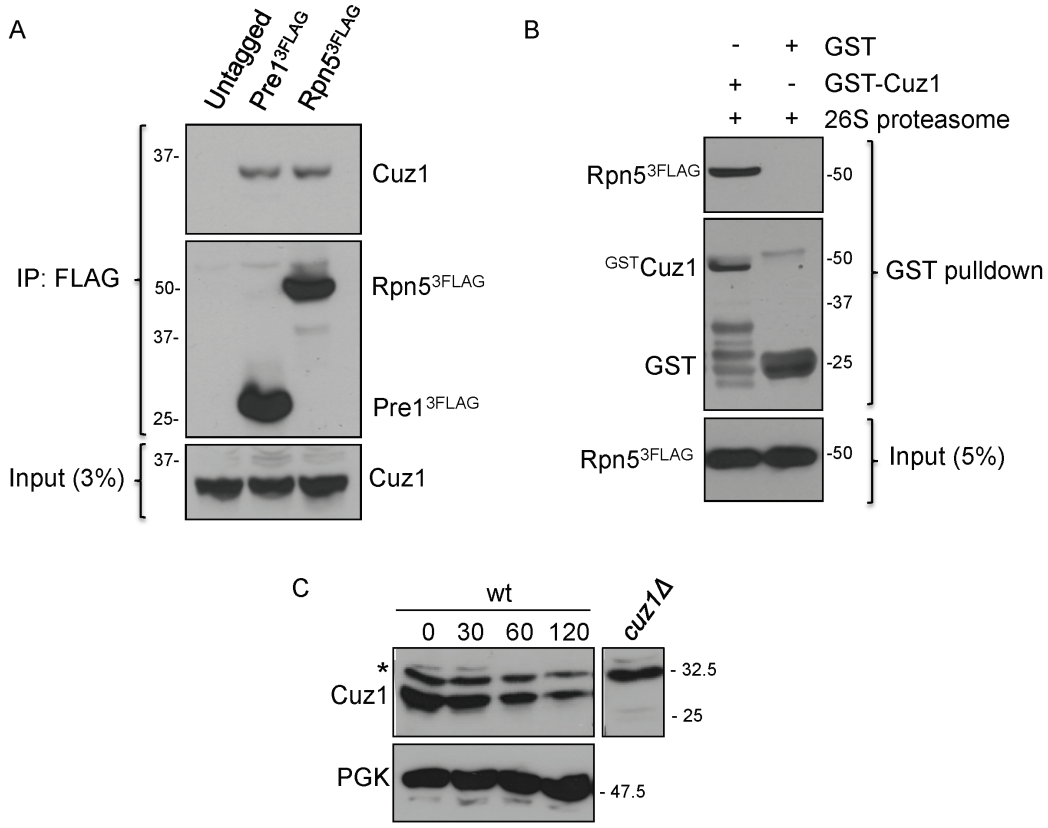


Figure 13- Physical interaction between Cuz1 and the proteasome. A) Cuz1 interacts *in vivo* with the 26S proteasome. Proteasomes were affinity-purified from extracts of yeast expressing either FLAG-tagged Pre1 (CP) or Rpn11 (RP) from the respective endogenous locus. The bound material was analyzed by anti-Cuz1 immunoblotting. B) Interaction between Cuz1 and 26S proteasomes *in vitro*. Recombinant GST-Cuz1 was incubated with yeast 26S proteasomes affinity-purified from an Rpn5-3FLAG-expressing strain. C) Cycloheximide chase analysis of Cuz1 degradation was followed using anti-Cuz1 antibody. PGK was used as a loading control and detected with anti-PGK antibodies. (*) indicates a non-specific band that cross-reacts with the anti-Cuz1 antibody.

A subset of the GST-Cuz1 deletion derivatives was used for pulldown assays with purified yeast RP. Whereas the UBL was necessary for interaction with Cdc48 and polyubiquitin (Figs. 7 and 12A), it was dispensable for RP binding (Fig. 14). A fragment containing the Zf_AN1 domain and the linker region was sufficient for maximal binding (GST-Cuz1$_{1-151}$). The Zf_AN1 domain itself does not appear to be sufficient for proteasome binding, but whether it is necessary remains to be determined.

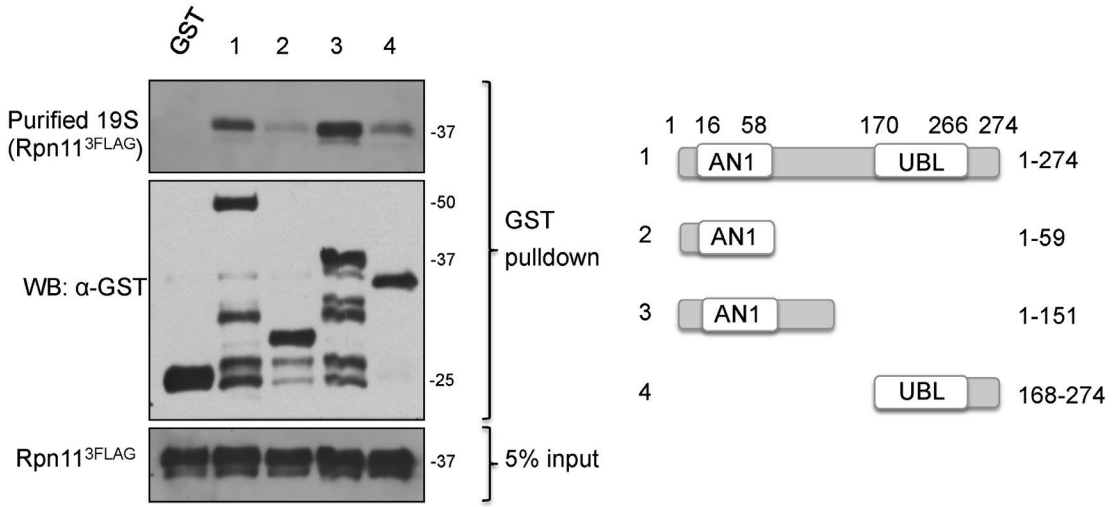


Figure 14- Domain requirements for the interaction of Cuz1 with the proteasome. The UBL domain is not required for Cuz1 interaction with proteasomes. Recombinant GST-Cuz1 constructs were incubated with purified 19S RP (purified from an Rpn11-3FLAG-expressing strain), and the proteins were subjected to GST-pulldown analysis.

The composition of the *S. cerevisiae* proteasome has been thoroughly analyzed, and Cuz1 association had not been reported (92,237). It is likely that the amount of Cuz1 on the proteasome is strongly sub-stoichiometric and its interaction highly dynamic. Nevertheless, in support of the physiological relevance of the Cuz1-proteasome interaction, deletion of *CUZ1* enhanced the growth defects of both *rpt6* (*cim3-1*), and *rpt1* (*cim5-1*) temperature-sensitive mutants, as well as yeast lacking *RPN9* (Fig. 15).

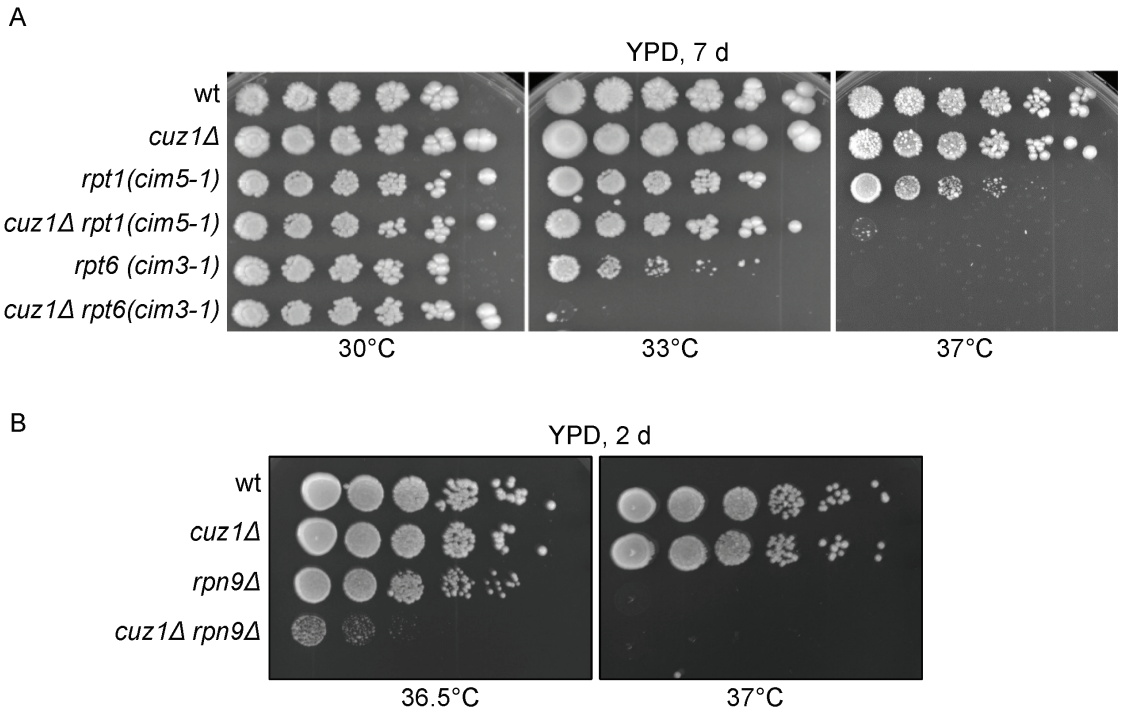


Figure 15- Growth assays reveal genetic interactions between *cuz1Δ* and mutations in proteasome subunit genes. Six-fold serial dilutions of cultures were spotted onto YPD plates. Deletion of *CUZ1* exacerbates the growth defects of *cim3-1* and *cim5-1* (A) (M. Funakoshi data) and also *rpn9Δ* (B) yeast.

Interaction of Cuz1 and the proteasome is stimulated by exposure of cells to arsenite- Arsenite induces protein misfolding and causes accumulation of polyubiquitinated conjugates *in vivo* (206,238). *CUZ1* mRNA levels are elevated

upon exposure of cells to arsenite (205), and we detected a modest increase in FLAG-Cuz1 protein levels when cells were exposed to 0.2 mM As_2O_3 for 2 h (Fig. 16A). It is noteworthy that both mammalian AIRAP and p97 (Cdc48) display increased binding to the proteasome upon arsenite stress (206,222). Based on these results, we tested whether arsenite might affect interaction of Cuz1 with proteasomes in yeast. In the presence of arsenite, we observed a marked increase in Cuz1 binding to proteasomes immunoprecipitated from yeast extracts; the increase greatly exceeded the modest overall increase of Cuz1 levels in the treated cells (Fig. 16A). When normalized to the amount of precipitated Rpn11-3FLAG, a ~10-fold increase in Cuz1-proteasome association was seen following arsenite treatment (Fig. 16B). The increase in this association was supported by LC-MS/MS analysis (Table 2), where addition of arsenite to cells before FLAG-Cuz1 purification led to the identification of 15 of the 19 RP subunits (but no CP subunits). This result also implies that Cuz1 might bind to the 19S rather than the 20S.

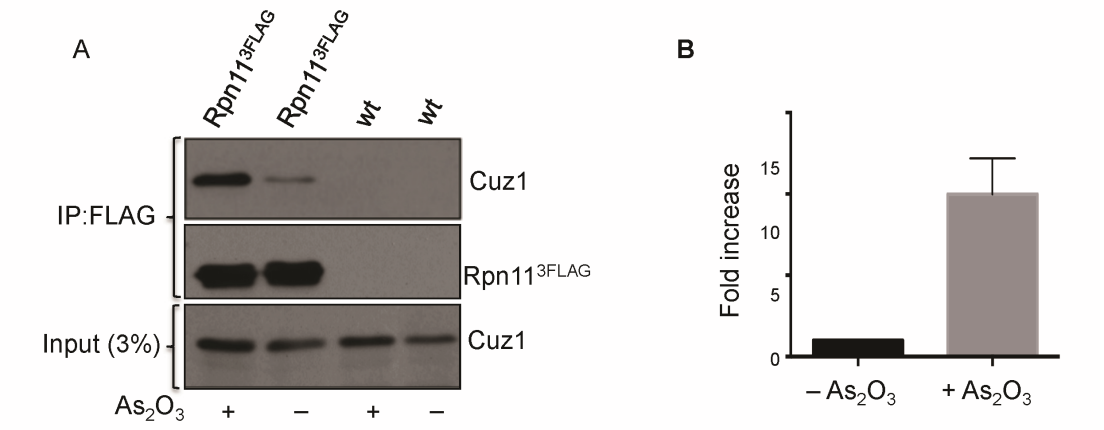


Figure 16. Exposure of yeast to arsenite enhances proteasome association with Cuz1. A) FLAG-tagged Rpn11 was immunoprecipitated and the amount of co-purifying Cuz1 was quantified. A representative experiment is shown at left. Cuz1 values were normalized to the levels of precipitated Rpn11-3FLAG. The quantification at right is derived from three independent experiments and shows the normalized fold-increase of coprecipitated Cuz1; error bars denote standard deviations.

Cuz1 plays a role in cellular defense against arsenite- One possible explanation for part of the increase in the levels of Cuz1 at the proteasome is the increased *CUZ1* transcription induced by Rpn4, which presumably leads to the modest observed increase in celular Cuz1 protein levels (Fig. 16A, input) The Rpn4 transcription factor upregulates the levels of genes possessing PACE elements in their promoter region, a group of genes that includes *CUZ1*.

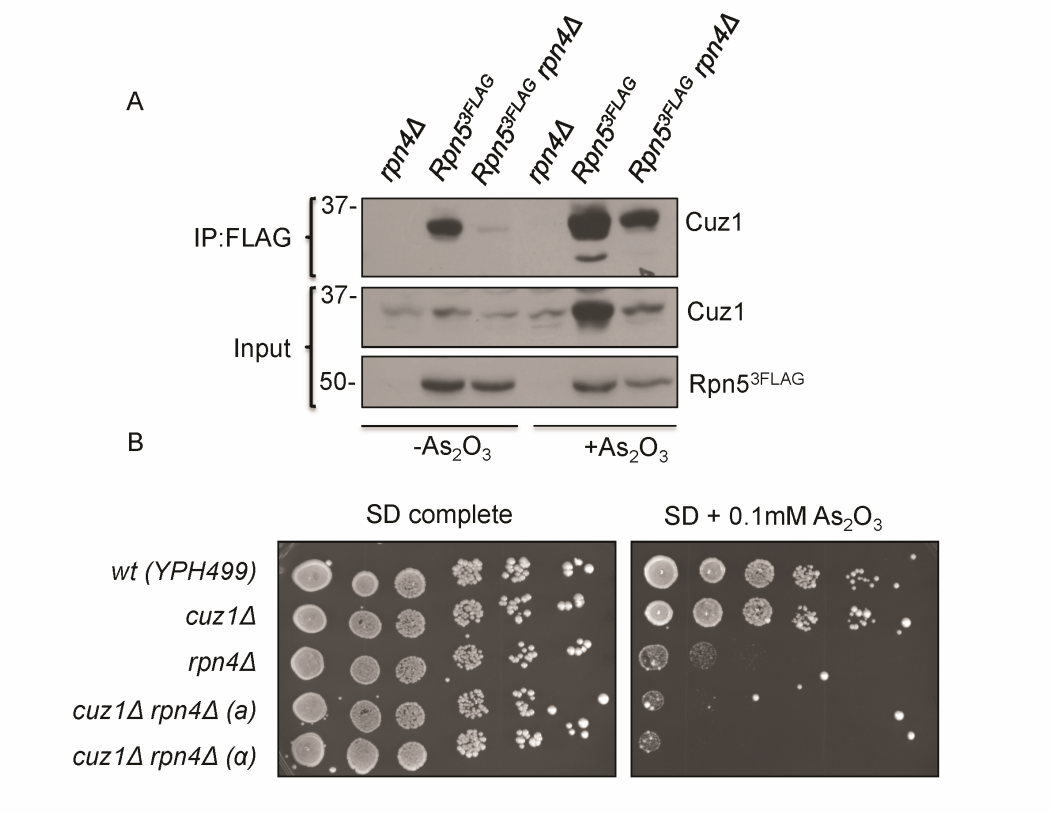


Figure 17- A) Increased association of Cuz1 with the proteasome upon arsenite treatment is partially dependent on Rpn4. Proteasomes were affinity-purified from extracts of *RPN4* and *rpn4Δ* yeast expressing FLAG-tagged Rpn5 . The bound material was analyzed by anti-Cuz1 immunoblotting. B) Growth assays reveal genetic interactions of *cuz1Δ* with *rpn4Δ*. Six-fold serial dilutions of cultures were spotted onto plates (SD minimal medium or SD with 0.1 mM As_2O_3).

To test the influence of Rpn4 on proteasome-Cuz1 interaction, we measured the amount of Cuz1 copurifying with proteasomes from untreated cells or cells

treated with arsenite. When arsenite was added, increased levels of bound Cuz1 were found even in the absence of Rpn4, although not as high as in wild type (Fig. 17A, compare lanes 3 and 6). This indicates that the observed increase is not due solely to the Rpn4-mediated response. Moreover deletion of *CUZ1* weakly enhanced the already strong growth defect of *rpn4Δ* yeast in the presence of arsenite, consistent with Cuz1 playing a role in cell's defense against the metalloid (Fig. 17B).

Loss of Cuz1 alone caused no reduction in cell growth in the presence of arsenite, but when *cuz1Δ cim5-1* cells were grown at high temperature, arsenite exacerbated the already slow growth resulting from the *cim5-1* mutation (Fig. 18A). This supports the potential biological importance of Cuz1 in promoting proteasome function in arsenite resistance.

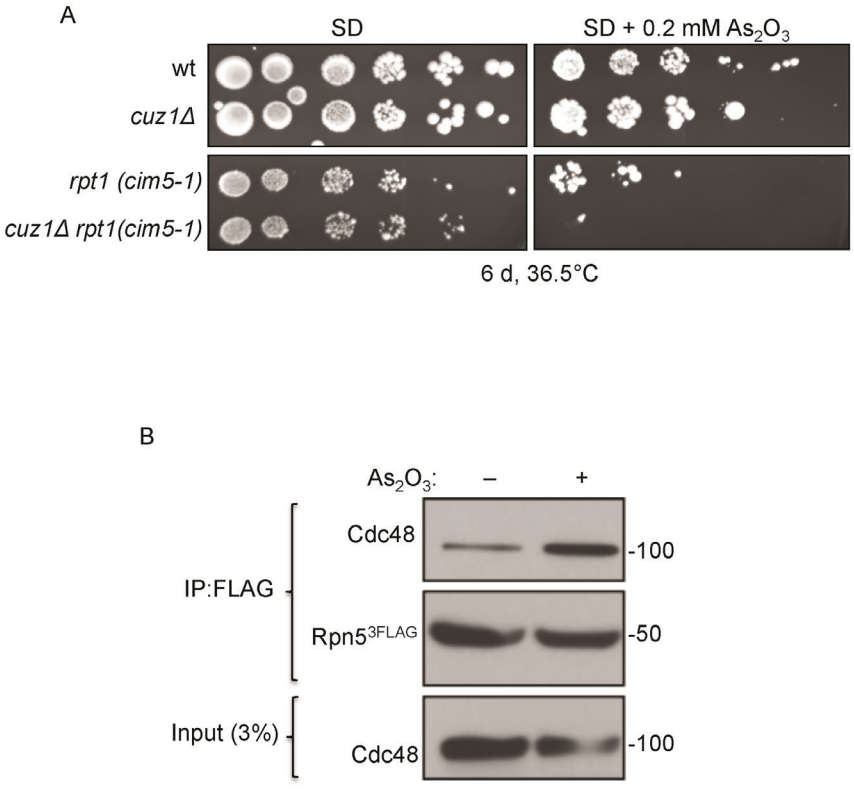


Figure 18- A) In the presence of arsenite, deletion of *CUZ1* worsens the growth defect of a temperature-sensitive *cim5-1* mutant at high temperature. B) Cdc48 interaction with the proteasome is enhanced by arsenite. Yeast expressing

proteasomes containing Rpn5-3FLAG were treated or not with arsenite for 30 minutes. Cell extracts were then prepared, and proteasomes and associated proteins were were immunoprecipitated with anti-FLAG resin. Co-purified Cdc48 was analyzed by anti-Cdc48 immunoblotting.

Finally, because arsenite increases the association of mammalian p97 with the proteasome (222), we checked whether this was also true for Cdc48. In fact, Cdc48 co-precipitation with the proteasome increased even after only a 30 min exposure to the metalloid (Fig. 18B). These data suggest that Cdc48 and the proteasome have linked functions in promoting arsenite resistance.

Because I observed increases in both Cuz1 and Cdc48 association with the proteasome, I considered the possibility that Cuz1 promotes the recruitment of Cdc48 to the proteasome. Arguing against this hypothesis, loss of Cuz1 caused a very small but reproducible increase in Cdc48 interaction with proteasomes either with or without arsenite stress (Fig. 19A). This suggests that Cuz1 may act as a possible dissociation factor for Cdc48 rather than a recruiting factor.

Cuz1 affects the interaction of polyubiquitinated proteins with the proteasome- Our results point to a possible role for Cuz1 in the release of polyubiquitinated conjugates from Cdc48 to downstream components of the UPS (Fig. 12B). To test this, we asked whether Cuz1 affected the levels of conjugates interacting with the proteasome. A parallel increase in polyubiquitinated proteins associated with the proteasome was observed when *CUZ1* is deleted (Fig. 19B). These data are consistent with the participation of Cuz1 in the transfer of polyubiquitin conjugates from Cdc48 to the proteasome.

In contrast, no effect on the levels of ubiquitinated conjugates at the proteasome were detected in *yor052Δ* yeast cells (Fig. 19B). Nevertheless, deletion of both *CUZ1* and *YOR052C* appeared to confer a slight growth advantage in arsenite, suggesting that these proteins are involved in cellular arsenite response, possibly having complementary roles (Fig. 20).

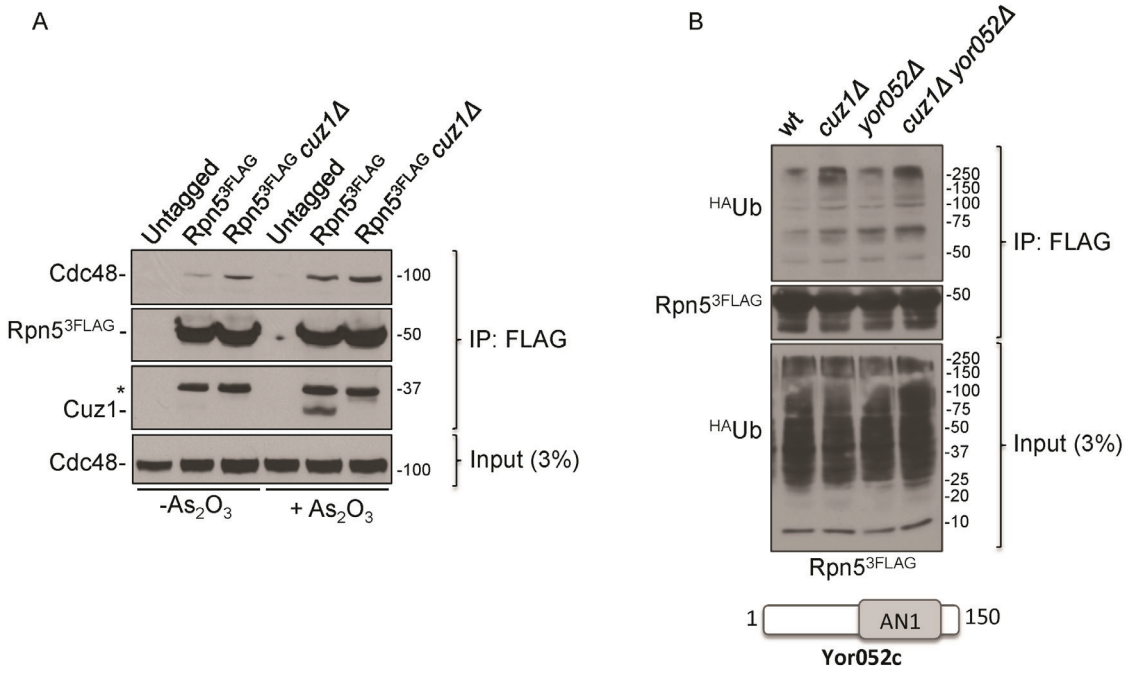


Figure 19- Cuz1 affects the interaction of polyubiquitinated substrates with proteasomes. A) A slight increase in Cdc48 bound to the proteasome is observed in the absence of Cuz1, both in the presence and absence of arsenite. FLAG tagged proteasomes were immunoprecipitated, and the amount of bound Cdc48 was analyzed by anti-Cdc48 immunoblotting. A nonspecific band is indicated by an asterisk. B) An increase in polyubiquitinated proteins on the proteasome is observed when *CUZ1* is deleted. After exposing cells for 2 h to 0.2 mM arsenite, the cells were lysed and proteasomes were immunoprecipitated. Copurified polyubiquitinated proteins were analyzed by anti-HA immunoblot analysis.

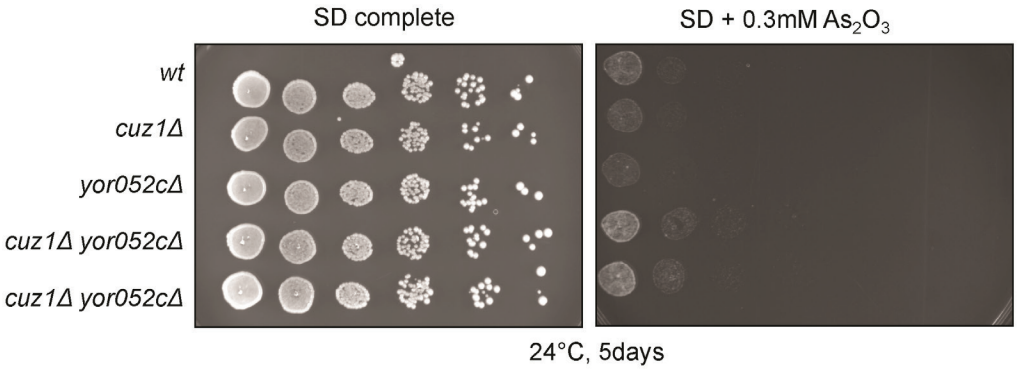


Figure 20- Deletion of both *CUZ1* and *YOR052C* confers a growth advantage under arsenite stress. Six-fold serial dilutions of the indicated strains were spotted onto plates (SD minimal medium or SD with 0.3 mM As_2O_3).

CUZ1 deletion does not affect proteasome stability or activity- A simple explanation for the accumulation of polyubiquitinated conjugates bound to the proteasome upon *CUZ1* deletion could be that the proteolytic activity of the proteasome was attenuated. Proteasomes containing AIRAP showed increased activity towards the fluorogenic peptide Suc-LLVY-AMC (206). We tested proteasome stability and activity by immunoblot analysis and in gel substrate overlay assays. Contrary to findings regarding AIRAP, no obvious differences were detected between *cuz1Δ* and WT proteasomes in response to arsenite (Fig. 21).

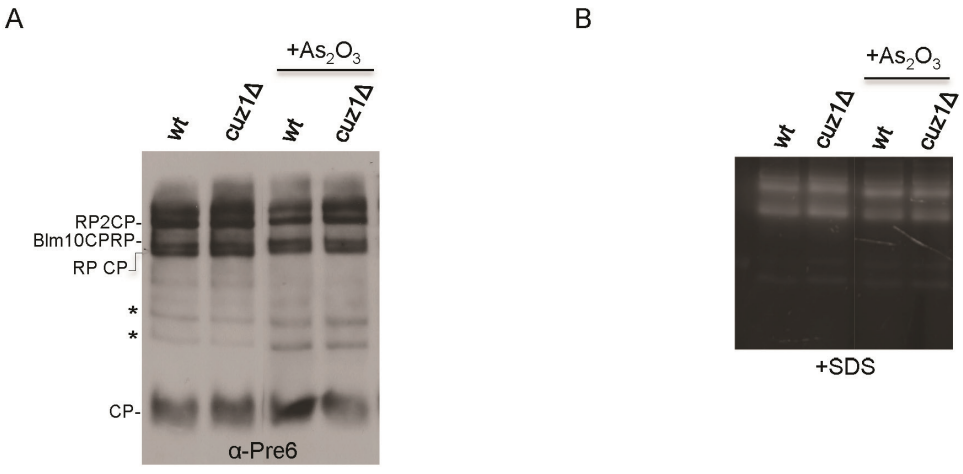
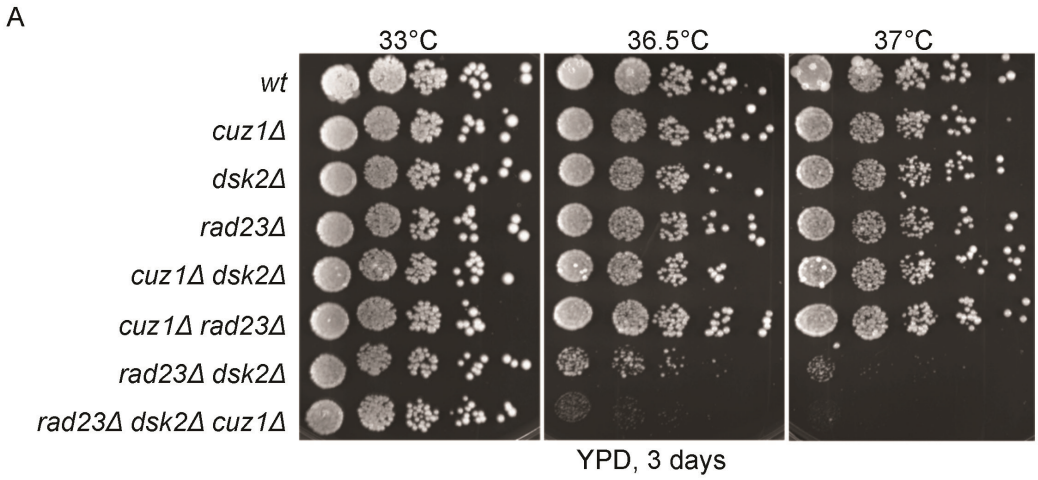


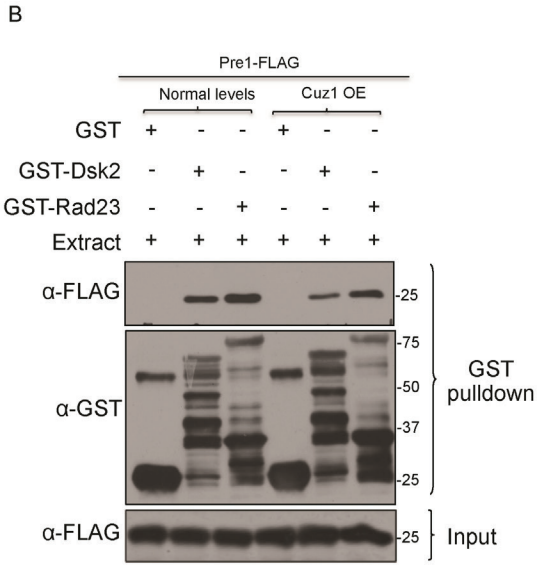
Figure 21- A) Native gel separation of proteins from yeast whole-cell extracts followed by immunoblot analysis. CP, the 20S core particle; RP, the 19S regulatory particle; RP2CP, the intact 26S proteasome; RPCP, singly RP-capped 20S particle; BLM10CPRP, proteasome hybrid; (*) 26S proteasome intermediates. B) Gel overlay with the fluorogenic substrate Suc-LLVY-AMC to test proteasomal activity.

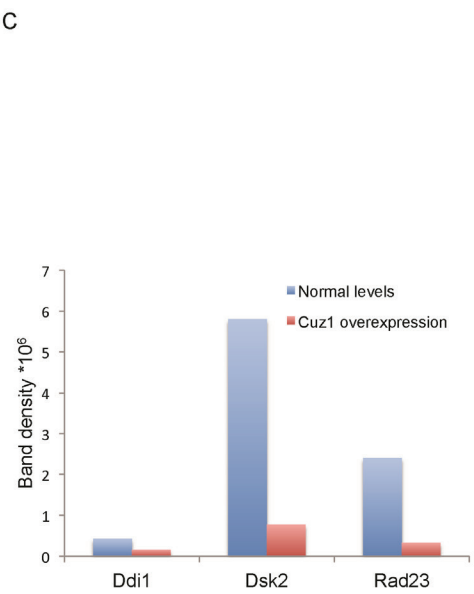
Cuz1 affects the interaction of shuttle factors with the proteasome- As previously described, proteasome shuttle factors are a group of proteins involved in the delivery of polyubiquitinated substrates to the proteasome. Interestingly, deletion of *CUZ1* exacerbated the growth defect of *rad23Δ dsk2Δ* yeast (Fig. 22A). No differences were observed for the double mutants *rad23Δ cuz1Δ* or *dsk2Δ cuz1Δ*, consistent with the redundant and overlapping functions of these shuttle factors. Deletion of *DDI1* was not tested in these growth assays.

Next we sought to analyze the influence of Cuz1 in the interaction of the shuttle factors with the proteasome. Upon Cuz1 overexpression we observed a small but reproducible decrease in the levels of Rad23, Dsk2 and Ddi1 at the proteasome (Fig. 22B and C) and a concomitant decrease in the levels of polyubiquitinated substrates interacting with it (Fig. 22D). Conversely, proteasomes purified from a *cuz1Δ* strain had greater levels of bound polyubiquitinated conjugates, as noted earlier (Fig. 19B).

Cuz1 therefore affects the interaction of both shuttle factors and ubiquitinated conjugates with the proteasome. Direct competition between shuttle factors and Cuz1 for the same site(s) on the proteasome could potentially explain these results. Rpn1 is the major docking site for shuttle factors. However, when I tested for a direct interaction between recombinant *E. coli*-produced Cuz1 and Rpn1 proteins, no binding was detected (data not shown). It remains possible that Cuz1 binds to another proteasome site that nevertheless interferes with Rpn1-shuttle factor association or that the recombinant Rpn1 or Cuz1 lacked a conformation or posttranslational modification critical to their interaction on the proteasome in yeast cells.


A
33°C
36.5°C
37°C
wt
cuz1Δ
dsk2Δ
rad23Δ
cuz1Δ dsk2Δ
cuz1Δ rad23Δ
rad23Δ dsk2Δ
rad23Δ dsk2Δ cuz1Δ
YPD, 3 days


B
Pre1-FLAG
Normal levels
Cuz1 OE
GST
GST-Dsk2
GST-Rad23
Extract
α-FLAG
α-GST
α-FLAG
GST pulldown
Input
-25
-75
-50
-37
-25
-25


C
Band density *10^6
Normal levels
Cuz1 overexpression
Ddi1
Dsk2
Rad23

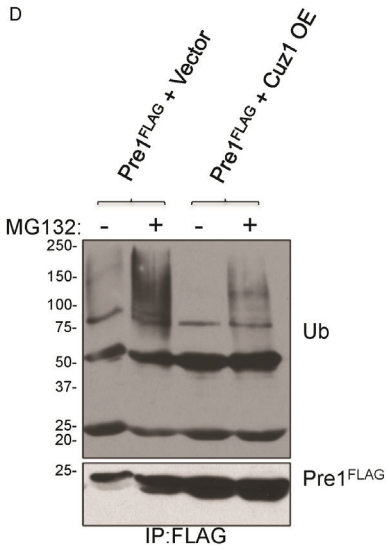

D
Pre1FLAG + Vector
Pre1FLAG + Cuz1 OE
MG132:
Ub
Pre1FLAG
IP:FLAG

Figure 22- Cuz1 affects the interaction of shuttle factors and polyubiquitinated proteins with the proteasome. A) Growth assays reveal genetic interactions of *CUZ1* with shuttle factors *RAD23 and DSK2*. Six-fold serial dilutions of cultures were spotted onto YPD plates and incubated at the indicated temperatures. B) Recombinant GST-Rad23 and GST-Dsk2 were incubated with extracts from yeast endogenous or overexpressed Cuz1. GST pulldown assays were performed to assess the amount of bound proteasome. C) Experiments were performed as in B) but GST-Ddi1 was also used. D) Proteasomes were purified from strains transformed with either empty vector or a vector with Cuz1 under a GAL promoter expression. Proteasome-bound ubiquitinated substrates were detected using anti-Ub antibody.

DISCUSSION

In this study we have described a novel arsenite-inducible yeast protein, Cuz1, which associates under normal growth conditions primarily with Cdc48 (Fig. 6A). Upon cellular exposure to arsenite, which activates multiple stress-response pathways (207), a ~10-fold increase in Cuz1-proteasome association occurs (Fig. 16). Cells are normally exposed to several environmental stresses and adaptation is vital to ensure their survival. We provide evidence that Cuz1 is involved in cell's response to arsenite (chapter 4, Fig. 17B, 18A and 20).

Cuz1 provides the first reported functional connection between the extremely widespread AN1-type Zn finger (Zf_AN1) motif and the phylogenetically conserved Cdc48 AAA-ATPase. Interestingly, a very recent report described an interaction between the AIRAP-L Zf_AN1 protein and p97 in mammalian cells; this interaction was suggested to regulate the translocation of some secreted proteins into the ER (229). The Zf_AN1 domain of Cuz1 is not required for Cdc48 binding but appears to contribute to proteasome association (Figs. 7 and 14). Conversely, a highly divergent UBL domain near the C-terminus of Cuz1 is required for its binding to Cdc48 but not the proteasome. Cuz1 plays an ancillary or partially redundant role in the degradation of UPS substrates that depend on Cdc48 (Fig. 9). Protein-protein interaction data and

other results suggest possible roles for Cuz1 in promoting polyubiquitinated substrate release from Cdc48 (Fig. 12B).

The Zf_AN1 domain has an extremely broad distribution in both eukaryotes and archaea, with a few examples in bacteria, presumably due to lateral gene transfer (239). Most remarkably, this domain is part of many highly divergent multidomain proteins, but most, if not all, are linked to membrane-localized proteolytic systems. When we performed sequence searches with the Zf_AN1 domain, it was found in a wide range of euryarchaeota species and several thaumarchaeota. The domain most commonly found to be part of archaeal Zf_AN1 proteins was a rhomboid-related protease domain. Rhomboid proteases are polytopic membrane proteins bearing a protease active site within the lipid bilayer (239). It may be relevant in this context that some eukaryotic rhomboid and pseudorhomboid proteins function in ERAD (240,241).

In eukaryotes, Zf_AN1 domains are commonly part of proteins that also contain ubiquitin, polyubiquitin, or ubiquitin-like sequences; however, the domain order in the protein is often permuted relative to Cuz1. For example, the fungus *Rhizopus oryzae* has a protein (Genbank EIE85715) with two N-terminal, near-exact ubiquitin repeats, which should be cleavable by deubiquitinating enzymes, and a C-terminal Zf_AN1 domain. The joining of divergent ubiquitin and UBL domains to Zf_AN1 motifs may be an example of convergent evolution based on their phylogenetic distribution and differences in Zf_AN1 and ubiquitin/UBL sequence; this might have been driven by a common role for many Zf_AN1 domain proteins in proteasome binding. Like Cuz1, the human AIRAP/ZFAND2A and AIRAPL/ZFAND2B proteins both bind 26S proteasomes, as does their C. elegans ortholog, AIP-1 (206,207). Our data with yeast Cuz1 (Fig. 14) and domain swaps with the AIRAP and AIRAPL proteins (206,207) both suggest that the Zf_AN1 domain participates in proteasome association. Finally, a subfamily of Zf_AN1 proteins also contain A20 zinc-finger domains, which in some cases have been shown to have ubiquitin ligase activity (242). In plants these proteins are associated with stress responses (243), while in mammals they are usually involved in the immune system (244,245). It is likely that Zf_AN1 proteins have widespread and disparate functions in the UPS.

Additional support for links between Cuz1 and both proteasomes and Cdc48 can be found in available genomic and proteomic databases. Cluster analysis of aggregated RNA microarray data (http://www.yeastgenome.org/) shows that *CUZ1* transcriptional regulation clusters very closely with that of *UBX4*, which encodes a UBX protein that has been suggested to promote dissociation of ubiquitinated proteins from Cdc48 based on results similar to those reported here for Cuz1 (149). Another gene with a similar transcription profile is *UFD1*. Of the 25 genes most closely correlated transcriptionally with *CUZ1* (in addition to *YOR052C*), 19 are proteasome subunit genes. This co-regulation is consistent with the fact that *CUZ1*, *YOR052C*, and proteasome genes all have upstream *PACE* sequences, the target for the Rpn4 transcription factor. Large-scale protein interaction studies using yeast two-hybrid or mass spectrometry approaches also identified, among other proteins, Cdc48, Ubx1, and Npl4 (http://www.yeastgenome.org/). Although not pursued further, these earlier genomic and proteomic studies are fully consistent with our findings.

Cdc48 adaptors bind to the ATPase in both mutually exclusive and interdependent fashion. Ubx1 and Npl4-Ufd1 do not bind the same Cdc48 hexamer (146,151), and conversely, certain UBX proteins only bind to Cdc48 if Npl4-Ufd1 is also present (246,247). Intriguingly, the mass spectrometry analysis of Cuz1-associated proteins identified Ubx1 as well as Npl4 and Ufd1 (Table 1 and 2). Moreover, mutations in either adaptor when combined with loss of Cuz1 cause enhanced growth defects (Fig. 10). This suggests that Cuz1 is part of distinct Cdc48-adaptor complexes. A C-terminal GFP fusion of Cuz1 localizes diffusely throughout the cell, with a slight concentration in the nucleus (248), so Cuz1 would be well placed to function in multiple Cdc48 complexes.

As is true for the majority of Cdc48 cofactors, Cuz1 interacts directly with the N-terminal domain of the ATPase (Fig. 6C). Notably, addition of ATP to Cuz1-Cdc48 binding reactions attenuates their association (Fig. 6A), and this was also apparent in our LC-MS/MS samples with ATP supplementation (Table 2). ATP has been reported to modulate the recruitment of other cofactors to Cdc48. ATP binding to the p97 D1 domain enhances interaction of the Npl4-Ufd1 heterodimer with the N-terminal domain of p97 (249), and disassembly of Ufd2-Rad23 complexes by Cdc48 depends

on Cdc48 binding to Ufd2 and ATP (223). Ubx2 interaction with Cdc48 is strongly decreased in the presence of ATP (250). Cuz1 occupancy of Cdc48 may similarly be tied to nucleotide-dependent conformation changes or specific steps of the ATPase catalytic cycle. A recent study suggests that Cdc48 may directly control proteolytic activity of the eukaryotic 20S proteasome (251); Cuz1 might modulate this potential Cdc48 function as well.

Many Cdc48 cofactors interact with polyubiquitin-protein conjugates, some of them directly. For example, Ubx1, Ubx2 and Ubx5 are UBA-UBX proteins in which the UBA domain mediates polyubiquitin binding (145). Cdc48, Npl4 and Ufd1 also all have ubiquitin-binding sites (232,252,253). Cuz1 interacts in vivo with polyubiquitinated proteins, but this is likely to be indirect (Fig. 11); this is not unexpected given that Cuz1 lacks any known ubiquitin-binding motif. The binding behavior of a series of Cuz1 deletion variants is identical for Cdc48 and polyubiquitinated species in cell extracts, consistent with the possibility that interaction between Cuz1 and polyubiquitin occurs in the context of a Cdc48 complex (Figs. 7 and 12A). While it is known that recruitment of polyubiquitinated substrates to Cdc48 is largely mediated by the Npl4-Ufd1 dimer (232), their release to downstream components is less well understood. Ubx4 has been identified as one possible releasing factor (149) and Vms1 as another (156). As observed upon deletion of these factors in the earlier studies, loss of Cuz1 also leads to an increase in the levels of polyubiquitinated substrates associated with Cdc48 (Fig. 12B). Therefore, Cuz1 might have a related mechanism of action (Fig. 23).

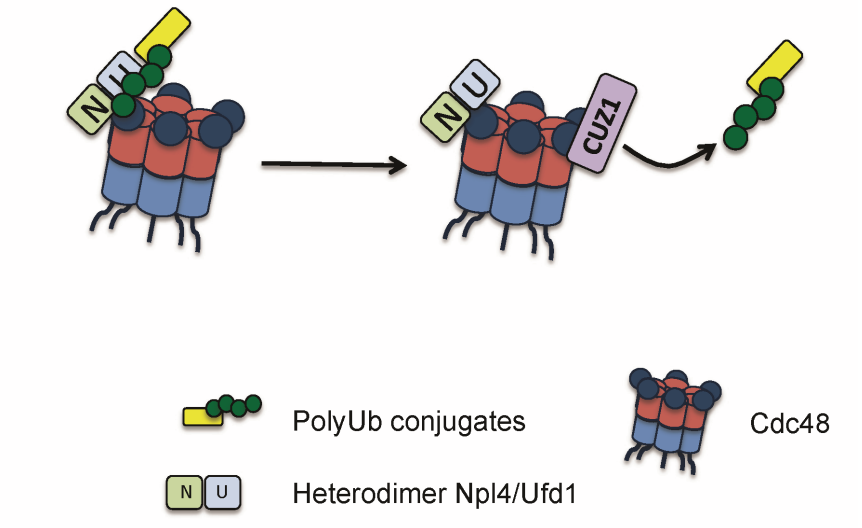


Figure 23- Schematic view of Cuz1 role in the context of Cdc48. Cuz1 is involved in the release of polyUb conjugates from Cdc48.

The exact mechanistic function of Cuz1, like that of most other Cdc48 cofactors, remains to be determined. Since Cuz1 interacts with both Cdc48 and the proteasome we can envision 3 possible scenarios: 1) a functional ternary complex composed of Cdc48, Cuz1 and the proteasome, 2) Cuz1 with distinct functions in complex with either Cdc48 or the proteasome or 3) Cuz1 sequentially binding Cdc48 and then the proteasome with an integrated function (Fig. 24).

Based on the genetic interactions we observe between mutations in Cuz1 and Npl4-Ufd1 (Fig. 10) and their co-purification (Tables 1 and 2), Cuz1 might work with Npl4-Ufd1 on a subset of Cdc48 complexes. Cuz1 function in these complexes remains unclear, but it may stimulate ATP binding or hydrolysis by Cdc48, inducing conformation changes that weaken ubiquitinated substrate binding. This would facilitate substrate transfer to downstream components like the proteasome or Rad23 or related shuttle factors.

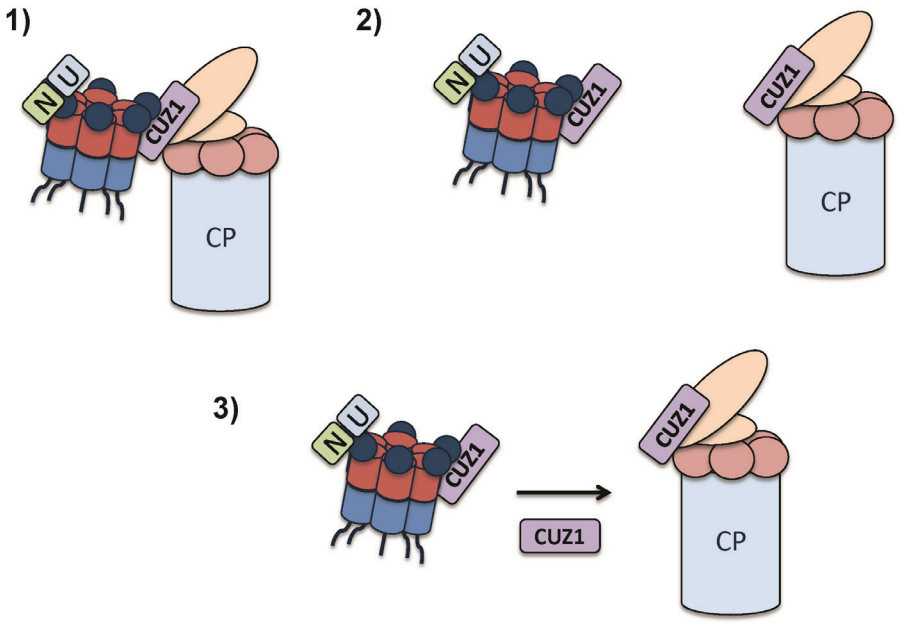


Figure 24- Possible scenarios for Cuz1 function in complex with 26S proteasome and Cdc48. The exact role of Cuz1 remains elusive, we detected the formation of Cuz1-Cdc48 and Cuz1-26S proteasome complex but we cannot exclude the 1) formation of a ternary complex Cuz1-Cdc48-26S proteasome with an integrated function. 2) Cuz1 associates with Cdc48 and the proteasome separately and exerts distinct functions in complex with either one or the other. 3) Cuz1 sequentially binding Cdc48 and then the proteasome with an integrated function.

Ufd2 binds to both Cdc48 and Rad23 and may recruit Rad23 to Cdc48-bound substrates (223). Following release from Ufd2, Rad23 and related shuttle proteins can bind the proteasome via their UBL domain interaction with Rpn1. In the absence of Cuz1, the Cdc48 complex may tend to remain bound to polyubiquitinated substrates and accompany them to the proteasome. Alternatively, Cuz1 might enhance the transfer of certain ubiquitin conjugates from Cdc48 directly to the proteasome. One intriguing possibility is that Cuz1 might be involved in promoting Cdc48-proteasome dissociation as suggested by the increased levels of Cdc48 interacting with the proteasome in the absence of *CUZ1* (Fig.19A). Arsenite may alter

Cuz1 itself in a way that impacts these processes, or the metalloid might act more indirectly, such as by modifying the cellular pool of ubiquitin conjugates.

Recent work by Hanna and colleagues (254) provides further information about Cuz1's role. They have confirmed our observation that Cuz1 interacts with both Cdc48 and the proteasome, although they tried and failed to obtain the ternary complex. In addition they have shown that Cuz1 directly interacts with Rpn2. In contrast to our results, they observed a direct interaction between Cuz1 and K48-linked polyubiquitin chains of 2 to 7 molecules. We explain the observed difference by the more stringent conditions used in our pulldown assay (Fig. 11B). The GST-Cuz1 binding to free chains observed by Hanna et al. appeared to be only slightly greater than the background binding to GST, which would be consistent with this interpretation. This result opens the interesting possibility that Cuz1, like the shuttle factors, might be escorting the polyubiquitinated conjugates from Cdc48 to the proteasome which would fit nicely the fact that *CUZ1* deletion causes an accumulation of conjugates at Cdc48 (Fig. 12B). However, it does not explain why the levels of ubiquitinated conjugates increase at the proteasome upon *CUZ1* deletion (Fig. 19B). It might be that the other shuttle factors substitute for Cuz1 function.

CHAPTER 4- SUMMARY, CONCLUSIONS AND FUTURE PERSPECTIVES

The primary goal of this thesis was to find and characterize novel components in the UPS. The UPS is the major route for the regulated degradation of proteins inside of a cell, so it is not a surprise that perturbation of this system leads to failure of cellular homeostasis and has been implicated in the pathogenesis of multiple diseases. Therefore gaining new insight into UPS mechanisms is key to new therapeutic approaches for the treatment of these diseases (22).

In this thesis I have characterized a new yeast gene, *YNL155w*, that was renamed *CUZ1* for Cdc48-associated UBL/Zn-finger protein-1. Several pieces of evidence pointed to a role for Cuz1 in the UPS, namely: 1) the existence of PACE elements in its promoter region; 2) a UBL domain in its C-terminal region combined with an N-terminal AN1 zinc-finger domain that was first identified in a ubiquitin-like protein from *Xenopus laevis* (228); 3) bioinformatic analysis suggesting a link between Cuz1 and ERAD (255); and 4) Cuz1 was thought to be AIRAP's ortholog, based on the fact that both are encoded by arsenite-inducible genes and they both possess AN1 zinc-finger domains (256).

My first approach to characterize this protein was a proteomic analysis of Cuz1 binding partners. Cdc48, a highly abundant hexameric AAA-ATPase, was identified as a major Cuz1 binding partner. Cdc48 activity is required for several different cellular pathways through its association with different ancillary proteins. I started by characterizing the interaction between Cuz1 and Cdc48. First, I validated this interaction *in vivo* and *in vitro*. I detected a direct interaction between Cuz1 and Cdc48, suggesting that this protein could be a novel Cdc48 cofactor. This possibility was further supported by the finding that ATP influences the interaction between Cuz1 and Cdc48, which is consistent with what had been observed for several other Cdc48 cofactors (249). It remains to be determined whether this modulation of Cuz1-Cdc48 association is due to ATP binding or hydrolysis. Moreover, I was able to determine that the N-terminal region of Cdc48, which is involved in the interaction with most known Cdc48 cofactors, is sufficient for binding Cuz1. Binding of Cuz1 to Cdc48 requires the Cuz1 ubiquitin-related domain (UBL). The exact determinants for

binding within the UBL should be addressed in future studies. For example, while the Cuz1 UBL does not contain exact motif matches to ubiquitin-binding segments in UBX domains or Npl4, there are some weak similarities that might indicate a similar mode of binding.

Interestingly, our LC-MS/MS results also revealed that Cuz1 copurifies with several other Cdc48 cofactors: Npl4-Ufd1, Ubx1/Shp1 and also Ubx2. In addition, we detected growth defects in *ubx1Δ cuz1Δ* strain and also in a *ufd1-2 cuz1Δ* mutant, which is consistent with our mass spectrometry data. As previously described, the Npl4-Ufd1 and Ubx1 Cdc48 adaptors bind to the ATPase in a mutually exclusive fashion. It would be interesting to test the implication that Cuz1 binds to at least two different Cdc48-adaptor complexes.

Our results indicate that Cuz1 affects protein degradation. Specifically, we have determined that Cuz1 plays an auxiliary or partially redundant role in $Cdc48^{Npl4\text{-}Ufd1}$-dependent protein degradation by the UPS. Cuz1 might work with Npl4-Ufd1 on a subset of Cdc48 substrates, so further investigation into potential substrates would be key to understanding the physiological role of Cuz1. In our mass spectrometry data, several well know Cdc48-dependent proteasome substrates were identified (fructose-1,6-bisphosphatase, Fbp1, and the largest subunit of RNA polymerase II, Rbp1), so these proteins would be good candidates for analysis.

Many Cdc48 cofactors act to recruit polyubiquitinated proteins to it. This prompted me to investigate if this was the case for Cuz1. In fact, Cuz1 is able to bind polyubiquitinated conjugates *in vivo*, but I failed to prove direct interaction with K48-linked chains. Contrary to our results, Hanna *et al* observed direct interaction with preference for chains of 4 or more ubiquitin molecules; this specificity argues against unspecific binding, although the binding appeared to be weak. Moreover, they argued that Cuz1 is a zinc-dependent ubiquitin binding protein due to loss of binding from treatment with the strong metal-binding phenanthroline compound. A likely explanation is that the direct interaction is weak and therefore the stringent conditions used in our pulldown abrogated this interaction, but there is also an indirect interaction that we are observing in the context of the Cdc48 complex and that therefore depends on the exact same fragment required for the interaction between

Cdc48 and Cuz1. More sensitive and quantitative techniques should be used to address ubiquitin binding by Cuz1. Notably, deletion of Cuz1 from cells causes an accumulation of ubiquitinated conjugates on Cdc48, consistent with a possible role in the release of the conjugates to downstream components. Most Cdc48 cofactors are poorly characterized and the exact mechanistic role of Cuz1 in complex with Cdc48 requires further study.

Our LC-MS/MS results also revealed that Cuz1 copurifies with several proteasome subunits. Therefore we focused on characterizing the interaction between Cuz1 and the proteasome, as well as exploring a possible role for Cuz1 in the cell's defense against arsenite. First we validated the interaction between Cuz1 and the 26S proteasome *in vivo* and *in vitro* and determined that a fragment that includes the AN1 zinc finger domain is required for interaction to occur. Interestingly, all the identified peptides from the MS analysis corresponded to 19S subunits, which would be consistent with Cuz1 binding the regulatory particle rather than the core particle. In fact we observed direct binding between Cuz1 and purified 19S regulatory particles, and *Hanna et al* determined that Cuz1 binds directly to the 19S base subunit Rpn2.

Cells are often exposed to metals and metalloids that can challenge protein homeostasis, but the exact effect of these compounds on the proteome as well as the mechanisms of toxicity are not fully understood. Arsenite exposure promotes an accumulation of misfolded proteins and causes accumulation of polyubiquitinated conjugates *in vivo* (206,238). There are several AN1 zinc finger domain-containing proteins whose interaction is modulated by arsenite, namely AIRAP, AIP-1 and AIRAP-L (206,207,229). This compelled us to test the influence of arsenite on the interaction of Cuz1 with the proteasome.

Arsenite exposure of yeast cells increases the interaction of Cuz1 with the proteasome, but the exact mechanism by which the metalloid exerts this effect is not clear. The transcription factor Rpn4 partially mediates this response by upregulating the transcription of *CUZ1* as well as other PACE element-regulated genes. The accumulation of Cuz1 at the proteasome might be partially explained by the overall increase of intracellular levels of Cuz1, although it is modest, but even when *RPN4*

was deleted, I still observed an increase in the levels of proteasome-bound Cuz1. Mechanisms involved in this effect should be addressed in more detail. Arsenite interacts directly with thiolates of vicinal cysteines in target proteins. It is possible that direct interaction of arsenite with Cuz1 cysteines (like those of the AN1 zinc finger domain) might alter its binding properties to the proteasome. This hypothesis could be addressed with Cuz1 cysteine mutants.

One open question is whether this arsenite effect is specific. Hanna et al. tested whether a strain lacking *CUZ1* would display a growth defect in the presence of antimony and saw a very weak growth defect of the mutant relative to WT cells. Other metalloids could be tested as well as oxidative stress-inducing treatments such as diamide or H_2O_2.

My work also provided evidence for Cuz1 involvement in the cellular response to arsenite. The data support the potential biological importance of Cuz1 in promoting proteasome function in arsenite resistance. Upon arsenite stress, there is an accumulation of polyubiquitinated proteins. The cell's response to this stress might include $Cdc48^{Cuz1}$ enhanced interaction with the proteasome during delivery of polyubiquitinated proteins to the proteasome.

I determined that Cuz1 seems to be involved in the release of conjugates from Cdc48 to downstream factors of the UPS system, either to the proteasome directly or to shuttle factors. In agreement with this hypothesis, I have found genetic interactions between *CUZ1* and two of the shuttle factors: *RAD23* and *DSK2*. Moreover, either overexpression or deletion of *CUZ1* affects the levels of proteasome-bound polyubiquitinated conjugates. Cuz1 might compete with Rad23, Dsk2 and Ddi1 for access to the proteasome; we observed a decrease in the levels of these shuttle factors bound to the proteasome upon *CUZ1* overexpression. These results support the possibility that Cuz1 is involved in the delivery of polyubiquitinated to the proteasome. Future work should focus on addressing whether the shuttle factors and Cuz1 might be functionally redundant, affecting the same substrates or instead working separately, regulating a non-overlapping set of substrates although the fact that both Rad23 and Dsk2 work with UFD and ERAD pathways argues against this last possibility.

Another interesting possibility suggested by our results is the existence of a transient ternary complex between Cdc48, the 26S proteasome and Cuz1. Cdc48 has been suggested to directly interact with either the 20S or with full 26S proteasomes through its 19S particle (81,222,251). Cdc48/p97 interaction with the proteasome had been previously reported upon arsenite exposure or other stresses (222). This raises the possibility that Cuz1 plays a role together with Cdc48 at the proteasome that will promote arsenite resistance. The existence and physiological relevance of such a hypothetical complex would need to be investigated.

In conclusion, this work provides the characterization of a novel protein, Cuz1, involved in Cdc48 function in the ubiquitin-proteasome system. Much of the machinery of the UPS has been identified, but key parts of the system remain poorly understood. Prominent among the open questions is the mechanism by which polyubiquitinated proteins are released by E3 ligases and transferred to ubiquitin receptors and the proteasome for degradation. We were able to infer a function for Cuz1 somewhere in this chain of events, but as with most Cdc48 cofactors, further work to assess its exact mechanistic role will be necessary.

BIBLIOGRAPHY

1. Goldberg, A. L., and Dice, J. F. (1974) Intracellular protein degradation in mammalian and bacterial cells. *Annual review of biochemistry* **43**, 835-869
2. Reinstein, E., and Ciechanover, A. (2006) Narrative review: protein degradation and human diseases: the ubiquitin connection. *Annals of internal medicine* **145**, 676-684
3. Rock, K. L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., and Goldberg, A. L. (1994) Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. *Cell* **78**, 761-771
4. De Duve, C., Gianetto, R., Appelmans, F., and Wattiaux, R. (1953) Enzymic content of the mitochondria fraction. *Nature* **172**, 1143-1144
5. Rabinovitz, M., and Fisher, J. M. (1964) Characteristics of the Inhibition of Hemoglobin Synthesis in Rabbit Reticulocytes by Threo-Alpha-Amino-Beta-Chlorobutyric Acid. *Biochim Biophys Acta* **91**, 313-322
6. Etlinger, J. D., and Goldberg, A. L. (1977) A soluble ATP-dependent proteolytic system responsible for the degradation of abnormal proteins in reticulocytes. *Proceedings of the National Academy of Sciences of the United States of America* **74**, 54-58
7. Ciehanover, A., Hod, Y., and Hershko, A. (1978) A heat-stable polypeptide component of an ATP-dependent proteolytic system from reticulocytes. *Biochemical and biophysical research communications* **81**, 1100-1105
8. Wilkinson, K. D., Urban, M. K., and Haas, A. L. (1980) Ubiquitin is the ATP-dependent proteolysis factor I of rabbit reticulocytes. *The Journal of biological chemistry* **255**, 7529-7532
9. Ciechanover, A., Heller, H., Elias, S., Haas, A. L., and Hershko, A. (1980) ATP-dependent conjugation of reticulocyte proteins with the polypeptide required for protein degradation. *Proceedings of the National Academy of Sciences of the United States of America* **77**, 1365-1368

10. Hershko, A., Ciechanover, A., and Rose, I. A. (1979) Resolution of the ATP-dependent proteolytic system from reticulocytes: a component that interacts with ATP. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 3107-3110
11. Kloetzel, P. M. (2001) Antigen processing by the proteasome. *Nature reviews. Molecular cell biology* **2**, 179-187
12. Lam, Y. A., Pickart, C. M., Alban, A., Landon, M., Jamieson, C., Ramage, R., Mayer, R. J., and Layfield, R. (2000) Inhibition of the ubiquitin-proteasome system in Alzheimer's disease. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 9902-9906
13. Jentsch, S., McGrath, J. P., and Varshavsky, A. (1987) The yeast DNA repair gene RAD6 encodes a ubiquitin-conjugating enzyme. *Nature* **329**, 131-134
14. Reed, S. I. (2006) The ubiquitin-proteasome pathway in cell cycle control. *Results and problems in cell differentiation* **42**, 147-181
15. Muratani, M., and Tansey, W. P. (2003) How the ubiquitin-proteasome system controls transcription. *Nature reviews. Molecular cell biology* **4**, 192-201
16. Wilkinson, K. D. (1999) Ubiquitin-dependent signaling: the role of ubiquitination in the response of cells to their environment. *The Journal of nutrition* **129**, 1933-1936
17. Hamilton, A. M., and Zito, K. (2013) Breaking it down: the ubiquitin proteasome system in neuronal morphogenesis. *Neural plasticity* **2013**, 196848
18. Coux, O., Tanaka, K., and Goldberg, A. L. (1996) Structure and functions of the 20S and 26S proteasomes. *Annual review of biochemistry* **65**, 801-847
19. Magill, L., Walker, B., and Irvine, A. E. (2003) The proteasome: a novel therapeutic target in haematopoietic malignancy. *Hematology* **8**, 275-283
20. Dahlmann, B. (2007) Role of proteasomes in disease. *BMC biochemistry* **8 Suppl 1**, S3
21. Naiki, H., and Nagai, Y. (2009) Molecular pathogenesis of protein misfolding diseases: pathological molecular environments versus quality control systems against misfolded proteins. *Journal of biochemistry* **146**, 751-756

22. Nalepa, G., Rolfe, M., and Harper, J. W. (2006) Drug discovery in the ubiquitin-proteasome system. *Nature reviews. Drug discovery* **5**, 596-613
23. Hideshima, T., Richardson, P., Chauhan, D., Palombella, V. J., Elliott, P. J., Adams, J., and Anderson, K. C. (2001) The proteasome inhibitor PS-341 inhibits growth, induces apoptosis, and overcomes drug resistance in human multiple myeloma cells. *Cancer research* **61**, 3071-3076
24. Vijay-Kumar, S., Bugg, C. E., Wilkinson, K. D., and Cook, W. J. (1985) Three-dimensional structure of ubiquitin at 2.8 A resolution. *Proc Natl Acad Sci U S A* **82**, 3582-3585
25. Sigismund, S., Polo, S., and Di Fiore, P. P. (2004) Signaling through monoubiquitination. *Curr Top Microbiol Immunol* **286**, 149-185
26. Peng, J., Schwartz, D., Elias, J. E., Thoreen, C. C., Cheng, D., Marsischky, G., Roelofs, J., Finley, D., and Gygi, S. P. (2003) A proteomics approach to understanding protein ubiquitination. *Nat Biotechnol* **21**, 921-926
27. Kim, H. T., Kim, K. P., Lledias, F., Kisselev, A. F., Scaglione, K. M., Skowyra, D., Gygi, S. P., and Goldberg, A. L. (2007) Certain pairs of ubiquitin-conjugating enzymes (E2s) and ubiquitin-protein ligases (E3s) synthesize nondegradable forked ubiquitin chains containing all possible isopeptide linkages. *J Biol Chem* **282**, 17375-17386
28. Thrower, J. S., Hoffman, L., Rechsteiner, M., and Pickart, C. M. (2000) Recognition of the polyubiquitin proteolytic signal. *The EMBO journal* **19**, 94-102
29. Xu, P., Duong, D. M., Seyfried, N. T., Cheng, D., Xie, Y., Robert, J., Rush, J., Hochstrasser, M., Finley, D., and Peng, J. (2009) Quantitative proteomics reveals the function of unconventional ubiquitin chains in proteasomal degradation. *Cell* **137**, 133-145
30. Gandre, S., and Kahana, C. (2002) Degradation of ornithine decarboxylase in Saccharomyces cerevisiae is ubiquitin independent. *Biochem Biophys Res Commun* **293**, 139-144

31. Spence, J., Sadis, S., Haas, A. L., and Finley, D. (1995) A ubiquitin mutant with specific defects in DNA repair and multiubiquitination. *Mol Cell Biol* **15**, 1265-1273
32. Haas, A. L., Warms, J. V., Hershko, A., and Rose, I. A. (1982) Ubiquitin-activating enzyme. Mechanism and role in protein-ubiquitin conjugation. *The Journal of biological chemistry* **257**, 2543-2548
33. Ciechanover, A., Heller, H., Katz-Etzion, R., and Hershko, A. (1981) Activation of the heat-stable polypeptide of the ATP-dependent proteolytic system. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 761-765
34. Burroughs, A. M., Jaffee, M., Iyer, L. M., and Aravind, L. (2008) Anatomy of the E2 ligase fold: implications for enzymology and evolution of ubiquitin/Ub-like protein conjugation. *J Struct Biol* **162**, 205-218
35. Aravind, L., and Koonin, E. V. (2000) The U box is a modified RING finger - a common domain in ubiquitination. *Current biology : CB* **10**, R132-134
36. Ohi, M. D., Vander Kooi, C. W., Rosenberg, J. A., Chazin, W. J., and Gould, K. L. (2003) Structural insights into the U-box, a domain associated with multi-ubiquitination. *Nature structural biology* **10**, 250-255
37. Sadowski, M., and Sarcevic, B. (2010) Mechanisms of mono- and poly-ubiquitination: Ubiquitination specificity depends on compatibility between the E2 catalytic core and amino acid residues proximal to the lysine. *Cell division* **5**, 19
38. Varshavsky, A. (1991) Naming a targeting signal. *Cell* **64**, 13-15
39. Ravid, T., and Hochstrasser, M. (2008) Diversity of degradation signals in the ubiquitin-proteasome system. *Nature reviews. Molecular cell biology* **9**, 679-690
40. Barnes, J. A., and Gomes, A. V. (1995) PEST sequences in calmodulin-binding proteins. *Molecular and cellular biochemistry* **149-150**, 17-27
41. Mukhopadhyay, D., and Riezman, H. (2007) Proteasome-independent functions of ubiquitin in endocytosis and signaling. *Science* **315**, 201-205

42. Haglund, K., and Dikic, I. (2005) Ubiquitylation and cell signaling. *EMBO J* **24**, 3353-3359
43. Ha, B. H., and Kim, E. E. (2008) Structures of proteases for ubiqutin and ubiquitin-like modifiers. *BMB reports* **41**, 435-443
44. Petroski, M. D., and Deshaies, R. J. (2005) Function and regulation of cullin-RING ubiquitin ligases. *Nat Rev Mol Cell Biol* **6**, 9-20
45. Liakopoulos, D., Doenges, G., Matuschewski, K., and Jentsch, S. (1998) A novel protein modification pathway related to the ubiquitin system. *EMBO J* **17**, 2208-2214
46. Lammer, D., Mathias, N., Laplaza, J. M., Jiang, W., Liu, Y., Callis, J., Goebl, M., and Estelle, M. (1998) Modification of yeast Cdc53p by the ubiquitin-related protein rub1p affects function of the SCFCdc4 complex. *Genes Dev* **12**, 914-926
47. Read, M. A., Brownell, J. E., Gladysheva, T. B., Hottelet, M., Parent, L. A., Coggins, M. B., Pierce, J. W., Podust, V. N., Luo, R. S., Chau, V., and Palombella, V. J. (2000) Nedd8 modification of cul-1 activates SCF(beta(TrCP))-dependent ubiquitination of IkappaBalpha. *Mol Cell Biol* **20**, 2326-2333
48. Kawakami, T., Chiba, T., Suzuki, T., Iwai, K., Yamanaka, K., Minato, N., Suzuki, H., Shimbara, N., Hidaka, Y., Osaka, F., Omata, M., and Tanaka, K. (2001) NEDD8 recruits E2-ubiquitin to SCF E3 ligase. *EMBO J* **20**, 4003-4012
49. Liu, J., Furukawa, M., Matsumoto, T., and Xiong, Y. (2002) NEDD8 modification of CUL1 dissociates p120(CAND1), an inhibitor of CUL1-SKP1 binding and SCF ligases. *Mol Cell* **10**, 1511-1518
50. Owerbach, D., McKay, E. M., Yeh, E. T., Gabbay, K. H., and Bohren, K. M. (2005) A proline-90 residue unique to SUMO-4 prevents maturation and sumoylation. *Biochem Biophys Res Commun* **337**, 517-520
51. Rodriguez, M. S., Dargemont, C., and Hay, R. T. (2001) SUMO-1 conjugation in vivo requires both a consensus modification motif and nuclear targeting. *J Biol Chem* **276**, 12654-12659

52. Bylebyl, G. R., Belichenko, I., and Johnson, E. S. (2003) The SUMO isopeptidase Ulp2 prevents accumulation of SUMO chains in yeast. *J Biol Chem* **278**, 44113-44120
53. Tatham, M. H., Jaffray, E., Vaughan, O. A., Desterro, J. M., Botting, C. H., Naismith, J. H., and Hay, R. T. (2001) Polymeric chains of SUMO-2 and SUMO-3 are conjugated to protein substrates by SAE1/SAE2 and Ubc9. *J Biol Chem* **276**, 35368-35374
54. Minty, A., Dumont, X., Kaghad, M., and Caput, D. (2000) Covalent modification of p73alpha by SUMO-1. Two-hybrid screening with p73 identifies novel SUMO-1-interacting proteins and a SUMO-1 interaction motif. *J Biol Chem* **275**, 36316-36323
55. Song, J., Durrin, L. K., Wilkinson, T. A., Krontiris, T. G., and Chen, Y. (2004) Identification of a SUMO-binding motif that recognizes SUMO-modified proteins. *Proc Natl Acad Sci U S A* **101**, 14373-14378
56. Kerscher, O., Felberbaum, R., and Hochstrasser, M. (2006) Modification of proteins by ubiquitin and ubiquitin-like proteins. *Annu Rev Cell Dev Biol* **22**, 159-180
57. Geiss-Friedlander, R., and Melchior, F. (2007) Concepts in sumoylation: a decade on. *Nat Rev Mol Cell Biol* **8**, 947-956
58. Uzunova, K., Gottsche, K., Miteva, M., Weisshaar, S. R., Glanemann, C., Schnellhardt, M., Niessen, M., Scheel, H., Hofmann, K., Johnson, E. S., Praefcke, G. J., and Dohmen, R. J. (2007) Ubiquitin-dependent proteolytic control of SUMO conjugates. *J Biol Chem* **282**, 34167-34175
59. Prudden, J., Pebernard, S., Raffa, G., Slavin, D. A., Perry, J. J., Tainer, J. A., McGowan, C. H., and Boddy, M. N. (2007) SUMO-targeted ubiquitin ligases in genome stability. *EMBO J* **26**, 4089-4101
60. Tatham, M. H., Geoffroy, M. C., Shen, L., Plechanovova, A., Hattersley, N., Jaffray, E. G., Palvimo, J. J., and Hay, R. T. (2008) RNF4 is a poly-SUMO-specific E3 ubiquitin ligase required for arsenic-induced PML degradation. *Nat Cell Biol* **10**, 538-546

61. Sugawara, K., Suzuki, N. N., Fujioka, Y., Mizushima, N., Ohsumi, Y., and Inagaki, F. (2004) The crystal structure of microtubule-associated protein light chain 3, a mammalian homologue of Saccharomyces cerevisiae Atg8. *Genes Cells* **9**, 611-618
62. Suzuki, N. N., Yoshimoto, K., Fujioka, Y., Ohsumi, Y., and Inagaki, F. (2005) The crystal structure of plant ATG12 and its biological implication in autophagy. *Autophagy* **1**, 119-126
63. Mizushima, N., Noda, T., Yoshimori, T., Tanaka, Y., Ishii, T., George, M. D., Klionsky, D. J., Ohsumi, M., and Ohsumi, Y. (1998) A protein conjugation system essential for autophagy. *Nature* **395**, 395-398
64. Shintani, T., Mizushima, N., Ogawa, Y., Matsuura, A., Noda, T., and Ohsumi, Y. (1999) Apg10p, a novel protein-conjugating enzyme essential for autophagy in yeast. *EMBO J* **18**, 5234-5241
65. Ichimura, Y., Kirisako, T., Takao, T., Satomi, Y., Shimonishi, Y., Ishihara, N., Mizushima, N., Tanida, I., Kominami, E., Ohsumi, M., Noda, T., and Ohsumi, Y. (2000) A ubiquitin-like system mediates protein lipidation. *Nature* **408**, 488-492
66. Hanada, T., Noda, N. N., Satomi, Y., Ichimura, Y., Fujioka, Y., Takao, T., Inagaki, F., and Ohsumi, Y. (2007) The Atg12-Atg5 conjugate has a novel E3-like activity for protein lipidation in autophagy. *J Biol Chem* **282**, 37298-37302
67. Furukawa, K., Mizushima, N., Noda, T., and Ohsumi, Y. (2000) A protein conjugation system in yeast with homology to biosynthetic enzyme reaction of prokaryotes. *J Biol Chem* **275**, 7462-7465
68. Van der Veen, A. G., Schorpp, K., Schlieker, C., Buti, L., Damon, J. R., Spooner, E., Ploegh, H. L., and Jentsch, S. (2011) Role of the ubiquitin-like protein Urm1 as a noncanonical lysine-directed protein modifier. *Proc Natl Acad Sci U S A* **108**, 1763-1770
69. Wang, F., Liu, M., Qiu, R., and Ji, C. (2011) The dual role of ubiquitin-like protein Urm1 as a protein modifier and sulfur carrier. *Protein Cell* **2**, 612-619
70. Bochtler, M., Ditzel, L., Groll, M., Hartmann, C., and Huber, R. (1999) The proteasome. *Annu Rev Biophys Biomol Struct* **28**, 295-317

71. Hershko, A., and Ciechanover, A. (1998) The ubiquitin system. *Annual review of biochemistry* **67**, 425-479
72. Tanaka, K. (2009) The proteasome: overview of structure and functions. *Proceedings of the Japan Academy. Series B, Physical and biological sciences* **85**, 12-36
73. Groll, M., Ditzel, L., Lowe, J., Stock, D., Bochtler, M., Bartunik, H. D., and Huber, R. (1997) Structure of 20S proteasome from yeast at 2.4 A resolution. *Nature* **386**, 463-471
74. Groll, M., Bajorek, M., Kohler, A., Moroder, L., Rubin, D. M., Huber, R., Glickman, M. H., and Finley, D. (2000) A gated channel into the proteasome core particle. *Nature structural biology* **7**, 1062-1067
75. Groll, M., and Huber, R. (2003) Substrate access and processing by the 20S proteasome core particle. *The international journal of biochemistry & cell biology* **35**, 606-616
76. Seemuller, E., Lupas, A., Stock, D., Lowe, J., Huber, R., and Baumeister, W. (1995) Proteasome from Thermoplasma acidophilum: a threonine protease. *Science* **268**, 579-582
77. Brannigan, J. A., Dodson, G., Duggleby, H. J., Moody, P. C., Smith, J. L., Tomchick, D. R., and Murzin, A. G. (1995) A protein catalytic framework with an N-terminal nucleophile is capable of self-activation. *Nature* **378**, 416-419
78. Basler, M., Kirk, C. J., and Groettrup, M. (2013) The immunoproteasome in antigen processing and other immunological functions. *Current opinion in immunology* **25**, 74-80
79. Stadtmueller, B. M., and Hill, C. P. (2011) Proteasome activators. *Molecular cell* **41**, 8-19
80. Huang, L., and Chen, C. H. (2009) Proteasome regulators: activators and inhibitors. *Current medicinal chemistry* **16**, 931-939
81. Barthelme, D., and Sauer, R. T. (2012) Identification of the Cdc48*20S proteasome as an ancient AAA+ proteolytic machine. *Science* **337**, 843-846

82. Lehmann, A., Niewienda, A., Jechow, K., Janek, K., and Enenkel, C. (2010) Ecm29 fulfils quality control functions in proteasome assembly. *Molecular cell* **38**, 879-888

83. Lander, G. C., Estrin, E., Matyskiela, M. E., Bashore, C., Nogales, E., and Martin, A. (2012) Complete subunit architecture of the proteasome regulatory particle. *Nature* **482**, 186-191

84. Lasker, K., Forster, F., Bohn, S., Walzthoeni, T., Villa, E., Unverdorben, P., Beck, F., Aebersold, R., Sali, A., and Baumeister, W. (2012) Molecular architecture of the 26S proteasome holocomplex determined by an integrative approach. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 1380-1387

85. Smith, D. M., Chang, S. C., Park, S., Finley, D., Cheng, Y., and Goldberg, A. L. (2007) Docking of the proteasomal ATPases' carboxyl termini in the 20S proteasome's alpha ring opens the gate for substrate entry. *Molecular cell* **27**, 731-744

86. Tomko, R. J., Jr., Funakoshi, M., Schneider, K., Wang, J., and Hochstrasser, M. (2010) Heterohexameric ring arrangement of the eukaryotic proteasomal ATPases: implications for proteasome structure and assembly. *Molecular cell* **38**, 393-403

87. Matyskiela, M. E., Lander, G. C., and Martin, A. (2013) Conformational switching of the 26S proteasome enables substrate degradation. *Nature structural & molecular biology* **20**, 781-788

88. Kajava, A. V. (2002) What curves alpha-solenoids? Evidence for an alpha-helical toroid structure of Rpn1 and Rpn2 proteins of the 26 S proteasome. *The Journal of biological chemistry* **277**, 49791-49798

89. Elsasser, S., Gali, R. R., Schwickart, M., Larsen, C. N., Leggett, D. S., Muller, B., Feng, M. T., Tubing, F., Dittmar, G. A., and Finley, D. (2002) Proteasome subunit Rpn1 binds ubiquitin-like protein domains. *Nature cell biology* **4**, 725-730

90. Rosenzweig, R., Bronner, V., Zhang, D., Fushman, D., and Glickman, M. H. (2012) Rpn1 and Rpn2 coordinate ubiquitin processing factors at proteasome. *The Journal of biological chemistry* **287**, 14659-14671
91. Wang, X., Chen, C. F., Baker, P. R., Chen, P. L., Kaiser, P., and Huang, L. (2007) Mass spectrometric characterization of the affinity-purified human 26S proteasome complex. *Biochemistry* **46**, 3553-3565
92. Verma, R., Chen, S., Feldman, R., Schieltz, D., Yates, J., Dohmen, J., and Deshaies, R. J. (2000) Proteasomal proteomics: identification of nucleotide-sensitive proteasome-interacting proteins by mass spectrometric analysis of affinity-purified proteasomes. *Molecular biology of the cell* **11**, 3425-3439
93. Leggett, D. S., Hanna, J., Borodovsky, A., Crosas, B., Schmidt, M., Baker, R. T., Walz, T., Ploegh, H., and Finley, D. (2002) Multiple associated proteins regulate proteasome structure and function. *Molecular cell* **10**, 495-507
94. Besche, H. C., Haas, W., Gygi, S. P., and Goldberg, A. L. (2009) Isolation of mammalian 26S proteasomes and p97/VCP complexes using the ubiquitin-like domain from HHR23B reveals novel proteasome-associated proteins. *Biochemistry* **48**, 2538-2549
95. Tomko, R. J., Jr., and Hochstrasser, M. (2011) Incorporation of the Rpn12 subunit couples completion of proteasome regulatory particle lid assembly to lid-base joining. *Molecular cell* **44**, 907-917
96. Estrin, E., Lopez-Blanco, J. R., Chacon, P., and Martin, A. (2013) Formation of an intricate helical bundle dictates the assembly of the 26S proteasome lid. *Structure* **21**, 1624-1635
97. Tomko, R. J., Jr., and Hochstrasser, M. (2014) The Intrinsically Disordered Sem1 Protein Functions as a Molecular Tether during Proteasome Lid Biogenesis. *Molecular cell*
98. Sakata, E., Bohn, S., Mihalache, O., Kiss, P., Beck, F., Nagy, I., Nickell, S., Tanaka, K., Saeki, Y., Forster, F., and Baumeister, W. (2012) Localization of the proteasomal ubiquitin receptors Rpn10 and Rpn13 by electron cryomicroscopy. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 1479-1484

99. Deveraux, Q., Ustrell, V., Pickart, C., and Rechsteiner, M. (1994) A 26 S protease subunit that binds ubiquitin conjugates. *The Journal of biological chemistry* **269**, 7059-7061

100. Husnjak, K., Elsasser, S., Zhang, N., Chen, X., Randles, L., Shi, Y., Hofmann, K., Walters, K. J., Finley, D., and Dikic, I. (2008) Proteasome subunit Rpn13 is a novel ubiquitin receptor. *Nature* **453**, 481-488

101. Lam, Y. A., Lawson, T. G., Velayutham, M., Zweier, J. L., and Pickart, C. M. (2002) A proteasomal ATPase subunit recognizes the polyubiquitin degradation signal. *Nature* **416**, 763-767

102. Zhang, N., Wang, Q., Ehlinger, A., Randles, L., Lary, J. W., Kang, Y., Haririnia, A., Storaska, A. J., Cole, J. L., Fushman, D., and Walters, K. J. (2009) Structure of the s5a:k48-linked diubiquitin complex and its interactions with rpn13. *Molecular cell* **35**, 280-290

103. Fu, H., Reis, N., Lee, Y., Glickman, M. H., and Vierstra, R. D. (2001) Subunit interaction maps for the regulatory particle of the 26S proteasome and the COP9 signalosome. *The EMBO journal* **20**, 7096-7107

104. Fu, H., Sadis, S., Rubin, D. M., Glickman, M., van Nocker, S., Finley, D., and Vierstra, R. D. (1998) Multiubiquitin chain binding and protein degradation are mediated by distinct domains within the 26 S proteasome subunit Mcb1. *The Journal of biological chemistry* **273**, 1970-1981

105. Matiuhin, Y., Kirkpatrick, D. S., Ziv, I., Kim, W., Dakshinamurthy, A., Kleifeld, O., Gygi, S. P., Reis, N., and Glickman, M. H. (2008) Extraproteasomal Rpn10 restricts access of the polyubiquitin-binding protein Dsk2 to proteasome. *Molecular cell* **32**, 415-425

106. Kim, H. T., Kim, K. P., Uchiki, T., Gygi, S. P., and Goldberg, A. L. (2009) S5a promotes protein degradation by blocking synthesis of nondegradable forked ubiquitin chains. *The EMBO journal* **28**, 1867-1877

107. Hamazaki, J., Iemura, S., Natsume, T., Yashiroda, H., Tanaka, K., and Murata, S. (2006) A novel proteasome interacting protein recruits the deubiquitinating enzyme UCH37 to 26S proteasomes. *The EMBO journal* **25**, 4524-4536

108. Yao, T., Song, L., Xu, W., DeMartino, G. N., Florens, L., Swanson, S. K., Washburn, M. P., Conaway, R. C., Conaway, J. W., and Cohen, R. E. (2006) Proteasome recruitment and activation of the Uch37 deubiquitinating enzyme by Adrm1. *Nature cell biology* **8**, 994-1002

109. Lam, Y. A., Xu, W., DeMartino, G. N., and Cohen, R. E. (1997) Editing of ubiquitin conjugates by an isopeptidase in the 26S proteasome. *Nature* **385**, 737-740

110. Schreiner, P., Chen, X., Husnjak, K., Randles, L., Zhang, N., Elsasser, S., Finley, D., Dikic, I., Walters, K. J., and Groll, M. (2008) Ubiquitin docking at the proteasome through a novel pleckstrin-homology domain interaction. *Nature* **453**, 548-552

111. Chen, L., Shinde, U., Ortolan, T. G., and Madura, K. (2001) Ubiquitin-associated (UBA) domains in Rad23 bind ubiquitin and promote inhibition of multi-ubiquitin chain assembly. *EMBO reports* **2**, 933-938

112. Bertolaet, B. L., Clarke, D. J., Wolff, M., Watson, M. H., Henze, M., Divita, G., and Reed, S. I. (2001) UBA domains of DNA damage-inducible proteins interact with ubiquitin. *Nature structural biology* **8**, 417-422

113. Funakoshi, M., Sasaki, T., Nishimoto, T., and Kobayashi, H. (2002) Budding yeast Dsk2p is a polyubiquitin-binding protein that can interact with the proteasome. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 745-750

114. Su, V., and Lau, A. F. (2009) Ubiquitin-like and ubiquitin-associated domain proteins: significance in proteasomal degradation. *Cellular and molecular life sciences : CMLS* **66**, 2819-2833

115. Elsasser, S., Chandler-Militello, D., Muller, B., Hanna, J., and Finley, D. (2004) Rad23 and Rpn10 serve as alternative ubiquitin receptors for the proteasome. *The Journal of biological chemistry* **279**, 26817-26822

116. Rao, H., and Sastry, A. (2002) Recognition of specific ubiquitin conjugates is important for the proteolytic functions of the ubiquitin-associated domain proteins Dsk2 and Rad23. *The Journal of biological chemistry* **277**, 11691-11695

117. Lambertson, D., Chen, L., and Madura, K. (1999) Pleiotropic defects caused by loss of the proteasome-interacting factors Rad23 and Rpn10 of Saccharomyces cerevisiae. *Genetics* **153**, 69-79
118. Brignone, C., Bradley, K. E., Kisselev, A. F., and Grossman, S. R. (2004) A post-ubiquitination role for MDM2 and hHR23A in the p53 degradation pathway. *Oncogene* **23**, 4121-4129
119. Dai, R. M., and Li, C. C. (2001) Valosin-containing protein is a multi-ubiquitin chain-targeting factor required in ubiquitin-proteasome degradation. *Nature cell biology* **3**, 740-744
120. Verma, R., Aravind, L., Oania, R., McDonald, W. H., Yates, J. R., 3rd, Koonin, E. V., and Deshaies, R. J. (2002) Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome. *Science* **298**, 611-615
121. Amerik, A. Y., and Hochstrasser, M. (2004) Mechanism and function of deubiquitinating enzymes. *Biochimica et biophysica acta* **1695**, 189-207
122. Hurley, J. H., Lee, S., and Prag, G. (2006) Ubiquitin-binding domains. *The Biochemical journal* **399**, 361-372
123. Yao, T., and Cohen, R. E. (2002) A cryptic protease couples deubiquitination and degradation by the proteasome. *Nature* **419**, 403-407
124. Hanna, J., Hathaway, N. A., Tone, Y., Crosas, B., Elsasser, S., Kirkpatrick, D. S., Leggett, D. S., Gygi, S. P., King, R. W., and Finley, D. (2006) Deubiquitinating enzyme Ubp6 functions noncatalytically to delay proteasomal degradation. *Cell* **127**, 99-111
125. Lee, B. H., Lee, M. J., Park, S., Oh, D. C., Elsasser, S., Chen, P. C., Gartner, C., Dimova, N., Hanna, J., Gygi, S. P., Wilson, S. M., King, R. W., and Finley, D. (2010) Enhancement of proteasome activity by a small-molecule inhibitor of USP14. *Nature* **467**, 179-184
126. Peth, A., Besche, H. C., and Goldberg, A. L. (2009) Ubiquitinated proteins activate the proteasome by binding to Usp14/Ubp6, which causes 20S gate opening. *Molecular cell* **36**, 794-804

127. Sakata, E., Stengel, F., Fukunaga, K., Zhou, M., Saeki, Y., Forster, F., Baumeister, W., Tanaka, K., and Robinson, C. V. (2011) The catalytic activity of Ubp6 enhances maturation of the proteasomal regulatory particle. *Molecular cell* **42**, 637-649
128. Lowe, J., Stock, D., Jap, B., Zwickl, P., Baumeister, W., and Huber, R. (1995) Crystal structure of the 20S proteasome from the archaeon T. acidophilum at 3.4 A resolution. *Science* **268**, 533-539
129. Smith, D. M., Fraga, H., Reis, C., Kafri, G., and Goldberg, A. L. (2011) ATP binds to proteasomal ATPases in pairs with distinct functional effects, implying an ordered reaction cycle. *Cell* **144**, 526-538
130. Smith, D. M., Kafri, G., Cheng, Y., Ng, D., Walz, T., and Goldberg, A. L. (2005) ATP binding to PAN or the 26S ATPases causes association with the 20S proteasome, gate opening, and translocation of unfolded proteins. *Molecular cell* **20**, 687-698
131. Benaroudj, N., and Goldberg, A. L. (2000) PAN, the proteasome-activating nucleotidase from archaebacteria, is a protein-unfolding molecular chaperone. *Nature cell biology* **2**, 833-839
132. Benaroudj, N., Zwickl, P., Seemuller, E., Baumeister, W., and Goldberg, A. L. (2003) ATP hydrolysis by the proteasome regulatory complex PAN serves multiple functions in protein degradation. *Molecular cell* **11**, 69-78
133. Rabl, J., Smith, D. M., Yu, Y., Chang, S. C., Goldberg, A. L., and Cheng, Y. (2008) Mechanism of gate opening in the 20S proteasome by the proteasomal ATPases. *Molecular cell* **30**, 360-368
134. Frohlich, K. U., Fries, H. W., Rudiger, M., Erdmann, R., Botstein, D., and Mecke, D. (1991) Yeast cell cycle protein CDC48p shows full-length homology to the mammalian protein VCP and is a member of a protein family involved in secretion, peroxisome formation, and gene expression. *J Cell Biol* **114**, 443-453
135. Song, C., Wang, Q., and Li, C. C. (2003) ATPase activity of p97-valosin-containing protein (VCP). D2 mediates the major enzyme activity, and D1

contributes to the heat-induced activity. *The Journal of biological chemistry* **278**, 3648-3655

136. Wang, Q., Song, C., and Li, C. C. (2003) Hexamerization of p97-VCP is promoted by ATP binding to the D1 domain and required for ATPase and biological activities. *Biochemical and biophysical research communications* **300**, 253-260
137. Wang, Q., Song, C., Yang, X., and Li, C. C. (2003) D1 ring is stable and nucleotide-independent, whereas D2 ring undergoes major conformational changes during the ATPase cycle of p97-VCP. *The Journal of biological chemistry* **278**, 32784-32793
138. Moir, D., Stewart, S. E., Osmond, B. C., and Botstein, D. (1982) Cold-sensitive cell-division-cycle mutants of yeast: isolation, properties, and pseudoreversion studies. *Genetics* **100**, 547-563
139. Ghislain, M., Dohmen, R. J., Levy, F., and Varshavsky, A. (1996) Cdc48p interacts with Ufd3p, a WD repeat protein required for ubiquitin-mediated proteolysis in Saccharomyces cerevisiae. *The EMBO journal* **15**, 4884-4899
140. Baek, G. H., Cheng, H., Choe, V., Bao, X., Shao, J., Luo, S., and Rao, H. (2013) Cdc48: A Swiss Army Knife of Cell Biology. *Journal of amino acids* **2013**, 183421
141. Dreveny, I., Kondo, H., Uchiyama, K., Shaw, A., Zhang, X., and Freemont, P. S. (2004) Structural basis of the interaction between the AAA ATPase p97/VCP and its adaptor protein p47. *The EMBO journal* **23**, 1030-1039
142. Buchberger, A., Howard, M. J., Proctor, M., and Bycroft, M. (2001) The UBX domain: a widespread ubiquitin-like module. *Journal of molecular biology* **307**, 17-24
143. Decottignies, A., Evain, A., and Ghislain, M. (2004) Binding of Cdc48p to a ubiquitin-related UBX domain from novel yeast proteins involved in intracellular proteolysis and sporulation. *Yeast* **21**, 127-139
144. Hartmann-Petersen, R., Wallace, M., Hofmann, K., Koch, G., Johnsen, A. H., Hendil, K. B., and Gordon, C. (2004) The Ubx2 and Ubx3 cofactors direct

Cdc48 activity to proteolytic and nonproteolytic ubiquitin-dependent processes. *Current biology : CB* **14**, 824-828

145. Schuberth, C., Richly, H., Rumpf, S., and Buchberger, A. (2004) Shp1 and Ubx2 are adaptors of Cdc48 involved in ubiquitin-dependent protein degradation. *EMBO reports* **5**, 818-824
146. Kondo, H., Rabouille, C., Newman, R., Levine, T. P., Pappin, D., Freemont, P., and Warren, G. (1997) p47 is a cofactor for p97-mediated membrane fusion. *Nature* **388**, 75-78
147. Uchiyama, K., Jokitalo, E., Kano, F., Murata, M., Zhang, X., Canas, B., Newman, R., Rabouille, C., Pappin, D., Freemont, P., and Kondo, H. (2002) VCIP135, a novel essential factor for p97/p47-mediated membrane fusion, is required for Golgi and ER assembly in vivo. *The Journal of cell biology* **159**, 855-866
148. Neuber, O., Jarosch, E., Volkwein, C., Walter, J., and Sommer, T. (2005) Ubx2 links the Cdc48 complex to ER-associated protein degradation. *Nature cell biology* **7**, 993-998
149. Alberts, S. M., Sonntag, C., Schafer, A., and Wolf, D. H. (2009) Ubx4 modulates cdc48 activity and influences degradation of misfolded proteins of the endoplasmic reticulum. *The Journal of biological chemistry* **284**, 16082-16089
150. Verma, R., Oania, R., Fang, R., Smith, G. T., and Deshaies, R. J. (2011) Cdc48/p97 mediates UV-dependent turnover of RNA Pol II. *Molecular cell* **41**, 82-92
151. Meyer, H. H., Shorter, J. G., Seemann, J., Pappin, D., and Warren, G. (2000) A complex of mammalian ufd1 and npl4 links the AAA-ATPase, p97, to ubiquitin and nuclear transport pathways. *The EMBO journal* **19**, 2181-2192
152. Hitchcock, A. L., Krebber, H., Frietze, S., Lin, A., Latterich, M., and Silver, P. A. (2001) The conserved npl4 protein complex mediates proteasome-dependent membrane-bound transcription factor activation. *Molecular biology of the cell* **12**, 3226-3241

153. Bruderer, R. M., Brasseur, C., and Meyer, H. H. (2004) The AAA ATPase p97/VCP interacts with its alternative co-factors, Ufd1-Npl4 and p47, through a common bipartite binding mechanism. *The Journal of biological chemistry* **279**, 49609-49616
154. Isaacson, R. L., Pye, V. E., Simpson, P., Meyer, H. H., Zhang, X., Freemont, P. S., and Matthews, S. (2007) Detailed structural insights into the p97-Npl4-Ufd1 interface. *The Journal of biological chemistry* **282**, 21361-21369
155. Heo, J. M., Livnat-Levanon, N., Taylor, E. B., Jones, K. T., Dephoure, N., Ring, J., Xie, J., Brodsky, J. L., Madeo, F., Gygi, S. P., Ashrafi, K., Glickman, M. H., and Rutter, J. (2010) A stress-responsive system for mitochondrial protein degradation. *Molecular cell* **40**, 465-480
156. Tran, J. R., Tomsic, L. R., and Brodsky, J. L. (2011) A Cdc48p-associated factor modulates endoplasmic reticulum-associated degradation, cell stress, and ubiquitinated protein homeostasis. *The Journal of biological chemistry* **286**, 5744-5755
157. Stapf, C., Cartwright, E., Bycroft, M., Hofmann, K., and Buchberger, A. (2011) The general definition of the p97/valosin-containing protein (VCP)-interacting motif (VIM) delineates a new family of p97 cofactors. *The Journal of biological chemistry* **286**, 38670-38678
158. Schuberth, C., and Buchberger, A. (2008) UBX domain proteins: major regulators of the AAA ATPase Cdc48/p97. *Cellular and molecular life sciences : CMLS* **65**, 2360-2371
159. Dantuma, N. P., and Hoppe, T. (2012) Growing sphere of influence: Cdc48/p97 orchestrates ubiquitin-dependent extraction from chromatin. *Trends in cell biology* **22**, 483-491
160. Dargemont, C., and Ossareh-Nazari, B. (2012) Cdc48/p97, a key actor in the interplay between autophagy and ubiquitin/proteasome catabolic pathways. *Biochimica et biophysica acta* **1823**, 138-144
161. Wolf, D. H., and Stolz, A. (2012) The Cdc48 machine in endoplasmic reticulum associated protein degradation. *Biochimica et biophysica acta* **1823**, 117-124

162. Meyer, H. H., Wang, Y., and Warren, G. (2002) Direct binding of ubiquitin conjugates by the mammalian p97 adaptor complexes, p47 and Ufd1-Npl4. *The EMBO journal* **21**, 5645-5652
163. den Besten, W., Verma, R., Kleiger, G., Oania, R. S., and Deshaies, R. J. (2012) NEDD8 links cullin-RING ubiquitin ligase function to the p97 pathway. *Nature structural & molecular biology* **19**, 511-516, S511
164. Nishimasu, R., Komori, H., Higuchi, Y., Nishimasu, H., and Hiroaki, H. (2010) Crystal structure of a PFU-PUL domain pair of Saccharomyces cerevisiae Doa1/Ufd3. *The Kobe journal of medical sciences* **56**, E125-139
165. Kakizuka, A. (2008) Roles of VCP in human neurodegenerative disorders. *Biochemical Society transactions* **36**, 105-108
166. Watts, G. D., Wymer, J., Kovach, M. J., Mehta, S. G., Mumm, S., Darvish, D., Pestronk, A., Whyte, M. P., and Kimonis, V. E. (2004) Inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia is caused by mutant valosin-containing protein. *Nature genetics* **36**, 377-381
167. Schroder, R., Watts, G. D., Mehta, S. G., Evert, B. O., Broich, P., Fliessbach, K., Pauls, K., Hans, V. H., Kimonis, V., and Thal, D. R. (2005) Mutant valosin-containing protein causes a novel type of frontotemporal dementia. *Annals of neurology* **57**, 457-461
168. Higashiyama, H., Hirose, F., Yamaguchi, M., Inoue, Y. H., Fujikake, N., Matsukage, A., and Kakizuka, A. (2002) Identification of ter94, Drosophila VCP, as a modulator of polyglutamine-induced neurodegeneration. *Cell death and differentiation* **9**, 264-273
169. Hirabayashi, M., Inoue, K., Tanaka, K., Nakadate, K., Ohsawa, Y., Kamei, Y., Popiel, A. H., Sinohara, A., Iwamatsu, A., Kimura, Y., Uchiyama, Y., Hori, S., and Kakizuka, A. (2001) VCP/p97 in abnormal protein aggregates, cytoplasmic vacuoles, and cell death, phenotypes relevant to neurodegeneration. *Cell death and differentiation* **8**, 977-984
170. Maattanen, P., Gehring, K., Bergeron, J. J., and Thomas, D. Y. (2010) Protein quality control in the ER: the recognition of misfolded proteins. *Seminars in cell & developmental biology* **21**, 500-511

171. Kostova, Z., and Wolf, D. H. (2003) For whom the bell tolls: protein quality control of the endoplasmic reticulum and the ubiquitin-proteasome connection. *The EMBO journal* **22**, 2309-2317
172. Ellgaard, L., Molinari, M., and Helenius, A. (1999) Setting the standards: quality control in the secretory pathway. *Science* **286**, 1882-1888
173. Sommer, T., and Wolf, D. H. (1997) Endoplasmic reticulum degradation: reverse protein flow of no return. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **11**, 1227-1233
174. Brodsky, J. L., and McCracken, A. A. (1999) ER protein quality control and proteasome-mediated protein degradation. *Seminars in cell & developmental biology* **10**, 507-513
175. Plemper, R. K., and Wolf, D. H. (1999) Retrograde protein translocation: ERADication of secretory proteins in health and disease. *Trends in biochemical sciences* **24**, 266-270
176. Rubenstein, E. M., Kreft, S. G., Greenblatt, W., Swanson, R., and Hochstrasser, M. (2012) Aberrant substrate engagement of the ER translocon triggers degradation by the Hrd1 ubiquitin ligase. *J Cell Biol* **197**, 761-773
177. Jarosch, E., Taxis, C., Volkwein, C., Bordallo, J., Finley, D., Wolf, D. H., and Sommer, T. (2002) Protein dislocation from the ER requires polyubiquitination and the AAA-ATPase Cdc48. *Nat Cell Biol* **4**, 134-139
178. Rabinovich, E., Kerem, A., Frohlich, K. U., Diamant, N., and Bar-Nun, S. (2002) AAA-ATPase p97/Cdc48p, a cytosolic chaperone required for endoplasmic reticulum-associated protein degradation. *Mol Cell Biol* **22**, 626-634
179. Carlson, E. J., Pitonzo, D., and Skach, W. R. (2006) p97 functions as an auxiliary factor to facilitate TM domain extraction during CFTR ER-associated degradation. *EMBO J* **25**, 4557-4566
180. (1991) Guide to yeast genetics and molecular biology. *Methods Enzymol* **194**, 1-863

181. Chiu, J., March, P. E., Lee, R., and Tillett, D. (2004) Site-directed, Ligase-Independent Mutagenesis (SLIM): a single-tube methodology approaching 100% efficiency in 4 h. *Nucleic Acids Res* **32**, e174
182. Storici, F., and Resnick, M. A. (2006) The delitto perfetto approach to in vivo site-directed mutagenesis and chromosome rearrangements with synthetic oligonucleotides in yeast. *Methods Enzymol* **409**, 329-345
183. Chen, P., Johnson, P., Sommer, T., Jentsch, S., and Hochstrasser, M. (1993) Multiple ubiquitin-conjugating enzymes participate in the in vivo degradation of the yeast MAT alpha 2 repressor. *Cell* **74**, 357-369
184. Chen, P., and Hochstrasser, M. (1995) Biogenesis, structure and function of the yeast 20S proteasome. *The EMBO journal* **14**, 2620-2630
185. Braun, S., Matuschewski, K., Rape, M., Thoms, S., and Jentsch, S. (2002) Role of the ubiquitin-selective CDC48(UFD1/NPL4)chaperone (segregase) in ERAD of OLE1 and other substrates. *The EMBO journal* **21**, 615-621
186. Sikorski, R. S., and Hieter, P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. *Genetics* **122**, 19-27
187. Ghislain, M., Udvardy, A., and Mann, C. (1993) S. cerevisiae 26S protease mutants arrest cell division in G2/metaphase. *Nature* **366**, 358-362
188. Hochstrasser, M., Ellison, M. J., Chau, V., and Varshavsky, A. (1991) The short-lived MAT alpha 2 transcriptional regulator is ubiquitinated in vivo. *Proceedings of the National Academy of Sciences of the United States of America* **88**, 4606-4610
189. Hakes, D. J., and Dixon, J. E. (1992) New vectors for high level expression of recombinant proteins in bacteria. *Analytical biochemistry* **202**, 293-298
190. Ishii, T., Funakoshi, M., and Kobayashi, H. (2006) Yeast Pth2 is a UBL domain-binding protein that participates in the ubiquitin-proteasome pathway. *The EMBO journal* **25**, 5492-5503
191. Ng, D. T., Spear, E. D., and Walter, P. (2000) The unfolded protein response regulates multiple aspects of secretory and membrane protein biogenesis and endoplasmic reticulum quality control. *The Journal of cell biology* **150**, 77-88

192. Johnson, E. S., Bartel, B., Seufert, W., and Varshavsky, A. (1992) Ubiquitin as a degradation signal. *The EMBO journal* **11**, 497-505
193. Hochstrasser, M., and Funakoshi, M. (2012) Disulfide engineering to map subunit interactions in the proteasome and other macromolecular complexes. *Methods Mol Biol* **832**, 349-362
194. Xu, P., Duong, D. M., and Peng, J. (2009) Systematical optimization of reverse-phase chromatography for shotgun proteomics. *J Proteome Res* **8**, 3944-3950
195. Peng, J., Elias, J. E., Thoreen, C. C., Licklider, L. J., and Gygi, S. P. (2003) Evaluation of multidimensional chromatography coupled with tandem mass spectrometry (LC/LC-MS/MS) for large-scale protein analysis: the yeast proteome. *J Proteome Res* **2**, 43-50
196. Elsasser, S., Schmidt, M., and Finley, D. (2005) Characterization of the proteasome using native gel electrophoresis. *Methods in enzymology* **398**, 353-363
197. Schmidt, M., Hanna, J., Elsasser, S., and Finley, D. (2005) Proteasome-associated proteins: regulation of a proteolytic machine. *Biological chemistry* **386**, 725-737
198. Hanna, J., Meides, A., Zhang, D. P., and Finley, D. (2007) A ubiquitin stress response induces altered proteasome composition. *Cell* **129**, 747-759
199. Beyersmann, D., and Hartwig, A. (2008) Carcinogenic metal compounds: recent insight into molecular and cellular mechanisms. *Archives of toxicology* **82**, 493-512
200. Guo, H. R. (2007) Cancer risks associated with arsenic in drinking water. *Environmental health perspectives* **115**, A339-340; author reply A340-331
201. Shen, Z. X., Chen, G. Q., Ni, J. H., Li, X. S., Xiong, S. M., Qiu, Q. Y., Zhu, J., Tang, W., Sun, G. L., Yang, K. Q., Chen, Y., Zhou, L., Fang, Z. W., Wang, Y. T., Ma, J., Zhang, P., Zhang, T. D., Chen, S. J., Chen, Z., and Wang, Z. Y. (1997) Use of arsenic trioxide (As2O3) in the treatment of acute promyelocytic leukemia (APL): II. Clinical efficacy and pharmacokinetics in relapsed patients. *Blood* **89**, 3354-3360

202. Shi, H., Shi, X., and Liu, K. J. (2004) Oxidative mechanism of arsenic toxicity and carcinogenesis. *Molecular and cellular biochemistry* **255**, 67-78
203. Thorsen, M., Perrone, G. G., Kristiansson, E., Traini, M., Ye, T., Dawes, I. W., Nerman, O., and Tamas, M. J. (2009) Genetic basis of arsenite and cadmium tolerance in Saccharomyces cerevisiae. *BMC genomics* **10**, 105
204. Jacobson, T., Navarrete, C., Sharma, S. K., Sideri, T. C., Ibstedt, S., Priya, S., Grant, C. M., Christen, P., Goloubinoff, P., and Tamas, M. J. (2012) Arsenite interferes with protein folding and triggers formation of protein aggregates in yeast. *Journal of cell science* **125**, 5073-5083
205. Haugen, A. C., Kelley, R., Collins, J. B., Tucker, C. J., Deng, C., Afshari, C. A., Brown, J. M., Ideker, T., and Van Houten, B. (2004) Integrating phenotypic and expression profiles to map arsenic-response networks. *Genome biology* **5**, R95
206. Stanhill, A., Haynes, C. M., Zhang, Y., Min, G., Steele, M. C., Kalinina, J., Martinez, E., Pickart, C. M., Kong, X. P., and Ron, D. (2006) An arsenite-inducible 19S regulatory particle-associated protein adapts proteasomes to proteotoxicity. *Molecular cell* **23**, 875-885
207. Yun, C., Stanhill, A., Yang, Y., Zhang, Y., Haynes, C. M., Xu, C. F., Neubert, T. A., Mor, A., Philips, M. R., and Ron, D. (2008) Proteasomal adaptation to environmental stress links resistance to proteotoxicity with longevity in Caenorhabditis elegans. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 7094-7099
208. Elsasser, S., and Finley, D. (2005) Delivery of ubiquitinated substrates to protein-unfolding machines. *Nature cell biology* **7**, 742-749
209. Mu, D., Hsu, D. S., and Sancar, A. (1996) Reaction mechanism of human DNA repair excision nuclease. *The Journal of biological chemistry* **271**, 8285-8294
210. Prakash, S., and Prakash, L. (2000) Nucleotide excision repair in yeast. *Mutation research* **451**, 13-24

211. Jansen, L. E., Verhage, R. A., and Brouwer, J. (1998) Preferential binding of yeast Rad4.Rad23 complex to damaged DNA. *The Journal of biological chemistry* **273**, 33111-33114

212. Guzder, S. N., Sung, P., Prakash, L., and Prakash, S. (1998) Affinity of yeast nucleotide excision repair factor 2, consisting of the Rad4 and Rad23 proteins, for ultraviolet damaged DNA. *The Journal of biological chemistry* **273**, 31541-31546

213. Schauber, C., Chen, L., Tongaonkar, P., Vega, I., Lambertson, D., Potts, W., and Madura, K. (1998) Rad23 links DNA repair to the ubiquitin/proteasome pathway. *Nature* **391**, 715-718

214. Biggins, S., Ivanovska, I., and Rose, M. D. (1996) Yeast ubiquitin-like genes are involved in duplication of the microtubule organizing center. *The Journal of cell biology* **133**, 1331-1346

215. Zhang, D., Chen, T., Ziv, I., Rosenzweig, R., Matiuhin, Y., Bronner, V., Glickman, M. H., and Fushman, D. (2009) Together, Rpn10 and Dsk2 can serve as a polyubiquitin chain-length sensor. *Molecular cell* **36**, 1018-1033

216. White, R. E., Dickinson, J. R., Semple, C. A., Powell, D. J., and Berry, C. (2011) The retroviral proteinase active site and the N-terminus of Ddi1 are required for repression of protein secretion. *FEBS letters* **585**, 139-142

217. Clarke, D. J., Mondesert, G., Segal, M., Bertolaet, B. L., Jensen, S., Wolff, M., Henze, M., and Reed, S. I. (2001) Dosage suppressors of pds1 implicate ubiquitin-associated domains in checkpoint control. *Molecular and cellular biology* **21**, 1997-2007

218. Lustgarten, V., and Gerst, J. E. (1999) Yeast VSM1 encodes a v-SNARE binding protein that may act as a negative regulator of constitutive exocytosis. *Molecular and cellular biology* **19**, 4480-4494

219. Kaplun, L., Tzirkin, R., Bakhrat, A., Shabek, N., Ivantsiv, Y., and Raveh, D. (2005) The DNA damage-inducible UbL-UbA protein Ddi1 participates in Mec1-mediated degradation of Ho endonuclease. *Molecular and cellular biology* **25**, 5355-5362

220. Voloshin, O., Bakhrat, A., Herrmann, S., and Raveh, D. (2012) Transfer of Ho endonuclease and Ufo1 to the proteasome by the UbL-UbA shuttle protein, Ddi1, analysed by complex formation in vitro. *PloS one* **7**, e39210
221. Beskow, A., Grimberg, K. B., Bott, L. C., Salomons, F. A., Dantuma, N. P., and Young, P. (2009) A conserved unfoldase activity for the p97 AAA-ATPase in proteasomal degradation. *Journal of molecular biology* **394**, 732-746
222. Isakov, E., and Stanhill, A. (2011) Stalled proteasomes are directly relieved by P97 recruitment. *The Journal of biological chemistry* **286**, 30274-30283
223. Baek, G. H., Kim, I., and Rao, H. (2011) The Cdc48 ATPase modulates the interaction between two proteolytic factors Ufd2 and Rad23. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 13558-13563
224. Mannhaupt, G., Schnall, R., Karpov, V., Vetter, I., and Feldmann, H. (1999) Rpn4p acts as a transcription factor by binding to PACE, a nonamer box found upstream of 26S proteasomal and other genes in yeast. *FEBS Lett* **450**, 27-34
225. Xie, Y., and Varshavsky, A. (2001) RPN4 is a ligand, substrate, and transcriptional regulator of the 26S proteasome: a negative feedback circuit. *Proc Natl Acad Sci U S A* **98**, 3056-3061
226. Ju, D., and Xie, Y. (2004) Proteasomal degradation of RPN4 via two distinct mechanisms, ubiquitin-dependent and -independent. *J Biol Chem* **279**, 23851-23854
227. Burroughs, A. M., Iyer, L. M., and Aravind, L. (2011) Functional diversification of the RING finger and other binuclear treble clef domains in prokaryotes and the early evolution of the ubiquitin system. *Molecular bioSystems* **7**, 2261-2277
228. Linnen, J. M., Bailey, C. P., and Weeks, D. L. (1993) Two related localized mRNAs from Xenopus laevis encode ubiquitin-like fusion proteins. *Gene* **128**, 181-188
229. Glinka, T., Alter, J., Braunstein, I., Tzach, L., Wei Sheng, C., Geifman, S., Edelmann, M., Kessler, B., and Stanhill, A. (2013) Signal-peptide mediated translocation is regulated by a P97-AIRAPL complex. *The Biochemical journal*

230. Pharoah, P. D., Tsai, Y. Y., Ramus, S. J., Phelan, C. M., Goode, E. L., Lawrenson, K., Buckley, M., Fridley, B. L., Tyrer, J. P., Shen, H., Weber, R., Karevan, R., Larson, M. C., Song, H., Tessier, D. C., Bacot, F., Vincent, D., Cunningham, J. M., Dennis, J., Dicks, E., Australian Cancer, S., Australian Ovarian Cancer Study, G., Aben, K. K., Anton-Culver, H., Antonenkova, N., Armasu, S. M., Baglietto, L., Bandera, E. V., Beckmann, M. W., Birrer, M. J., Bloom, G., Bogdanova, N., Brenton, J. D., Brinton, L. A., Brooks-Wilson, A., Brown, R., Butzow, R., Campbell, I., Carney, M. E., Carvalho, R. S., Chang-Claude, J., Chen, Y. A., Chen, Z., Chow, W. H., Cicek, M. S., Coetzee, G., Cook, L. S., Cramer, D. W., Cybulski, C., Dansonka-Mieszkowska, A., Despierre, E., Doherty, J. A., Dork, T., du Bois, A., Durst, M., Eccles, D., Edwards, R., Ekici, A. B., Fasching, P. A., Fenstermacher, D., Flanagan, J., Gao, Y. T., Garcia-Closas, M., Gentry-Maharaj, A., Giles, G., Gjyshi, A., Gore, M., Gronwald, J., Guo, Q., Halle, M. K., Harter, P., Hein, A., Heitz, F., Hillemanns, P., Hoatlin, M., Hogdall, E., Hogdall, C. K., Hosono, S., Jakubowska, A., Jensen, A., Kalli, K. R., Karlan, B. Y., Kelemen, L. E., Kiemeney, L. A., Kjaer, S. K., Konecny, G. E., Krakstad, C., Kupryjanczyk, J., Lambrechts, D., Lambrechts, S., Le, N. D., Lee, N., Lee, J., Leminen, A., Lim, B. K., Lissowska, J., Lubinski, J., Lundvall, L., Lurie, G., Massuger, L. F., Matsuo, K., McGuire, V., McLaughlin, J. R., Menon, U., Modugno, F., Moysich, K. B., Nakanishi, T., Narod, S. A., Ness, R. B., Nevanlinna, H., Nickels, S., Noushmehr, H., Odunsi, K., Olson, S., Orlow, I., Paul, J., Pejovic, T., Pelttari, L. M., Permuth-Wey, J., Pike, M. C., Poole, E. M., Qu, X., Risch, H. A., Rodriguez-Rodriguez, L., Rossing, M. A., Rudolph, A., Runnebaum, I., Rzepecka, I. K., Salvesen, H. B., Schwaab, I., Severi, G., Shen, H., Shridhar, V., Shu, X. O., Sieh, W., Southey, M. C., Spellman, P., Tajima, K., Teo, S. H., Terry, K. L., Thompson, P. J., Timorek, A., Tworoger, S. S., van Altena, A. M., van den Berg, D., Vergote, I., Vierkant, R. A., Vitonis, A. F., Wang-Gohrke, S., Wentzensen, N., Whittemore, A. S., Wik, E., Winterhoff, B., Woo, Y. L., Wu, A. H., Yang, H. P., Zheng, W., Ziogas, A., Zulkifli, F., Goodman, M. T., Hall, P., Easton, D. F., Pearce, C. L., Berchuck, A., Chenevix-Trench, G., Iversen, E.,

Monteiro, A. N., Gayther, S. A., Schildkraut, J. M., and Sellers, T. A. (2013) GWAS meta-analysis and replication identifies three new susceptibility loci for ovarian cancer. *Nat Genet* **45**, 362-370, 370e361-362

231. Kelley, L. A., and Sternberg, M. J. (2009) Protein structure prediction on the Web: a case study using the Phyre server. *Nat Protoc* **4**, 363-371
232. Rape, M., Hoppe, T., Gorr, I., Kalocay, M., Richly, H., and Jentsch, S. (2001) Mobilization of processed, membrane-tethered SPT23 transcription factor by CDC48(UFD1/NPL4), a ubiquitin-selective chaperone. *Cell* **107**, 667-677
233. Stolz, A., Hilt, W., Buchberger, A., and Wolf, D. H. (2011) Cdc48: a power machine in protein degradation. *Trends Biochem Sci* **36**, 515-523
234. Johnson, E. S., Ma, P. C., Ota, I. M., and Varshavsky, A. (1995) A proteolytic pathway that recognizes ubiquitin as a degradation signal. *The Journal of biological chemistry* **270**, 17442-17456
235. Hiller, M. M., Finger, A., Schweiger, M., and Wolf, D. H. (1996) ER degradation of a misfolded luminal protein by the cytosolic ubiquitin-proteasome pathway. *Science* **273**, 1725-1728
236. Buchberger, A. (2010) Control of ubiquitin conjugation by cdc48 and its cofactors. *Subcell Biochem* **54**, 17-30
237. Guerrero, C., Milenkovic, T., Przulj, N., Kaiser, P., and Huang, L. (2008) Characterization of the proteasome interaction network using a QTAX-based tag-team strategy and protein interaction network analysis. *Proc Natl Acad Sci U S A* **105**, 13333-13338
238. Kirkpatrick, D. S., Dale, K. V., Catania, J. M., and Gandolfi, A. J. (2003) Low-level arsenite causes accumulation of ubiquitinated proteins in rabbit renal cortical slices and HEK293 cells. *Toxicol Appl Pharmacol* **186**, 101-109
239. Freeman, M. (2008) Rhomboid proteases and their biological functions. *Annual review of genetics* **42**, 191-210
240. Zettl, M., Adrain, C., Strisovsky, K., Lastun, V., and Freeman, M. (2011) Rhomboid family pseudoproteases use the ER quality control machinery to regulate intercellular signaling. *Cell* **145**, 79-91

241. Greenblatt, E. J., Olzmann, J. A., and Kopito, R. R. (2011) Derlin-1 is a rhomboid pseudoprotease required for the dislocation of mutant alpha-1 antitrypsin from the endoplasmic reticulum. *Nature structural & molecular biology* **18**, 1147-1152
242. Vij, S., and Tyagi, A. K. (2008) A20/AN1 zinc-finger domain-containing proteins in plants and animals represent common elements in stress response. *Functional & integrative genomics* **8**, 301-307
243. Mukhopadhyay, A., Vij, S., and Tyagi, A. K. (2004) Overexpression of a zinc-finger protein gene from rice confers tolerance to cold, dehydration, and salt stress in transgenic tobacco. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 6309-6314
244. Heyninck, K., and Beyaert, R. (2005) A20 inhibits NF-kappaB activation by dual ubiquitin-editing functions. *Trends in biochemical sciences* **30**, 1-4
245. Huang, J., Teng, L., Li, L., Liu, T., Li, L., Chen, D., Xu, L. G., Zhai, Z., and Shu, H. B. (2004) ZNF216 Is an A20-like and IkappaB kinase gamma-interacting inhibitor of NFkappaB activation. *The Journal of biological chemistry* **279**, 16847-16853
246. Schuberth, C., and Buchberger, A. (2005) Membrane-bound Ubx2 recruits Cdc48 to ubiquitin ligases and their substrates to ensure efficient ER-associated protein degradation. *Nature cell biology* **7**, 999-1006
247. Rumpf, S., and Jentsch, S. (2006) Functional division of substrate processing cofactors of the ubiquitin-selective Cdc48 chaperone. *Molecular cell* **21**, 261-269
248. Huh, W. K., Falvo, J. V., Gerke, L. C., Carroll, A. S., Howson, R. W., Weissman, J. S., and O'Shea, E. K. (2003) Global analysis of protein localization in budding yeast. *Nature* **425**, 686-691
249. Chia, W. S., Chia, D. X., Rao, F., Bar Nun, S., and Geifman Shochat, S. (2012) ATP binding to p97/VCP D1 domain regulates selective recruitment of adaptors to its proximal N-domain. *PloS one* **7**, e50490

250. Wilson, J. D., Liu, Y., Bentivoglio, C. M., and Barlowe, C. (2006) Sel1p/Ubx2p participates in a distinct Cdc48p-dependent endoplasmic reticulum-associated degradation pathway. *Traffic* **7**, 1213-1223

251. Barthelme, D., and Sauer, R. T. (2013) Bipartite determinants mediate an evolutionarily conserved interaction between Cdc48 and the 20S peptidase. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 3327-3332

252. Wang, B., Alam, S. L., Meyer, H. H., Payne, M., Stemmler, T. L., Davis, D. R., and Sundquist, W. I. (2003) Structure and ubiquitin interactions of the conserved zinc finger domain of Npl4. *The Journal of biological chemistry* **278**, 20225-20234

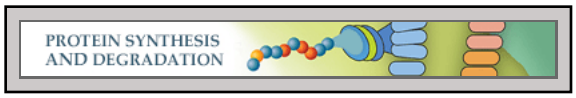
253. Ye, Y., Meyer, H. H., and Rapoport, T. A. (2003) Function of the p97-Ufd1-Npl4 complex in retrotranslocation from the ER to the cytosol: dual recognition of nonubiquitinated polypeptide segments and polyubiquitin chains. *The Journal of cell biology* **162**, 71-84

254. Hanna, J., Waterman, D., Isasa, M., Elsasser, S., Shi, Y., Gygi, S., and Finley, D. (2013) Cuz1/Ynl155w, a Zinc-Dependent Ubiquitin Binding Protein, Protects Cells from Metalloid-Induced Proteotoxicity. *The Journal of biological chemistry*

255. Venancio, T. M., Balaji, S., Iyer, L. M., and Aravind, L. (2009) Reconstructing the ubiquitin network: cross-talk with other systems and identification of novel functions. *Genome biology* **10**, R33

256. Brohee, S., Janky, R., Abdel-Sater, F., Vanderstocken, G., Andre, B., and van Helden, J. (2011) Unraveling networks of co-regulated genes on the sole basis of genome sequences. *Nucleic acids research* **39**, 6340-6358

Protein Synthesis and Degradation:



A Conserved Protein with AN1 Zinc Finger and Ubiquitin-like Domains Modulates Cdc48 (p97) Function in the Ubiquitin-Proteasome Pathway

Bebiana Sá-Moura, Minoru Funakoshi, Robert J. Tomko, Jr., R. Jürgen Dohmen, Zhiping Wu, Junmin Peng and Mark Hochstrasser

J. Biol. Chem. 2013, 288:33682-33696.
doi: 10.1074/jbc.M113.521088 originally published online October 11, 2013

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 47, pp. 33682–33696, November 22, 2013

A Conserved Protein with AN1 Zinc Finger and Ubiquitin-like Domains Modulates Cdc48 (p97) Function in the Ubiquitin-Proteasome Pathway*[S]

Received for publication, September 20, 2013, and in revised form, October 10, 2013 Published, JBC Papers in Press, October 11, 2013, DOI 10.1074/jbc.M113.521088

Bebiana Sá-Moura[‡§1], Minoru Funakoshi[‡2], Robert J. Tomko, Jr.[‡3], R. Jürgen Dohmen[¶], Zhiping Wu[‖], Junmin Peng[‖4], and Mark Hochstrasser[‡5]

From the [‡]Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06520, the [§]Graduate Program in Areas of Basic and Applied Biology, Abel Salazar Biomedical Sciences Institute, University of Porto, Porto 4099-002, Portugal, the [¶]Institute for Genetics, University of Cologne, Zülpicher Strasse 47, D-50674 Cologne, Germany, and the [‖]Departments of Structural Biology and Developmental Neurobiology, St. Jude Proteomics Facility, St. Jude Children's Research Hospital, Memphis, Tennessee 38105

Background: New regulators of the ubiquitin-proteasome system (UPS) were sought in yeast.
Results: Cuz1 (Cdc48-associated ubiquitin-like/zinc finger protein-1) interacts with the Cdc48/p97 ATPase and promotes endoplasmic reticulum-associated degradation.
Conclusion: Cuz1 is a highly conserved Cdc48 cofactor that also binds proteasomes, especially in cells exposed to arsenite.
Significance: This first characterization of the Cuz1 protein family links it to specific Cdc48/p97 complexes.

Regulated protein degradation mediated by the ubiquitin-proteasome system (UPS) is critical to eukaryotic protein homeostasis. Often vital to degradation of protein substrates is their disassembly, unfolding, or extraction from membranes. These processes are catalyzed by the conserved AAA-ATPase Cdc48 (also known as p97). Here we characterize the Cuz1 protein (Cdc48-associated UBL/zinc finger protein-1), encoded by a previously uncharacterized arsenite-inducible gene in budding yeast. Cuz1, like its human ortholog ZFAND1, has both an AN1-type zinc finger (Zf_AN1) and a divergent ubiquitin-like domain (UBL). We show that Cuz1 modulates Cdc48 function in the UPS. The two proteins directly interact, and the Cuz1 UBL, but not Zf_AN1, is necessary for binding to the Cdc48 N-terminal domain. Cuz1 also associates, albeit more weakly, with the proteasome, and the UBL is dispensable for this interaction. Cuz1-proteasome interaction is strongly enhanced by exposure of cells to the environmental toxin arsenite, and in a proteasome mutant, loss of Cuz1 enhances arsenite sensitivity. Whereas loss of Cuz1 alone causes only minor UPS degradation defects, its combination with mutations in the $Cdc48^{Npl4\text{-}Ufd1}$ complex leads to much greater impairment. Cuz1 helps limit the accumulation of ubiquitin conjugates on both the proteasome and Cdc48, suggesting a possible role in the transfer of ubiquitylated substrates from Cdc48 to the proteasome or in their release from these complexes.

The ubiquitin-proteasome system (UPS)[6] is an elaborate network of enzymes and proteins that ensures the specific and timely degradation of proteins in eukaryotic cells (1). Substrate proteins include both regulatory proteins that must be inactivated for proper cell and organismal function and quality control substrates such as misfolded or misassembled proteins (1). Attachment of ubiquitin polymers to substrates is generally required for their efficient recognition by the 26 S proteasome, a 2.6-MDa complex that ultimately degrades the substrate into short peptides and recycles ubiquitin (2).

The 26 S proteasome is composed of two major subcomplexes: the 20 S proteasome core particle (CP) and the 19 S regulatory particle (RP) (3, 4). The RP recognizes polyubiquitylated substrates, and uses a heterohexameric ring of AAA^+ ATPases to unfold substrates and translocate them into the central chamber of the CP where the proteolytic sites are located. Additional subunits in the RP function either as intrinsic receptors for polyubiquitin via their ubiquitin-binding domains or as binding sites for more mobile extrinsic ubiquitin receptors or shuttle factors such as Rad23 (5).

Much of the machinery of the UPS has been identified, but key parts of the system remain poorly understood. Prominent among the open questions is the mechanism by which polyubiquitylated proteins are released by E3 ligases and transferred to ubiquitin receptors and the proteasome for degradation. The highly conserved, multifunctional Cdc48 protein, also called valosin-containing protein or p97 in mammals (6), is an AAA-ATPase that forms a homohexameric ring (7). It has been shown to be required for UPS-mediated protein degradation of several classes of substrates, including those degraded by the endoplasmic reticulum-associated degradation (ERAD) and

* This work was supported, in whole or in part, by National Institutes of Health Grants GM083050 and GM046904 (to M. H.).
[S] This article contains supplemental Tables S1–S3.
[1] Supported by a Portuguese Foundation for Science and Technology Fellowship SFRH/BD/33538/2008.
[2] Present address: Marine Products Kimuraya Co., Ltd., 3307 Watari, Sakaiminato, Tottori 684-0072, Japan.
[3] Supported in part by an American Cancer Society postdoctoral fellowship.
[4] Supported by the American Lebanese Syrian Associated Charities.
[5] To whom correspondence should be addressed: 266 Whitney Ave., New Haven, CT 06520. Tel.: 203-432-5101; Fax: 203-432-5158; E-mail: mark.hochstrasser@yale.edu.

[6] The abbreviations used are: UPS, ubiquitin-proteasome system; CP, core particle; RP, regulatory particle; ERAD, endoplasmic reticulum-associated degradation; UFD, ubiquitin-fusion degradation; UBL, ubiquitin-like domain; Cuz1, Cdc48-associated UBL/zinc-finger protein-1; co-IP, co-immunoprecipitation.

ubiquitin-fusion degradation (UFD) branches of the UPS (8–11). The general mechanistic function of Cdc48 appears to be that of a "segregase," a protein that uses the energy of ATP hydrolysis to unfold or disassemble protein complexes or to extract proteins from membranes (12, 13).

Cdc48 consists of a globular N-terminal domain; two type II AAA (ATPases associated with diverse cellular activities) ATPase domains called D1 and D2, which share 40% sequence identity; and a disordered C-terminal tail (14, 15). Cdc48 interacts with a plethora of cofactors, and these proteins mediate the wide range of functions in which Cdc48 has been implicated. Members of the largest family of Cdc48 cofactors are related by the UBX (ubiquitin regulatory X) domain; there are seven UBX proteins in *Saccharomyces cerevisiae*. Structural determination of the UBX domain, which binds specifically to the Cdc48 N-terminal domain (16), revealed a β-grasp fold similar to that of ubiquitin (17).

The first characterized UBX domain-containing Cdc48 cofactor was p47 (called Ubx1 or Shp1 in yeast), which is required for homotypic membrane fusion in the nuclear envelope, Golgi, and ER (18, 19). Ubx2 is a transmembrane protein of the ER that helps recruit Cdc48 to ubiquitin-ligase complexes in the ER membrane (20), whereas Ubx5 appears to recruit Cdc48 complexes to chromatin sites of DNA repair (21). Precise functions for the other UBX proteins have not yet been as clearly defined (see "Discussion").

In the ERAD and UFD pathways, a key Cdc48 cofactor is the Npl4-Ufd1 heterodimer (22, 23). One Cdc48 hexamer interacts with one Npl4-Ufd1 heterodimer via a short binding site (BS1) in the C-terminal region of Ufd1 and a region in Npl4 with a similar fold to UBX and ubiquitin (the ubiquitin-D or UBX-related domain) (24). The $Cdc48^{Npl4\text{-}Ufd1}$ complex is required for the extraction of ERAD substrates from the ER membrane (25). Another Cdc48 cofactor, Vms1, has recently been shown to function in both ERAD and mitochondrial protein degradation (26, 27); in mitochondrial degradation, Vms1 is necessary to recruit Cdc48 to the mitochondrial membrane.

The physical and functional coordination between Cdc48 and the proteasome is still poorly understood. Here we describe a previously uncharacterized yeast protein, YNL155w, which associates with Cdc48 and contains both a conserved AN1-type zinc finger (Zf_AN1) domain and an extremely diverged C-terminal ubiquitin-like domain (UBL). Based on these features, we have named the protein Cdc48-associated UBL/zinc finger protein-1 (Cuz1). Cuz1 is highly conserved, with the most similar human protein being the uncharacterized ZFAND1 polypeptide. Cuz1 binds directly to Cdc48 and also to the proteasome; the latter interaction is strongly augmented in cells exposed to arsenite (As_2O_3). Deletion of *CUZ1* causes minor UPS degradation defects; however, when *cuz1*Δ is combined with mutations in the $Cdc48^{Npl4\text{-}Ufd1}$ complex, the proteolytic deficiency is enhanced. Loss of Cuz1 also increases the accumulation of polyubiquitin conjugates on the proteasome and Cdc48. These data indicate that Cuz1 is a novel Cdc48 cofactor that may promote transfer of ubiquitylated substrates from Cdc48 to the proteasome or facilitate the disassembly of Cdc48-polyubiquitin-substrate complexes on the proteasome.

EXPERIMENTAL PROCEDURES

Yeast Strains, Plasmids, and Plasmid Constructions—Yeast rich (YPD) and minimal (SD) media were prepared as described previously, and all yeast manipulations were carried out according to standard procedures (28). Yeast chromosomal gene deletions were made by PCR-mediated marker amplification and gene replacement in diploid cells; the resulting diploid heterozygotes were dissected to verify 2:2 marker segregation and to isolate haploid deletion strains. Complete lists of *Saccharomyces cerevisiae* strains and plasmids used in this study are presented in supplemental Tables S1 and S2, respectively.

The *YNL155W* (*CUZ1*) and *YOR052C* genes were isolated by PCR amplification from genomic yeast DNA, and inserted into various plasmids. The absence of mutations was verified by DNA sequencing of the entire inserts. Plasmid pRS314-FLAG-Cuz1 was derived from pRS314-Cuz1 using site-directed, ligase-independent mutagenesis (SLIM) (29). DNA sequences encoding His_6-Cdc48 or His_6-Cdc48(1–220) were PCR amplified from genomic yeast DNA using an oligonucleotide that introduced a His_6 tag and then cloned into pET42b using NdeI and XhoI restriction sites, which removed the sequence for the GST tag from the plasmid. DNA sequencing confirmed that the ORF contained no mutations. Plasmid pGEX-KT was used to express full-length Cuz1 and different Cuz1 deletion variants as GST fusions in *Escherichia coli*. *CUZ1* sequences were obtained by amplifying the desired DNA fragments from yeast genomic DNA and inserting them downstream of the GST coding sequence in pGEX-KT. To make pGEX-KT-Cuz1–4S, Cys to Ser codon mutations were introduced into the *CUZ1* sequence using two sequential QuikChange (Stratagene) site-directed mutagenesis reactions.

To fuse the endogenous *CUZ1* gene to an upstream FLAG epitope sequence and maintain the normal promoter sequences, we used the *delitto perfetto* methodology (30). After insertion of the CORE-I-SceI cassette from pGSKU, the FLAG coding sequence was amplified from pRS314-FLAG-Cuz1 using primers whose 5′ segments had 40 nucleotides of identity to sequences upstream and downstream, respectively, of the CORE cassette insertion. This PCR product was then transformed into yeast to replace the CORE cassette in *CUZ1* by homologous recombination. Correct recombination was verified by DNA sequencing and anti-FLAG immunoblotting.

Identification of Cuz1-binding Proteins by LC-MS/MS—Late log-phase 2-liter cultures of yeast cells were harvested by centrifugation. Cell pellets were washed with ice-cold water, centrifuged, flash frozen in liquid nitrogen, and stored at −80 °C. Cell lysis was achieved by grinding cells to a fine powder in liquid nitrogen (31). The powder was resuspended in a buffer containing 50 mM HEPES, pH 7.5, 200 mM NaCl, 10% glycerol, 0.5% Triton X-100 and Complete Protease Inhibitor tablets (Roche Applied Science). The extract was centrifuged for 25 min at 30,000 × *g* to remove cell debris. The protein concentration was determined using the BCA assay (Pierce), and 96 mg of protein extract (~40 ml) were mixed with 0.4 ml of FLAG-M2 antibody resin (50% slurry; Sigma). After 2 h rotating at 4 °C, the beads were washed four times with 10 ml of the resuspension buffer. Beads were resuspended in 0.6 ml of buffer and then transferred to a new tube to which 3× FLAG peptide

was added to a final concentration of 0.2 mg/ml. After incubation for 45 min at 4 °C, the batch eluate was concentrated using a Vivaspin 500 concentrator (MWCO 10,000 kDa; GE Healthcare). SDS-PAGE followed by silver staining was used to evaluate 10% of the concentrated eluate. The remainder was frozen with liquid nitrogen and used for liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis.

The mass spectrometry analysis was performed according to an optimized procedure for LC-MS/MS (32). Briefly, the immunoprecipitated proteins were resolved and excised from a Coomassie Blue-stained SDS gel and digested with trypsin. The extracted peptides were loaded on a C_{18} capillary column (75 μm inner diameter, 10 cm length, 2.7 μm HALO C18 resin, tip size 15 μm; New Objective, MA), and then eluted during a 60-min gradient of 10–40% solvent B (solvent A, 0.1% formic acid; solvent B, 70% acetonitrile, 0.1% formic acid, flow rate of 300 nl/min). The eluted peptides were analyzed on a hybrid LTQ Orbitrap Velos MS (ThermoFisher Scientific) with one MS survey scan and up to 10 data-dependent MS/MS scans. Acquired MS/MS spectra were searched against yeast Uniprot database using the SEQUEST algorithm. Searching parameters included mass tolerance of precursor ions (±20 ppm) and product ion (±0.5 Da), tryptic restriction, dynamic mass shifts for oxidized Met (+15.9949), two maximal modification sites, two maximal missed cleavages, as well as only *b* and *y* ions counted. To evaluate the false discovery rate during spectrum-peptide matching, all original protein sequences were reversed to generate a decoy database that was concatenated to the original database (33). Assigned peptides were grouped by charge state and then filtered by matching scores (XCorr and ΔCn) to reduce the protein false discovery rate to 1%.

Co-immunoprecipitation and Immunoblot Analyses—For co-immunoprecipitation (co-IP) experiments, cultures were grown at 30 °C to mid-logarithmic phase (A_{600} ~1); where indicated, As_2O_3 was added to a final concentration of 0.2 mM, and the cultures were then incubated with shaking for 2 h, except where indicated otherwise. Cells were harvested by centrifugation. To test the interaction of Cdc48 with Cuz1, lysates were prepared by resuspending cell pellets in co-IP buffer A (25 mM Tris-HCl, pH 8.0, 200 mM NaCl, 2 mM $MgCl_2$, 5% glycerol, 1% Triton X-100 and protease inhibitors) and, when indicated, 2 mM ATP. The resuspended cells were disrupted using glass beads in an MP Biomedicals FastPrep bead-beater followed by centrifugation at 21,000 × *g* for 10 min to remove cell debris. After protein quantification, 2.5 mg of protein extract were incubated with 50 μl of FLAG-M2 slurry for 2 h at 4 °C. The beads were washed three times with 1 ml of co-IP buffer and then resuspended in 25 μl of gel loading buffer. Proteins were resolved by SDS-PAGE and analyzed by immunoblotting.

To test for interaction of Cuz1 with proteasomes, the same co-IP methodology was used except for a modified co-IP buffer (co-IP buffer B, 50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 5 mM $MgCl_2$, 0.1% Triton X-100, 5 mM ATP and protease inhibitors). After protein binding, the resin was washed three times with 1 ml of PBS containing 0.2% Tween 20. To test the interaction of polyubiquitylated substrates with Cdc48, cells expressing V5-tagged Cdc48 were transformed with a plasmid expressing an HA-tagged ubiquitin gene under the control of the *CUP1* promoter (34). $CuSO_4$ was added to a final concentration of 0.1 mM when the cultures were diluted. Cells were resuspended in co-IP buffer B without ATP, lysed, and after protein quantification, 1.5 mg of protein was incubated with 40 μl of anti-V5-agarose (Sigma). After 2 h, the beads were washed 3 times with 1 ml of PBS containing 0.2% Tween 20. To test the interaction of the proteasome with polyubiquitylated substrates, cells were grown in the presence of $CuSO_4$ to logarithmic phase and exposed to As_2O_3 for 2 h. Extracts were prepared with co-IP buffer B without ATP; 1 mg of protein was incubated with anti-FLAG resin, which was then washed with PBS containing 0.2% Tween 20. For testing the interaction of Cdc48 with the proteasome, co-IP buffer C was used: 50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 5 mM $MgCl_2$, 0.5% Triton X-100.

Recombinant Protein Purification and in Vitro Binding Assays—Expression of GST and GST fusion proteins was induced in *E. coli* BL21(DE3) transformants by addition of 1 mM isopropyl 1-thio-β-D-galactopyranoside and overnight growth at 30 °C. The GST fusion proteins were purified with glutathione-agarose (ThermoScientific) and eluted with reduced glutathione according to the manufacturer's instructions. Expression of His_6-Cdc48 in *E. coli* Rosetta2 (DE3) pLysS cells was induced by adding 1 mM isopropyl 1-thio-β-D-galactopyranoside for 4 h at 30 °C. The recombinant protein was purified using HisPur Cobalt Resin (ThermoScientific) and eluted using a buffer containing 150 mM imidazole. All the purified recombinant proteins were dialyzed against 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM $MgCl_2$, and 10% glycerol.

For testing the interaction of Cdc48 with GST-Cuz1 and Cuz1 deletion derivatives, the purified proteins were used in a 1:1 molar ratio of Cuz1 monomer to Cdc48 monomer in GST pulldown assays. Binding reactions were incubated for 2 h at 4 °C in a final volume of 0.4 ml of co-IP buffer A. To examine binding of GST-Cuz1 and its deletion derivatives to purified 26 S proteasomes, proteasomes were first purified from yeast as in Ref. 35, and binding reactions were done in 19 S co-IP buffer. Finally, for measuring interaction of ubiquitylated substrates with GST-Cuz1 and its derivatives, recombinant proteins (3 μg) were incubated with 800 μg of yeast extract from cells overexpressing HA-tagged ubiquitin. Extract preparation as well as GST pulldowns were performed with co-IP buffer D: 50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 5 mM $MgCl_2$, 1% Triton X-100. After incubation for 2 h at 4 °C, beads were washed with the same buffer and proteins were eluted by boiling in gel loading buffer.

Cycloheximide Chase/Immunoblot and Pulse-Chase Analyses—For analysis of substrate degradation by cycloheximide chase/immunoblot assays, cultures were grown at room temperature (~23 °C) to logarithmic phase and switched for 1 h to 37 °C. Cycloheximide was added to a final concentration of 0.25 mg/ml, and 2.5 A_{600} equivalents of cells were harvested at each time point. The chase was performed at 37 °C. Cell pellets were resuspended in 0.1 ml of water plus 0.1 ml of 0.2 M NaOH and incubated for 5 min at room temperature. Cells were pelleted by centrifugation, resuspended in gel loading buffer, and heated to 100 °C for 5 min; the lysates were centrifuged for 2 min at 21,000 × *g* to remove cell debris.

Pulse-chase analysis was performed essentially as described previously (36). Cultures were grown at 23 °C to exponential phase; after washing, cells (~10 A_{600} eq) were incubated for 4 min at 28 °C and labeled with ~0.2 mCi of Tran^{35}S-label (MP Biomedicals) for 10 min and chased with excess cold methionine and cysteine at 28 °C. Immunoprecipitation was performed using anti-β-galactosidase antibody and protein A-agarose (Repligen). Immunoprecipitated proteins were separated by SDS-PAGE and analyzed by autoradiography using a Storm 860 PhosphorImager system and ImageQuant 5.2 software (Molecular Dynamics).

Antibodies—An anti-Cuz1 polyclonal antiserum was raised in rabbits and subsequently purified. Additional antibodies used in this study were anti-HA (Covance), anti-GST (Abcam), anti-FLAG (Sigma), anti-V5 (Invitrogen), anti-PGK (Invitrogen), anti-β-galactosidase (Millipore), anti-Tetra His (Qiagen), and anti-Cdc48 (a gift from Dr. Thomas Sommer, Berlin).

RESULTS

Yeast Cuz1/YNL155w and YOR052c as Potential UPS Factors—An initial bioinformatic search for uncharacterized *S. cerevisiae* genes that might function in the UPS led us to two genes, *YNL155W* and *YOR052C* (Fig. 1*A*). These genes are preceded by one or two PACE (proteasome-associated control element) sequences; such nonamer DNA elements (consensus: 5′-GGTGGCAAA-3′) are binding sites for the Rpn4 transcription factor and are found upstream of most proteasome genes as well as other genes involved in the UPS or in other stress response systems (37, 38). Rpn4 is required for normal levels of proteasome gene expression. The presence of upstream proteasome-associated control elements was our original search criterion. Both *YNL155W* and *YOR052C* encode predicted proteins with AN1-type zinc finger (Zf_AN1) domains, which coordinate a pair of zinc ions and are part of a widespread structural motif known as the treble-clef domain (39). Treble-clef domains include the RING and IBR domains, both sequence signatures of ubiquitin ligases. Similarity between YNL155w and YOR052c is restricted to the Zf_AN1 domains (46% sequence identity over 32 residues). Two mammalian Zf_AN1 proteins, ZFAND2A/AIRAP (zinc finger-AN1 domain/arsenite-inducible RNA-associated protein) and ZFAND2B/AIRAPL (AIRAP-like), were reported to bind the 26 S proteasome and may modulate its activity (40, 41). The *Caenorhabditis elegans* ortholog of AIRAPL, AIP-1 has been genetically linked to proteotoxic stress resistance and increased longevity (40).

Both YNL155w and YOR052c belong to the set of proteins in Cluster of Orthologous Groups 3582 (COG3582). More detailed sequence comparisons showed that the human ZFAND family member closest to YNL155w is ZFAND1, although the latter protein has an additional Zf_AN1 motif at residues 64–105 (Fig. 1*B*). Nothing is known about the biochemical function of ZFAND1, but ZFAND1 mutations have been linked to several cancers including ovarian carcinoma (42). YOR052c is a much more divergent protein, with only a low level of similarity to mammalian proteins. Human ZFAND1 has diverged substantially from AIRAP (28% identity over 140 residues) and AIRAPL (29% identity over 130 residues).

In addition to the AN1-type zinc finger, sequence and structural homology searches revealed that both YNL155w and ZFAND1 contain a UBL near the C terminus, which is not true of YOR052c, AIRAP, or AIRAPL. Using the Phyre2 structural modeling program (43), the YNL155w sequence between residues 161 and 265 could be readily fit to the structure of ubiquitin (90% confidence score over 79 residues). The two structures were aligned with a root mean square deviation of 2.15 Å over 64 core residues (Fig. 1*C*). Ramachandran plot analysis (MolProbity) of the modeled polypeptide showed 88% of the backbone conformations in allowed regions. Similar results were obtained with human ZFAND1 residues 150–250. Because of these structural features and the association of YNL155w with Cdc48 (next section), we named the YNL155w protein Cuz1 for Cdc48-associated UBL/Zn-finger protein-1.

A Proteomic Screen for Cuz1-binding Proteins—As a first step to determine potential functions for Cuz1 in the UPS, we searched for interactions of Cuz1 with other proteins. Toward this end, the chromosomal *CUZ1* locus was modified to encode an N terminally FLAG-tagged Cuz1 protein. FLAG-Cuz1 was purified under nondenaturing conditions on an anti-FLAG affinity resin with elution from the resin by excess 3× FLAG peptide. As a negative control, a parallel purification from yeast cells expressing untagged Cuz1 was used. A fraction of each eluate was first evaluated by SDS-PAGE and silver staining (Fig. 2*A*). The band corresponding to FLAG-Cuz1 was the most prominent species, and a second protein close to 100 kDa in mass was also seen in the FLAG-Cuz1 eluate and not in the untagged control.

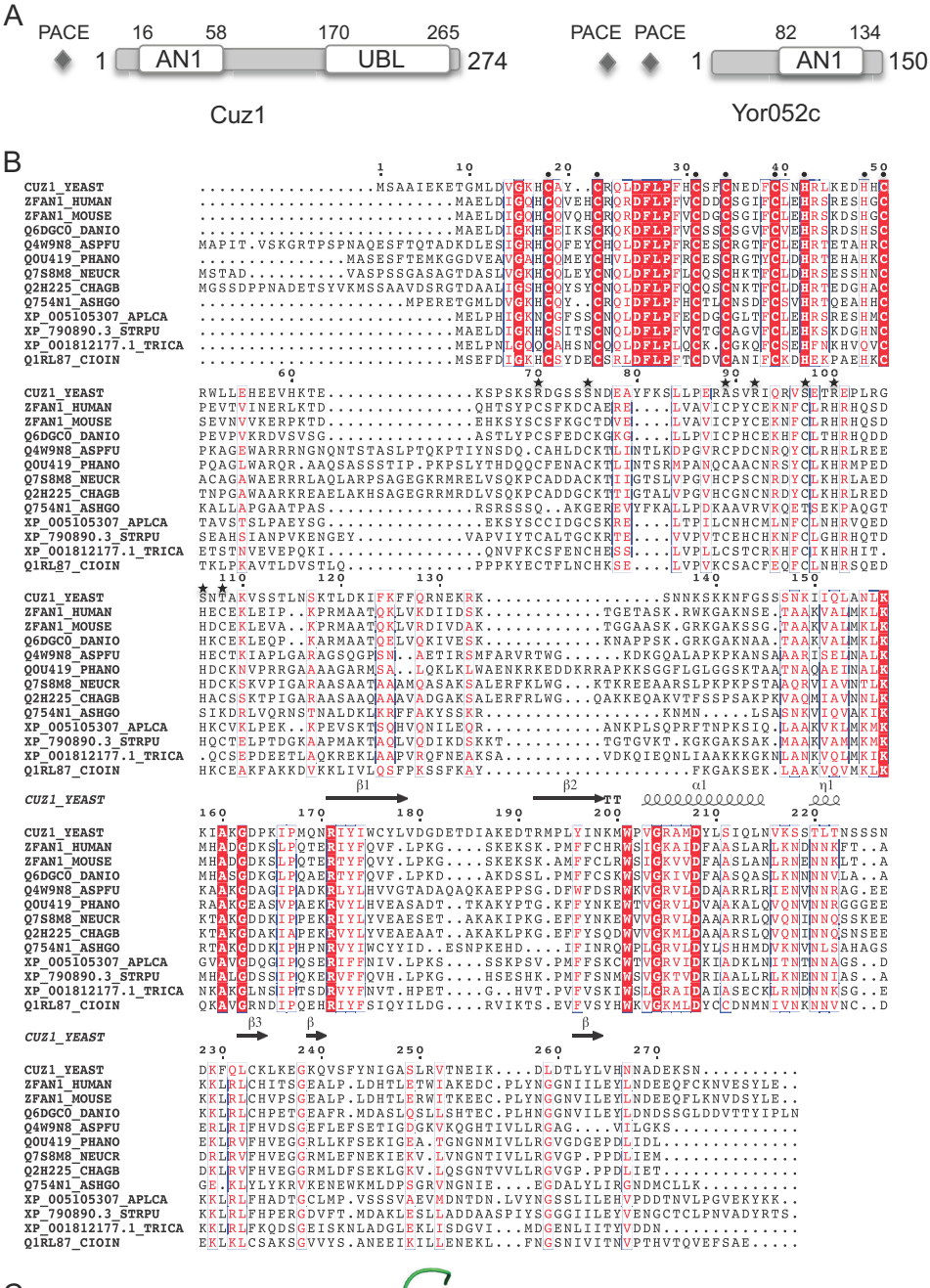
The remainder of each purified sample was analyzed by LC-MS/MS. Peptides from over 370 different proteins were identified in both the control (untagged Cuz1) and FLAG-Cuz1 preparations. Only those proteins represented by at least five times as many spectral counts in the tagged sample are shown in Fig. 2*B*. From this analysis, Cdc48 appeared to be the major Cuz1-interacting protein *in vivo*. Cdc48, with a predicted molecular mass of 92 kDa, is likely to be the protein migrating near the 100-kDa size standard in Fig. 2*A*. Notably, we also detected several proteins known to interact with Cdc48: Npl4, Ubx1/Shp1, and more weakly, Ubx2. Npl4 is a Cdc48 cofactor that is usually bound to the Cdc48 hexameric ring as part of an Npl4-Ufd1 heterodimer (12, 22). No Ufd1 peptides were detected in this sample but were detected in two subsequent purifications (supplemental Table S3).

Other proteins identified in the FLAG-Cuz1 purification are also likely to be significant. Ubiquitin was represented by more than eight times the number of spectral counts seen in the control purification (Fig. 2*B*). Ubiquitin-conjugate binding by Cuz1 (likely indirect) was verified by co-immunoprecipitation analysis (see Fig. 5, below). Rpn3, Rpn7, and Rpt3, all subunits of the proteasome RP, were also identified (interaction between Cuz1 and the proteasome was subsequently validated; see below). The remaining proteins in Fig. 2*B* were not pursued further.

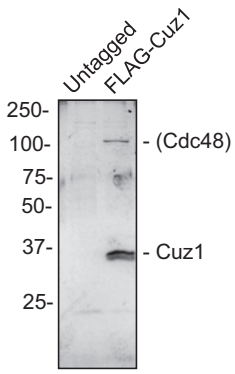
In summary, the mass spectrometry data suggest that Cuz1 functions primarily with the Cdc48 ATPase *in vivo*, possibly with multiple distinct Cdc48-cofactor complexes. The apparent association of Cuz1 with proteasomes and ubiquitin, together with its binding to Cdc48, indicates that Cuz1 may indeed act as a component of the UPS, as was originally sug-

gested by the UBL and Zf_AN1 domains in its polypeptide sequence.

Cuz1 Associates Directly with the Cdc48 AAA-ATPase—To validate the *in vivo* interaction of Cuz1 and Cdc48 that was suggested by the LC-MS/MS analysis, we fused the chromosomal copy of *CDC48* with a sequence encoding a V5 epitope tag. The tag on the essential Cdc48 protein caused no detectable growth defect. Using a strain that had both the chromosomal *CDC48-V5* and *FLAG-CUZ1* alleles, we generated whole cell extracts under nondenaturing conditions and immune-



A



B

	Spectral counts		Peptide counts				
Protein	Control	Tag	Control	Tag	Description	MW (kDa)	SC ratio
CDC48	11	2932	5	55	Cell division control protein 48	92	255
CUZ1	11	945	4	22	AN1-type zinc finger protein YNL155W	31	82.2
NPL4	0	20	0	5	Nuclear protein localization protein 4	66	40
UBX1	0	17	0	6	UBX domain-containing protein 1 (SHP1)	47	34
QNS1	4	98	3	17	Glutamine-dependent NAD(+) synthetase	81	21.8
RPN3	0	8	0	7	26S proteasome regulatory subunit RPN3	60	16
RPT3	0	8	0	4	26S protease regulatory subunit RPT3	48	16
RGI2	0	7	0	2	Respiratory growth induced protein 2	19	14
ERG9	0	6	0	2	Farnesyl pyrophosphate synthase	40	12
HXT6/7	0	5	0	3	High-affinity hexose transporters HXT6, HXT7	63	10
SPT5	0	5	0	2	Transcription elongation factor SPT5	116	10
RPL40B	12	102	3	4	Ubiquitin-60S ribosomal protein L40	15	8.2
RPS31	12	102	3	4	Ubiquitin-40S ribosomal protein S31	17	8.2
RPL40A	12	102	3	4	Ubiquitin-60S ribosomal proteinL40	15	8.2
UBI4	12	102	3	4	Polyubiquitin	43	8.2
PCK1	5	44	3	8	Phosphoenolpyruvate carboxykinase [ATP]	61	8
YGR250C	0	4	0	3	Uncharacterized RNA-binding protein YGR250C	89	8
UBX2	0	4	0	2	UBX domain-containing protein 2	67	8
VAR1	0	4	0	1	Ribosomal protein VAR1, mitochondrial	47	8
RPB2	2	18	2	9	RNA polymerase II second largest subunit B150	139	7.2
ARO1	36	224	17	44	Pentafunctional AROM polypeptide	175	6.1
RPB5	0	3	0	2	DNA-directed RNA polymerases I, II, and III subunit	25	6
FAS1	0	3	0	2	Fatty acid synthase subunit beta	229	6
RPN7	0	3	0	2	26S proteasome regulatory subunit RPN7	49	6
FPS1	0	3	0	2	Glycerol uptake/efflux facilitator protein	74	6
FBP1	0	3	0	2	Fructose-1,6-bisphosphatase	38	6
FRD1	0	3	0	2	Fumarate reductase	51	6
GAD1	2	14	2	6	Glutamate decarboxylase	66	5.6
RPB1	8	45	6	16	RNA polymerase II largest subunit B220	191	5.3

FIGURE 2. **Identification of Cuz1-binding proteins *in vivo*.** *A,* proteins from a strain expressing FLAG-Cuz1 from the chromosomal *CUZ1* locus, or an untagged control strain, were affinity purified on an anti-FLAG resin; 10% of the purified sample was resolved in a 10% SDS-PAGE gel followed by silver staining. *B,* the remaining sample from the immunopurification was subjected to LC-MS/MS analysis. Using spectral counts as a semi-quantitative index, the majority of proteins showed similar abundance in both samples (untagged *versus* FLAG-Cuz1). The table shows the proteins from the FLAG-Cuz1 purification that had a spectral count (*SC*) ratio ≥5-fold above the untagged control.

precipitated FLAG-Cuz1 and any associated proteins with anti-FLAG antibody beads. As seen in Fig. 3*A*, FLAG-Cuz1 efficiently co-precipitated the Cdc48-V5 protein. Interestingly, addition of 2 mM ATP to the extraction buffer consistently reduced the amount of co-precipitated Cdc48 protein (Fig. 3*A*, *lanes 3* and *5 versus lanes 2* and *4*). This effect of ATP was confirmed by LC-MS/MS analysis; by comparing Cdc48 spectral counts in FLAG-Cuz1 purifications in buffers with or with-

FIGURE 1. **Sequence features of yeast Cuz1/YNL155w and YOR052c.** *A,* schematic depicting domain organization of Cuz1 and Yor052c. Proteasome-associated control elements (*PACE*) are found upstream of the corresponding genes. *B,* Cuz1 is evolutionarily conserved, containing an AN1-type zinc finger (Zf_AN1) domain in its N-terminal region. Aligned proteins were chosen based on the phylogenetic tree of Cuz1 orthologs from the Phylome database. Alignments were performed with ClustalOmega and edited with ESPript. The expected zinc-coordinating residues of the N-terminal Zf_AN1 domain are indicated with a *filled circle* (●). Except for Cuz1 and Q754N1, all other proteins possess a second Zf_AN1 domain; the putative metal-coordinating residues of this second domain are marked with a *filled star* (★). Proteins are from the following species: *YEAST*, *S. cerevisiae*; *HUMAN*, *Homo sapiens*; *MOUSE*, *Mus musculus*; *DANIO*, *Danio rerio*; ASPFU, *Aspergillus fumigatus*; *PHANO*, *Phaeosphaeria nodorum SN15*; *NEUCR*, *Neurospora crassa*; *CHAGB*, *Chaetomium globosum*; *ASHGO*, *Ashbya gossypii*; *APLCA*, *Aplysia californica*; *STRPU*, *Strongylocentrotus purpuratus*; *TRICA*, *Tribolium castaneum*; *CIOIN*, *Ciona intestinalis.* Secondary structure elements were added using ESPript based on the Protein Data Bank file of the obtained model for the ubiquitin-like domain. *C,* Cuz1 contains a C-terminal UBL. Protein fold and three-dimensional structure predictions were obtained using Phyre2 (43). Model includes fragment 161–265 in *green* and aligned to ubiquitin (Protein Data Bank 1UBQ) in *blue* using PyMol. Phyre2 output model was predicted based on human ubiquilin 3 (d1yqba1).

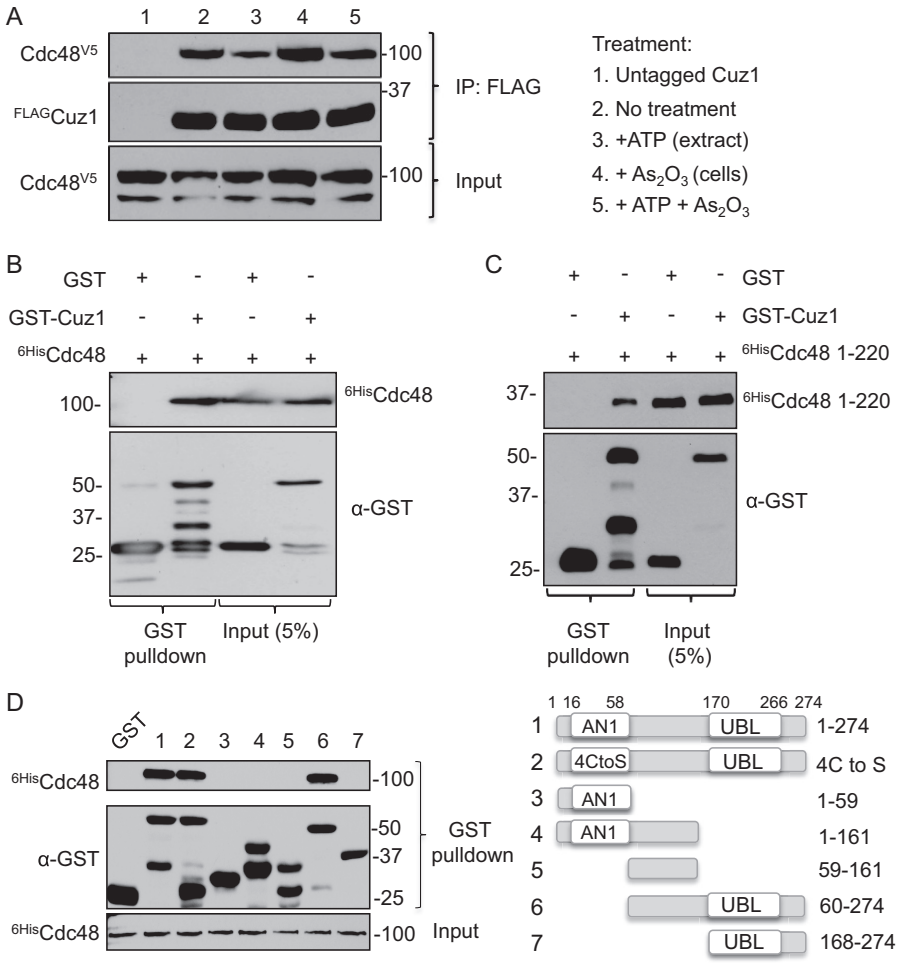


FIGURE 3. **Cuz1 interacts directly with Cdc48.** *A,* Cuz1 and Cdc48 associate *in vivo*, and ATP reduces their interaction. Cells expressed FLAG-tagged Cuz1 and Cdc48-V5 or, as a control, untagged Cuz1 and Cdc48-V5. Cultures were treated with 0.2 mM As_2O_3 for 2 h prior to lysis where indicated. ATP (2 mM) was added to the extracts where indicated. Following immunoprecipitation of FLAG-Cuz1, anti-V5 and anti-FLAG immunoblot analysis was performed. *B,* Cdc48 interaction with Cuz1 is direct. His_6-Cdc48, GST, and GST-Cuz1 were expressed in and purified from *E. coli*. GST pulldowns were followed by anti-His tag and anti-GST immunoblotting. *C,* Cuz1 interacts with N-terminal domain of Cdc48. Binding of GST-Cuz1 to His_6-Cdc48(1–220) was tested as described for the full-length construct in *B*. *D,* the Cuz1 Zf_AN1 domain is neither necessary nor sufficient for Cdc48 interaction, whereas the UBL domain is required. All proteins used in the GST pulldown analysis were purified from *E. coli*.

out added ATP, a greater than 2-fold reduction in counts was seen when ATP was added compared with extracts without the added nucleotide (supplemental Table S3).

To determine whether the association of Cuz1 with Cdc48 was direct or mediated by other proteins, *in vitro* binding assays were carried out using recombinant GST-Cuz1 and His_6-Cdc48 proteins both purified from *E. coli*. GST or GST-Cuz1 was immobilized on glutathione-agarose beads and incubated with His_6-Cdc48. GST-Cuz1, but not GST, was able to pull down His_6-Cdc48, indicating that their interaction did not require any other yeast proteins (Fig. 3*B*). Cdc48 interacts with its cofactors through either the Cdc48 N-terminal domain or its C-terminal tail, although the N-terminal domain is the predominant interaction site (44). Therefore, we asked whether a fragment encompassing the Cdc48 N-terminal domain (residues 1–220) would be sufficient for Cuz1 binding. As shown in Fig. 3*C*, this was in fact the case.

To identify which region(s) of Cuz1 was involved in Cdc48 binding, several GST-Cuz1 deletion variants were created (Fig. 3*D*). Neither the Cuz1 zinc finger domain by itself (GST-Cuz1(1–59)) nor a fragment consisting of the less conserved central region between the Zf_AN1 and UBL domains (GST-Cuz1(59–161)) showed detectable binding to Cdc48. Moreover, a fragment including both of these regions (GST-Cuz1(1–161)) also failed to pull down Cdc48. Conversely, mutation of four cysteines in Zf_AN1 to serines, which should eliminate zinc binding, did not impair binding to Cdc48 (4-Cys to Ser, construct 2). Together, these data indicate that the Cuz1 zinc finger is neither necessary nor sufficient for Cdc48 interaction.

Most of the known Cdc48 adaptors bind Cdc48 via a UBX domain, which has a ubiquitin-like (β-grasp) fold. The Cdc48 cofactor Npl4 binds to Cdc48 through a UBX-related domain (24). Cuz1 possesses a domain predicted to be a UBL (Fig. 1), and a C-terminal deletion that removed the UBL and little else prevented Cdc48 binding (Fig. 3*D*, GST-Cuz1(1–161)). This indicates that the Cuz1 UBL is necessary for Cdc48 binding.

We tested two N-terminal Cuz1 truncations to help narrow down the region that is sufficient for Cdc48 interaction. GST-Cuz1(60–274) and GST-Cuz1(168–274) were expressed at similar levels, but only the longer construct was able to bind

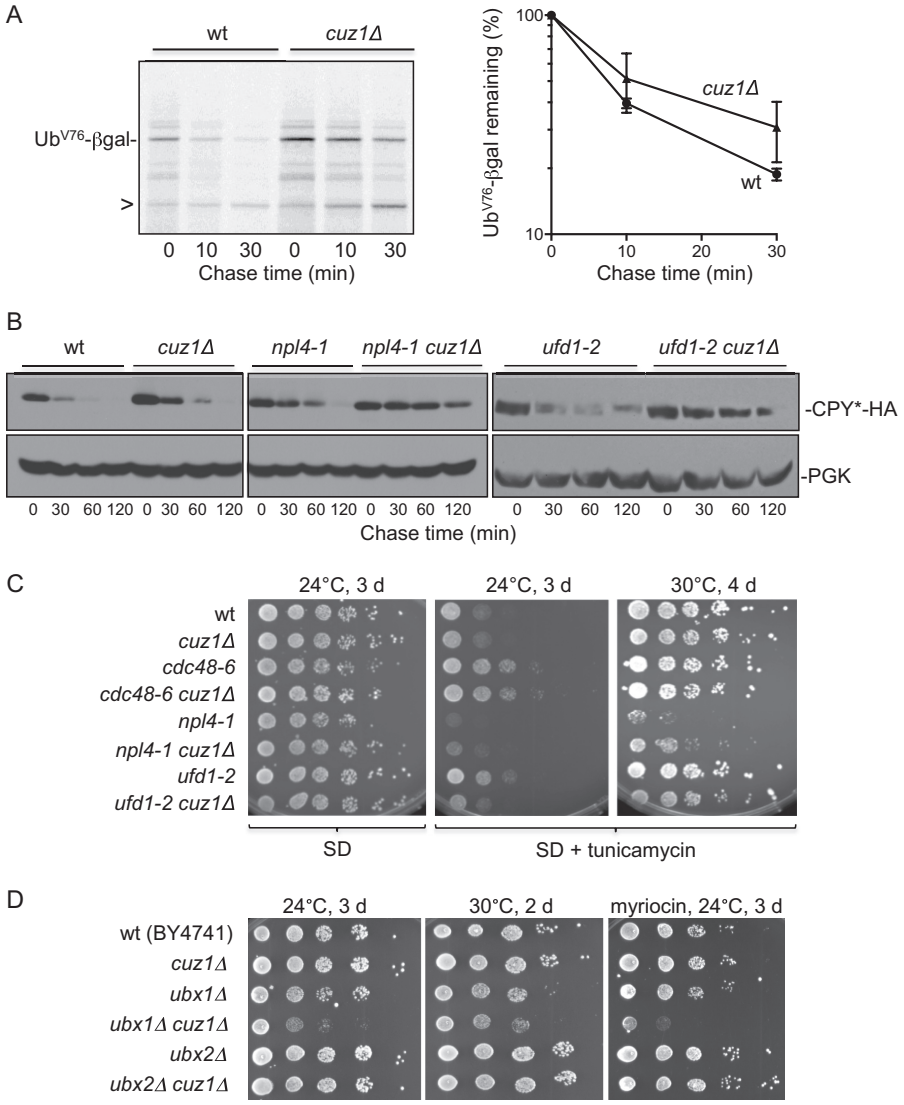


FIGURE 4. **Deletion of Cuz1 causes cellular protein degradation defects.** *A,* pulse-chase analysis of $Ub^{Val\text{-}76}$-β-gal in the indicated yeast strains. Representative autoradiograph of a gel is shown at *left*. *Arrowhead* indicates a 90-kDa degradation product observed with $Ub^{Val\text{-}76}$-β-gal degradation in yeast. Bands *above* the primary $Ub^{Val\text{-}76}$-β-gal are polyubiquitylated species. The *graph at right* shows the mean degradation rates observed from three independent experiments. *Error bars* represent S.E. *B,* degradation of CPY*-HA was analyzed by cycloheximide chase/immunoblot analysis. CPY*-HA was detected by anti-HA immunoblotting. As a loading control, the membrane was subsequently probed with anti-PGK antibodies (*bottom panels*). *C,* growth assays reveal genetic interactions of *cuz1*Δ with mutations in $Cdc48^{Npl4\text{-}Ufd1}$. 6-Fold serial dilutions of cultures were spotted onto plates (SD minimal medium or SD with 0.5 μg/ml of tunicamycin). The apparent growth advantage of *cdc48-6* and *cdc48-6 cuz1*Δ in medium containing tunicamycin could in principle be due to a low constitutive induction of the ER unfolded-protein response in these cells. *D,* double mutant analysis of *cuz1*Δ with different *UBX* gene deletions. Myriocin was used at 0.2 μg/ml. 10-Fold serial dilutions of cultures were spotted onto the plates. Negative genetic interactions between *cuz1*Δ and *ubx1*Δ were also observed in a different strain background (not shown).

Cdc48 (Fig. 3*D*, *constructs 6* and *7*). This confirmed the lack of a requirement for the Zf_AN1 domain for Cdc48 binding and suggested either that the UBL is not sufficient for binding or that additional sequences N-terminal to residue 168 are necessary for a fully functional UBL. Considered together, the deletion data indicate that the Cuz1 UBL is necessary for Cdc48 binding but may not be sufficient, whereas the zinc finger domain is neither necessary nor sufficient for this interaction.

Cuz1 Functions with Cdc48 in the Ubiquitin-Proteasome System—Cdc48 has a broad array of functions, but one of its best characterized roles is in protein degradation by the UPS (45). We used pulse-chase analyses to determine whether Cuz1 contributes to the degradation of the UFD substrate $Ub^{Val\text{-}76}$-β-galactosidase ($Ub^{Val\text{-}76}$-β-gal) (46). Deletion of *CUZ1* caused a very mild but reproducible slowdown in $Ub^{Val\text{-}76}$-β-gal degradation kinetics (Fig. 4*A*). We then combined the *cuz1*Δ allele with temperature-sensitive mutations in $Cdc48^{Npl4\text{-}Ufd1}$ components. The defects in $Ub^{Val\text{-}76}$-β-gal degradation were already sufficiently severe in these single mutants at the semi-permissive temperature of 28 °C that we could not detect any additional defect when the mutations were combined with *cuz1*Δ (not shown).

We also examined degradation of the classical ERAD substrate CPY*, a mutant derivative of the vacuolar carboxypeptidase Y enzyme that is retrotranslocated from the ER lumen for degradation by the cytoplasmic proteasome (47). Degradation of an HA-tagged CPY* protein at 37 °C appeared to be weakly impaired by loss of Cuz1 (Fig. 4*B*). Notably, when *cuz1*Δ was

combined with mutations in Npl4 or Ufd1, CPY*-HA degradation was further impeded (the *cdc48-6* single mutant was already strongly defective; not shown). These results indicate that Cuz1 has an auxiliary or partially redundant role in $Cdc48^{Npl4\text{-}Ufd1}$-dependent protein degradation by the UPS.

When growth of these same strains was examined under various conditions, distinct genetic interactions between *cuz1*Δ and different $Cdc48^{Npl4\text{-}Ufd1}$ mutations were observed. The *ufd1-2 cuz1*Δ double mutant grew more slowly than the *ufd1-2* single mutant in the presence of tunicamycin, an ER stress inducer (Fig. 4*C*). We did not see clear differences in growth when *cuz1*Δ was combined with *cdc48-6*, and in combination with *npl4-1*, an apparent increase in growth rate was seen relative to the *npl4-1* single mutant. This was seen with cells derived from two different *npl4-1 cuz1*Δ spores, but when *CUZ1* was reintroduced into these cells on a plasmid, no change in growth was seen, suggesting that the double mutants carried a cryptic suppressor of *npl4-1* (not shown). Collectively, the growth data suggest an overlap in Cuz1 and Ufd1 function, whereas the results with the *cdc48-6* and *npl4-1* mutants are not readily interpreted.

Because the Cdc48-binding Ubx1/Shp1 and Ubx2 proteins were found to copurify with Cuz1 based on LC-MS/MS, we tested for genetic interactions between *cuz1*Δ and deletions of all seven yeast *UBX* genes as well as the gene for the Cdc48 cofactor Vms1. For *ubx2*Δ–*ubx7*Δ and *vms1*Δ, no obvious differences in growth rate were seen under a variety of conditions when these alleles were combined with *cuz1*Δ (Fig. 4*D* and not shown). In contrast, a *ubx1*Δ *cuz1*Δ double mutant grew markedly slower than either single mutant at lower temperatures (24 °C), but this effect was attenuated at 30 °C and no longer detected at 36.5 °C (Fig. 4*D* and not shown). Membrane stressors such tunicamycin or the sphingolipid synthesis inhibitor myriocin, exacerbated the synthetic defect. The *ubx1*Δ *cuz1*Δ genetic interaction, together with the enhanced defects seen in the *ufd1-2 cuz1*Δ mutant, is consistent with our LC-MS/MS results and supports a function for Cuz1 in multiple Cdc48-adaptor complexes.

Cuz1 Interacts with Ubiquitylated Proteins in Vivo—Many Cdc48 cofactors help recruit or process polyubiquitylated protein substrates (48). This prompted us to investigate a possible interaction between Cuz1 and ubiquitylated proteins. GST-Cuz1 was immobilized on glutathione-agarose beads and incubated with whole cell extracts from yeast that expressed HA-tagged ubiquitin. GST and GST-Rad23 were used as negative and positive controls, respectively. GST-Cuz1 bound to high molecular mass ubiquitylated species that were visualized near the top of the resolving gel and in the stacking gel (Fig. 5*A*). The pattern of ubiquitin conjugates that bound was similar to those bound by the well characterized ubiquitin chain-binding GST-Rad23 protein, although the level of bound conjugates was lower than found with Rad23. No conjugates were detected when GST was used (Fig. 5*A*).

We tested whether the ubiquitylated protein-Cuz1 interaction was likely to be direct by performing the pulldown assay with purified polyubiquitin chains. Neither Lys^{48}-linked chains (chain lengths of 2 to 7 ubiquitins) nor Lys^{63}-linked tetraubiquitin chains bound detectably to GST-Cuz1, in contrast to GST-Rad23 (data not shown). Although it remains possible that Cuz1 directly interacts with ubiquitin chains of distinct linkages, it is more likely that the interactions seen in yeast lysates were indirect (or only occur in the context of a protein complex, such as one bearing additional ubiquitin-binding sites). Using the same Cuz1 deletion constructs described above for examining Cdc48 interactions, we mapped the region(s) in Cuz1 responsible for interaction with ubiquitylated proteins in yeast extracts (Fig. 5*B*). We observed the exact same binding behaviors as seen when Cdc48 interaction was evaluated (Fig. 3*D*). Specifically, binding did not require the Zf_AN1 domain, but association was observed with a C-terminal Cuz1 fragment including the UBL (GST-Cuz1(60–274)) but with no other truncations. A parsimonious explanation of these results is that Cdc48 mediates the interaction of ubiquitylated proteins with Cuz1, presumably in the context of Cdc48 complexes with one or more of its known cofactors.

These results raise the question of whether Cuz1 influences the interaction of Cdc48 with ubiquitylated substrates *in vivo*. We transformed a plasmid overexpressing HA-tagged ubiquitin into *cuz1*Δ and *CUZ1* strains carrying the chromosomal *CDC48-V5* allele. If Cuz1 were involved in the recruitment of substrates to Cdc48, loss of Cuz1 might reduce ubiquitin-conjugate interaction with the ATPase complex. Conversely, if Cuz1 were affecting substrate release from Cdc48, an increase in the level of ubiquitylated substrates on Cdc48 may be observed. In fact, when Cdc48-V5 was immunoprecipitated from extracts derived from *cuz1*Δ cells, there was a reproducible increase in the levels of high molecular mass ubiquitylated species that were co-precipitated (Fig. 5*C*). This suggests a potential role for Cuz1 in releasing ubiquitylated substrates from Cdc48 or in transferring them from Cdc48 to the proteasome or proteasome shuttle factors.

Cuz1 Binds to the Proteasome and Is Stimulated by Exposure of Cells to Arsenite—The preceding data raised the possibility that Cuz1 is involved in transferring ubiquitylated substrates from Cdc48 to the proteasome. This idea is supported by our mass spectrometry data, which revealed low levels of proteasome subunits co-purifying with Cuz1 (Fig. 2*B*). Moreover, previous studies had shown that AIRAP and AIRAPL, mammalian proteins related to Cuz1, can interact with the 26 S proteasome (40, 41). To determine whether Cuz1 interacts with the yeast 26 S proteasome, we affinity purified 26 S proteasomes from yeast cells that expressed proteasomes with a FLAG epitope tag on either the Pre1 (β4) subunit of the CP or the Rpn5 subunit of the RP. Anti-Cuz1 immunoblot analysis of the purified complexes revealed association of Cuz1 in both cases (Fig. 6*A*). Additionally, GST-tagged Cuz1 was purified from *E. coli* associated with isolated 26 S proteasome particles, suggesting that the Cuz1-proteasome interaction is direct (Fig. 6*B*). We noted that Cuz1 is a long-lived protein, so its association with the proteasome is unlikely to be as a substrate (data not shown). A subset of the GST-Cuz1 deletion derivatives described above was used for pulldown assays with purified yeast RP. In contrast to Cdc48 and polyubiquitin (Figs. 3*D* and 5*B*), the UBL was not necessary for RP binding (Fig. 6*C*). A fragment containing the Zf_AN1 domain and the linker region was sufficient for maximal binding (GST-Cuz1(1–151)). The Zf_AN1 domain itself

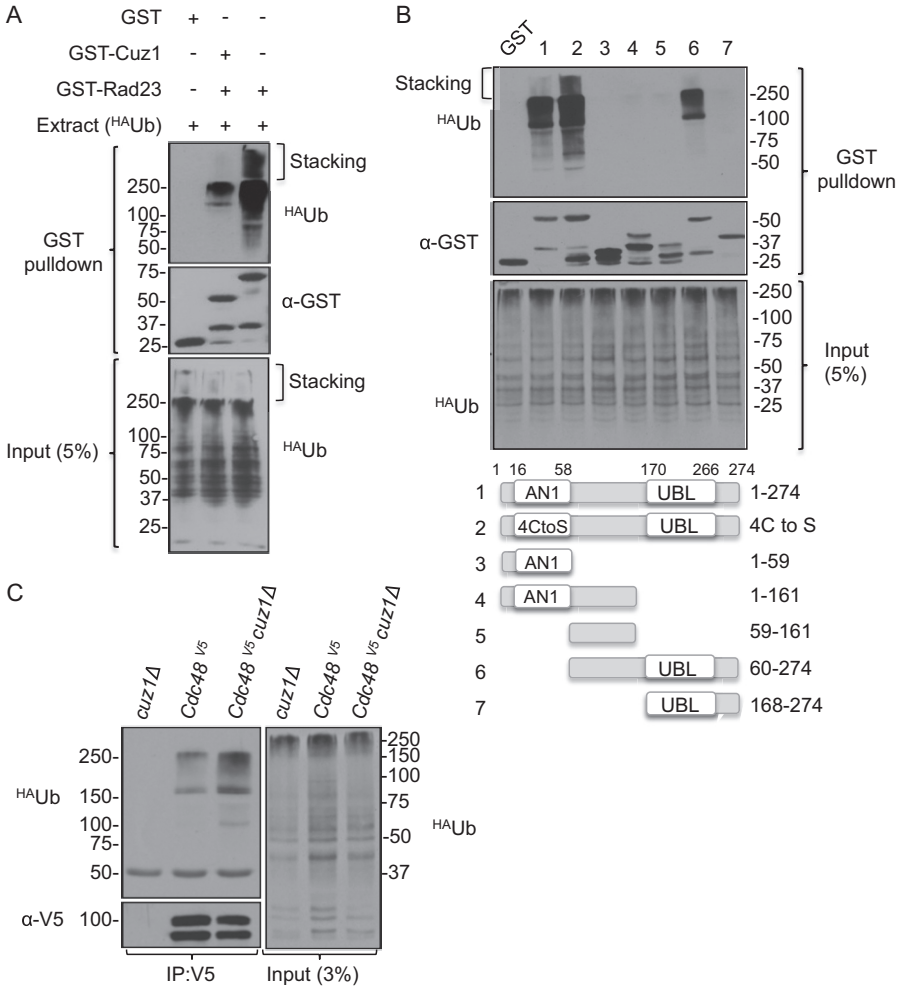


FIGURE 5. **Cuz1 associates with ubiquitin-protein conjugates *in vivo*.** *A,* recombinant GST-Cuz1 was incubated with extracts from yeast overexpressing HA-tagged ubiquitin, and protein eluted from the glutathione resin was analyzed by anti-HA and anti-GST immunoblotting. *B,* the Cuz1 UBL domain is required for ubiquitin-conjugate interaction, but the Zf_AN1 domain is neither necessary nor sufficient. The indicated GST-tagged constructs were used in GST pulldown assays performed as in *A*. *C,* loss of Cuz1 enhances association of polyubiquitinated conjugates with Cdc48 *in vivo*. Analysis was done with cultures of *CUZ1* or *cuz1*Δ cells expressing Cdc48-V5 from the endogenous *CDC48* locus and expressing HA-tagged ubiquitin from a plasmid.

does not appear to be sufficient for proteasome binding, but whether it is necessary remains to be determined.

The composition of the *S. cerevisiae* proteasome has been thoroughly analyzed, and Cuz1 association was not reported (49, 50). It is likely that the amount of Cuz1 on the proteasome is strongly substoichiometric and its interaction transient. Measurements of epitope-tagged Cuz1 levels in yeast compared with those of the proteasome and Cdc48 suggest that the latter proteins are present at 5–20 times the amount of Cuz1 (51, 52). Nevertheless, in support of the physiological relevance of the Cuz1-proteasome interaction, deletion of *CUZ1* enhanced the growth defects of both *rpt6* (*cim3-1*) and *rpt1* (*cim5-1*) temperature-sensitive mutants (Fig. 6*D*).

Arsenite is a well known inducer of protein misfolding and causes accumulation of polyubiquitylated conjugates *in vivo* (41, 53). *CUZ1* mRNA levels were reported to be elevated by exposure of cells to arsenite (54), and we detected a modest increase in FLAG-Cuz1 protein levels when cells were exposed to 0.2 mM As_2O_3 for 2 h (Fig. 7*A*, *input lanes*). It is noteworthy that both mammalian AIRAP and p97 (Cdc48) display increased binding to the proteasome upon arsenite stress (41, 55). Based on these results, we tested whether arsenite might affect interaction of Cuz1 with proteasomes in yeast. In the presence of arsenite, we observed a marked increase in Cuz1 binding to proteasomes immunoprecipitated from yeast extracts; the increase greatly exceeded the modest overall increase of Cuz1 levels in the treated cells (Fig. 7*A*). When normalized to the amount of precipitated FLAG-Rpn11, a ~10-fold increase in Cuz1-proteasome association was seen following arsenite treatment (Fig. 7*A*, *right*). The increase in this association was supported by LC-MS/MS analysis (supplemental Table S3). Addition of arsenite to cells before FLAG-Cuz1 purification led to the identification of 15 of the 19 RP subunits (but no CP subunits).

Loss of Cuz1 alone caused no reduction in cell growth in the presence of arsenite, but when *cuz1*Δ *cim5-1* cells grown at high temperature were examined, arsenite exacerbated the already slow growth seen in the *cim5-1* single mutant (Fig. 7*B*). This supports the potential biological importance of Cuz1 in promoting proteasome function in arsenite resistance. Finally, because arsenite seemed to increase association of mammalian p97 with the proteasome (55), we checked if this was also true

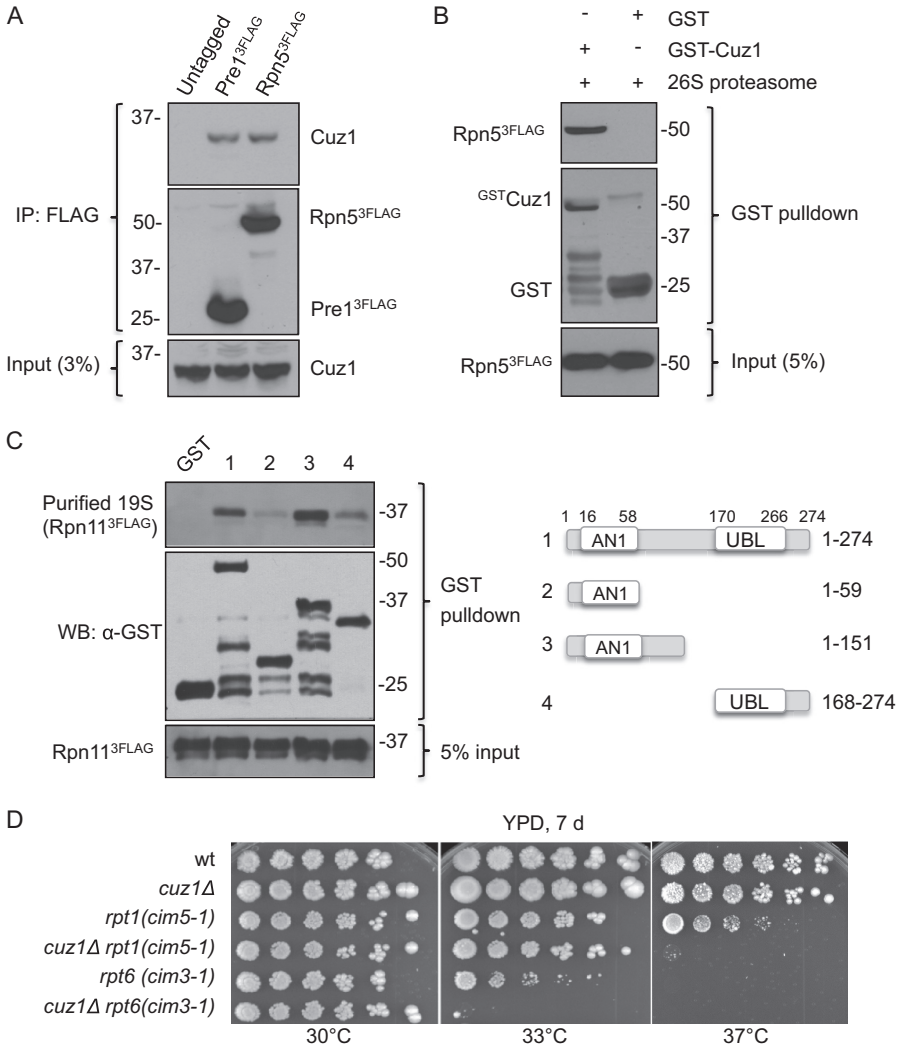


FIGURE 6. **Genetic and physical interactions between Cuz1 and the proteasome.** *A*, Cuz1 interacts *in vivo* with the 26 S proteasome. Proteasomes were affinity purified from extracts of yeast expressing either FLAG-tagged Pre1 (CP) or Rpn11 (RP) from the respective endogenous locus. The bound material was analyzed by anti-Cuz1 immunoblotting. *B,* interaction between Cuz1 and 26 S proteasomes *in vitro*. Recombinant GST-Cuz1 was incubated with yeast 26 S proteasomes affinity-purified from an Rpn5–3FLAG-expressing strain. *C,* the UBL domain is not required for Cuz1 interaction with proteasomes. Recombinant GST-Cuz1 constructs were mixed with purified proteasomal 19 S RP (purified from an Rpn11–3FLAG-expressing strain), and the proteins were subjected to GST pulldown analysis. *D,* deletion of *CUZ1* exacerbates the growth defects of *cim3-1* and *cim5-1* proteasome mutants. Serial dilutions of cultures were done as described in the legend to Fig. 4*C*.

for Cdc48. In fact, Cdc48 co-precipitation with the proteasome increased already after just a 30-min exposure to the metalloid (Fig. 7*C*). These data suggest that Cdc48 and the proteasome have linked functions in promoting arsenite resistance.

Interestingly, loss of Cuz1 caused a small but reproducible increase in Cdc48 interaction with proteasomes with and without arsenite stress (Fig. 8*A*). A parallel increase in polyubiquitylated proteins on the proteasome was observed when *CUZ1* was deleted (Fig. 8*B*). These data would be consistent with the participation of Cuz1 in either the transfer of polyubiquitin conjugates from Cdc48 to the proteasome, which would normally end with the release of Cdc48 from the proteasome, or, more directly, in Cdc48-proteasome dissociation.

DISCUSSION

In this study we have described a novel arsenite-inducible yeast protein, Cuz1, which associates under normal growth conditions primarily with Cdc48; however, upon cellular exposure to arsenite, which activates multiple stress-response pathways (40), a ~10-fold increase in Cuz1-proteasome association occurs (Fig. 7*A*). Cuz1 provides the first reported functional connection between the extremely widespread AN1-type zinc finger (Zf_AN1) motif and the phylogenetically conserved Cdc48 AAA-ATPase. The Zf_AN1 domain is not required for Cdc48 binding but appears to contribute to proteasome association. Conversely, a highly divergent UBL domain near the C terminus of Cuz1 is required for its binding to Cdc48 but not the proteasome. Cuz1 plays an ancillary or partially redundant role in the degradation of UPS substrates that depend on Cdc48. Protein-protein interaction data and other results suggest possible roles for Cuz1 in promoting polyubiquitylated substrate release from Cdc48 or the proteasome (or both).

The Zf_AN1 domain has an extremely broad distribution in both eukaryotes and archaea, with a few examples in bacteria,

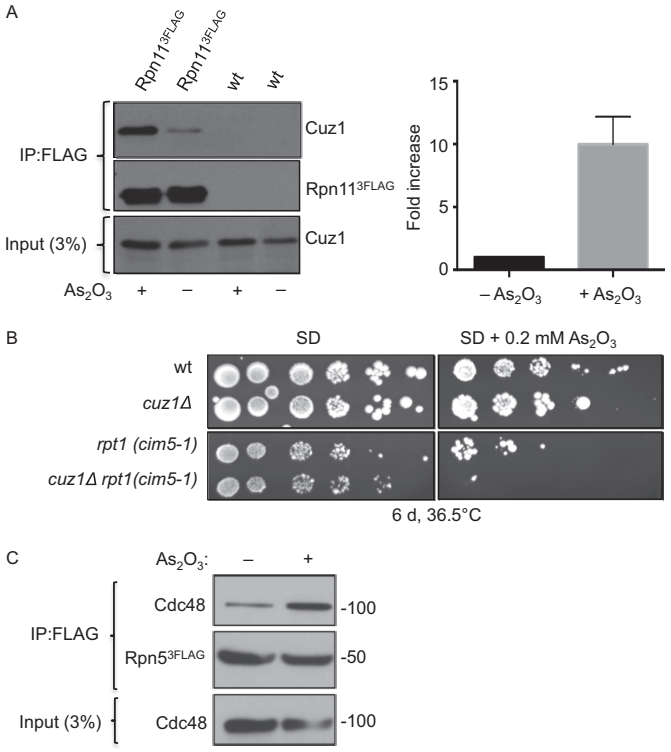


FIGURE 7. **Cell exposure to arsenite enhances proteasome association with Cuz1.** *A,* FLAG-tagged Rpn11 was immunoprecipitated and the amount of co-purifying Cuz1 was quantified using a Syngene G-box. A representative experiment is shown at the *left*. Cuz1 values were normalized to the levels of precipitated Rpn11–3FLAG. The quantification at the *right* is derived from three independent experiments and shows the normalized fold-increase of coprecipitated Cuz1; *error bars* denote S.D. *B,* in the presence of arsenite, deletion of *CUZ1* worsens the growth defect of a temperature-sensitive *cim5-1* mutant at high temperature. *C,* Cdc48 interaction with the proteasome is enhanced by arsenite. Proteasomes tagged with Rpn5–3FLAG were immunoprecipitated with anti-FLAG resin from extracts derived from cells grown in the presence of arsenite for 30 min. Co-purified Cdc48 levels was analyzed by anti-Cdc48 immunoblotting.

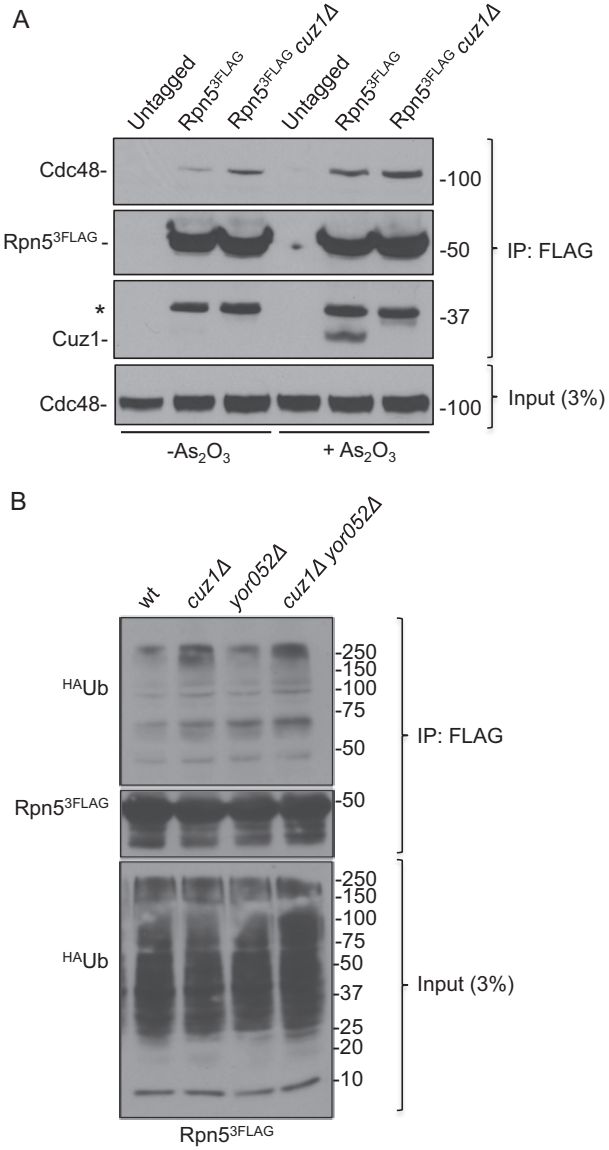


FIGURE 8. **Cuz1 affects the interaction of polyubiquitinated substrates with proteasomes.** *A,* a slight increase in Cdc48 bound to the proteasome is observed in the absence of Cuz1, both in the presence and absence of arsenite. FLAG-tagged proteasomes were immunoprecipitated, and the amount of bound Cdc48 was analyzed by anti-Cdc48 immunoblotting. An unspecific band is indicated with an *asterisk*. *B,* an increase in polyubiquitylated proteins on the proteasome is observed when *CUZ1* is deleted. After exposing cells for 2 h to 0.2 mM arsenite, cells were lysed and proteasomes were immunoprecipitated. Co-purified polyubiquitinated proteins were analyzed by anti-HA immunoblot analysis.

presumably due to lateral gene transfer (39). Most remarkably, this domain is part of many highly divergent multidomain proteins, but most, if not all, are linked to membrane-localized proteolytic systems. When we performed sequence searches with the Zf_AN1 domain, it was found in a wide range of euryarchaeota species and several thaumarchaeotes. The domain most commonly found to be part of archaeal Zf_AN1 proteins was a rhomboid-related protease domain. Rhomboid proteases are polytopic membrane proteins bearing a protease active site deep within the lipid bilayer (56). It may be relevant in this context that some eukaryotic rhomboid and pseudorhomboid proteins function in ERAD (57, 58).

In eukaryotes, Zf_AN1 domains are commonly part of proteins that also contain ubiquitin, polyubiquitin, or ubiquitin-like sequences; however, the domain order in the protein is often permuted relative to Cuz1. For example, the fungus *Rhizopus oryzae* has a protein (GenBank™ EIE85715) with two N-terminal, near-exact ubiquitin repeats, which should be cleavable by deubiquitylating enzymes, and a C-terminal Zf_AN1 domain. The joining of divergent ubiquitin and UBL domains to Zf_AN1 motifs may be an example of convergent evolution based on their phylogenetic distribution and differences in Zf_AN1 and ubiquitin/UBL sequence; this might have been driven by a common role for many Zf_AN1 domain proteins in proteasome binding. Like Cuz1, the human AIRAP/ZFAND2A and AIRAPL/ZFAND2B proteins both bind 26 S proteasomes, as does their *C. elegans* ortholog, AIP-1 (40, 41). Our data with yeast Cuz1 (Fig. 6) and domain swaps with the AIRAP and AIRAPL proteins (40, 41) both suggest that the Zf_AN1 domain participates in proteasome association. Finally, a subfamily of Zf_AN1 proteins also contain A20 zinc finger domains, which in some cases have been shown to have ubiquitin ligase activity (59). In plants these proteins are associated with stress responses (60), whereas in mammals they are usually involved in the immune system (61, 62). It is likely that

Zf_AN1 proteins have widespread and disparate functions in the UPS.

Additional support for links between Cuz1 and both proteasomes and Cdc48 can be found in available genomic and proteomic databases. Cluster analysis of aggregated RNA microarray data shows that *CUZ1* transcriptional regulation clusters very closely with that of *UBX4*, which encodes a UBX protein that has been suggested to promote dissociation of ubiquitinated proteins from Cdc48 based on results similar to those reported here for Cuz1 (63). Another gene with a similar transcription profile is *UFD1*. Of the 25 genes most closely correlated transcriptionally with *CUZ1* (in addition to *YOR052C*), 19 are proteasome subunit genes. This co-regulation is consistent with the fact that *CUZ1*, *YOR052C*, and proteasome genes all have upstream proteasome-associated control element sequences, the target for the Rpn4 transcription factor. Large scale protein interaction studies using yeast two-hybrid or mass spectrometry approaches also identified, among other proteins, Cdc48, Ubx1, and Npl4. Although not pursued further, these earlier genomic and proteomic studies are fully consistent with our findings.

Cdc48 adaptors bind to the ATPase in both mutually exclusive and interdependent fashion. Ubx1 and Npl4-Ufd1 do not bind the same Cdc48 hexamer (18, 22), and conversely, certain UBX proteins only bind to Cdc48 if Npl4-Ufd1 is also present (64, 65). Intriguingly, the mass spectrometry analysis of Cuz1-associated proteins identified Ubx1 as well as Npl4 and Ufd1 (Fig. 2*B* and supplemental Table S3). Moreover, mutations in either adaptor when combined with loss of Cuz1 cause enhanced growth defects (Fig. 4). This suggests that Cuz1 is part of distinct Cdc48-adaptor complexes. A C-terminal GFP fusion of Cuz1 localizes diffusely throughout the cell, with a slight concentration in the nucleus (66), so Cuz1 would be well placed to function in multiple Cdc48 complexes.

As is true for the majority of Cdc48 cofactors, Cuz1 interacts directly with the N-terminal domain of the ATPase (Fig. 3*C*). Notably, addition of ATP to Cuz1-Cdc48 binding reactions attenuates their association (Fig. 3*A*), and this was also apparent in our LC-MS/MS samples with ATP supplementation (supplemental Table S3). ATP has been reported to modulate the recruitment of other cofactors to Cdc48. ATP binding to the D1 domain enhances interaction of the Npl4-Ufd1 heterodimer with the N-terminal domain of p97 (67), and disassembly of Ufd2-Rad23 complexes by Cdc48 depends on Cdc48 binding to Ufd2 and ATP (68). Ubx2 interaction with Cdc48 is strongly decreased in the presence of ATP (69). Cuz1 occupancy of Cdc48 may similarly be tied to nucleotide-dependent conformation changes or specific steps of the ATPase catalytic cycle. It remains to be determined whether this modulation of Cuz1-Cdc48 association is due to ATP binding or hydrolysis. A recent study suggests that Cdc48 may directly control proteolytic activity of the eukaryotic 20 S proteasome (70); Cuz1 might modulate this potential Cdc48 function as well.

Many Cdc48 cofactors interact with polyubiquitin-protein conjugates, some of them directly. For example, Ubx1, Ubx2, and Ubx5 are UBA-UBX proteins in which the UBA domain mediates polyubiquitin binding (71). Cdc48, Npl4, and Ufd1 also all have ubiquitin-binding sites (12, 72, 73). Cuz1 interacts *in vivo* with polyubiquitylated proteins (Fig. 5*A*), but this is likely to be indirect; this is not unexpected given that Cuz1 lacks any known ubiquitin-binding motif. The binding behavior of a series of Cuz1 deletion variants is identical for Cdc48 and polyubiquitylated species in cell extracts, consistent with the possibility that interaction between Cuz1 and polyubiquitin occurs in the context of a Cdc48 complex. Although it is known that recruitment of polyubiquitinated substrates to Cdc48 is largely mediated by the Npl4-Ufd1 dimer (73), their release to downstream components is less well understood. Ubx4 has been identified as one possible releasing factor (61) and Vms1 as another (27). As observed upon deletion of these factors in the earlier studies, loss of Cuz1 also leads to an increase in the levels of polyubiquitylated substrates associated with Cdc48 (Fig. 5*C*). Therefore, Cuz1 might have a related mechanism of action. Moreover, *cuz1*Δ cells also accumulate higher levels of polyubiquitin conjugates on proteasomes (Fig. 8*B*).

The exact mechanistic function of Cuz1, like that of most other Cdc48 cofactors, remains to be determined. Based on the genetic interactions we observe between mutations in Cuz1 and Npl4-Ufd1 (Fig. 4) and their co-purification (Fig. 2*B* and supplemental Table S3), Cuz1 might work with Npl4-Ufd1 on a subset of Cdc48 complexes. Cuz1 function in these complexes remains unclear, but it may stimulate ATP binding or hydrolysis by Cdc48, inducing conformation changes that weaken ubiquitylated substrate binding. This would facilitate substrate transfer to Rad23 or related shuttle factors. Other factors such as Ufd2 bind to both Cdc48 and Rad23 and may recruit Rad23 to Cdc48-bound substrates (68). Following release from Ufd2, Rad23 and related shuttle proteins can bind the proteasome via their UBL domain interaction with Rpn1. In the absence of Cuz1, the Cdc48 complex may tend to remain bound to polyubiquitylated substrates and accompany them to the proteasome. Alternatively, Cuz1 might enhance the transfer of certain ubiquitin conjugates from Cdc48 directly to the proteasome or promote Cdc48-proteasome dissociation. Arsenite may alter Cuz1 itself in a way that impacts these processes, or the metalloid might act more indirectly, such as by modifying the cellular pool of ubiquitin conjugates. These ideas are testable and will be the subject of future studies.

REFERENCES

1. Ravid, T., and Hochstrasser, M. (2008) Diversity of degradation signals in the ubiquitin-proteasome system. *Nat. Rev. Mol. Cell Biol.* **9,** 679–690
2. Thrower, J. S., Hoffman, L., Rechsteiner, M., and Pickart, C. M. (2000) Recognition of the polyubiquitin proteolytic signal. *EMBO J.* **19,** 94–102
3. Peters, J. M., Franke, W. W., and Kleinschmidt, J. A. (1994) Distinct 19 S and 20 S subcomplexes of the 26 S proteasome and their distribution in the nucleus and the cytoplasm. *J. Biol. Chem.* **269,** 7709–7718
4. Tomko, R. J., Jr., and Hochstrasser, M. (2013) Molecular architecture and assembly of the eukaryotic proteasome. *Annu. Rev. Biochem.* **82,** 415–445
5. Schmidt, M., Hanna, J., Elsasser, S., and Finley, D. (2005) Proteasome-associated proteins. Regulation of a proteolytic machine. *Biol. Chem.* **386,** 725–737
6. Fröhlich, K. U., Fries, H. W., Rüdiger, M., Erdmann, R., Botstein, D., and Mecke, D. (1991) Yeast cell cycle protein CDC48p shows full-length homology to the mammalian protein VCP and is a member of a protein family involved in secretion, peroxisome formation, and gene expression. *J. Cell Biol.* **114,** 443–453
7. Peters, J. M., Walsh, M. J., and Franke, W. W. (1990) An abundant and ubiquitous homo-oligomeric ring-shaped ATPase particle related to the

putative vesicle fusion proteins Sec18p and NSF. *EMBO J.* **9,** 1757–1767
8. Ye, Y., Meyer, H. H., and Rapoport, T. A. (2001) The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. *Nature* **414,** 652–656
9. Rabinovich, E., Kerem, A., Fröhlich, K. U., Diamant, N., and Bar-Nun, S. (2002) AAA-ATPase p97/Cdc48p, a cytosolic chaperone required for endoplasmic reticulum-associated protein degradation. *Mol. Cell. Biol.* **22,** 626–634
10. Bays, N. W., Wilhovsky, S. K., Goradia, A., Hodgkiss-Harlow, K., and Hampton, R. Y. (2001) HRD4/NPL4 is required for the proteasomal processing of ubiquitinated ER proteins. *Mol. Biol. Cell* **12,** 4114–4128
11. Ghislain, M., Dohmen, R. J., Levy, F., and Varshavsky, A. (1996) Cdc48p interacts with Ufd3p, a WD repeat protein required for ubiquitin-mediated proteolysis in Saccharomyces cerevisiae. *EMBO J.* **15,** 4884–4899
12. Rape, M., Hoppe, T., Gorr, I., Kalocay, M., Richly, H., and Jentsch, S. (2001) Mobilization of processed, membrane-tethered SPT23 transcription factor by CDC48(UFD1/NPL4), a ubiquitin-selective chaperone. *Cell* **107,** 667–677
13. Braun, S., Matuschewski, K., Rape, M., Thoms, S., and Jentsch, S. (2002) Role of the ubiquitin-selective CDC48(UFD1/NPL4) chaperone (segregase) in ERAD of OLE1 and other substrates. *EMBO J.* **21,** 615–621
14. DeLaBarre, B., and Brunger, A. T. (2003) Complete structure of p97/valosin-containing protein reveals communication between nucleotide domains. *Nat. Struct. Biol.* **10,** 856–863
15. Huyton, T., Pye, V. E., Briggs, L. C., Flynn, T. C., Beuron, F., Kondo, H., Ma, J., Zhang, X., and Freemont, P. S. (2003) The crystal structure of murine p97/VCP at 3.6 Å. *J. Struct. Biol.* **144,** 337–348
16. Dreveny, I., Kondo, H., Uchiyama, K., Shaw, A., Zhang, X., and Freemont, P. S. (2004) Structural basis of the interaction between the AAA ATPase p97/VCP and its adaptor protein p47. *EMBO J.* **23,** 1030–1039
17. Buchberger, A., Howard, M. J., Proctor, M., and Bycroft, M. (2001) The UBX domain. A widespread ubiquitin-like module. *J. Mol. Biol.* **307,** 17–24
18. Kondo, H., Rabouille, C., Newman, R., Levine, T. P., Pappin, D., Freemont, P., and Warren, G. (1997) p47 is a cofactor for p97-mediated membrane fusion. *Nature* **388,** 75–78
19. Uchiyama, K., Jokitalo, E., Kano, F., Murata, M., Zhang, X., Canas, B., Newman, R., Rabouille, C., Pappin, D., Freemont, P., and Kondo, H. (2002) VCIP135, a novel essential factor for p97/p47-mediated membrane fusion, is required for Golgi and ER assembly *in vivo*. *J. Cell Biol.* **159,** 855–866
20. Neuber, O., Jarosch, E., Volkwein, C., Walter, J., and Sommer, T. (2005) Ubx2 links the Cdc48 complex to ER-associated protein degradation. *Nat. Cell. Biol.* **7,** 993–998
21. Verma, R., Oania, R., Fang, R., Smith, G. T., and Deshaies, R. J. (2011) Cdc48/p97 mediates UV-dependent turnover of RNA Pol II. *Mol. Cell* **41,** 82–92
22. Meyer, H. H., Shorter, J. G., Seemann, J., Pappin, D., and Warren, G. (2000) A complex of mammalian ufd1 and npl4 links the AAA-ATPase, p97, to ubiquitin and nuclear transport pathways. *EMBO J.* **19,** 2181–2192
23. Hitchcock, A. L., Krebber, H., Frietze, S., Lin, A., Latterich, M., and Silver, P. A. (2001) The conserved npl4 protein complex mediates proteasome-dependent membrane-bound transcription factor activation. *Mol. Biol. Cell* **12,** 3226–3241
24. Bruderer, R. M., Brasseur, C., and Meyer, H. H. (2004) The AAA ATPase p97/VCP interacts with its alternative co-factors, Ufd1-Npl4 and p47, through a common bipartite binding mechanism. *J. Biol. Chem.* **279,** 49609–49616
25. Jarosch, E., Taxis, C., Volkwein, C., Bordallo, J., Finley, D., Wolf, D. H., and Sommer, T. (2002) Protein dislocation from the ER requires polyubiquitination and the AAA-ATPase Cdc48. *Nat. Cell Biol.* **4,** 134–139
26. Heo, J. M., Livnat-Levanon, N., Taylor, E. B., Jones, K. T., Dephoure, N., Ring, J., Xie, J., Brodsky, J. L., Madeo, F., Gygi, S. P., Ashrafi, K., Glickman, M. H., and Rutter, J. (2010) A stress-responsive system for mitochondrial protein degradation. *Mol. Cell* **40,** 465–480
27. Tran, J. R., Tomsic, L. R., and Brodsky, J. L. (2011) A Cdc48p-associated factor modulates endoplasmic reticulum-associated degradation, cell stress, and ubiquitinated protein homeostasis. *J. Biol. Chem.* **286,** 5744–5755
28. Guthrie, C., and Fink, G. R. (eds) (1991) Guide to yeast genetics and molecular biology. *Methods Enzymol.* **194,** 1–863
29. Chiu, J., March, P. E., Lee, R., and Tillett, D. (2004) Site-directed, ligase-independent mutagenesis (SLIM). A single-tube methodology approaching 100% efficiency in 4 h. *Nucleic Acids Res.* **32,** e174
30. Storici, F., and Resnick, M. A. (2006) The *delitto perfetto* approach to *in vivo* site-directed mutagenesis and chromosome rearrangements with synthetic oligonucleotides in yeast. *Methods Enzymol.* **409,** 329–345
31. Hochstrasser, M., and Funakoshi, M. (2012) Disulfide engineering to map subunit interactions in the proteasome and other macromolecular complexes. *Methods Mol. Biol.* **832,** 349–362
32. Xu, P., Duong, D. M., and Peng, J. (2009) Systematical optimization of reverse-phase chromatography for shotgun proteomics. *J. Proteome Res.* **8,** 3944–3950
33. Peng, J., Elias, J. E., Thoreen, C. C., Licklider, L. J., and Gygi, S. P. (2003) Evaluation of multidimensional chromatography coupled with tandem mass spectrometry (LC/LC-MS/MS) for large-scale protein analysis. The yeast proteome. *J. Proteome Res.* **2,** 43–50
34. Hochstrasser, M., Ellison, M. J., Chau, V., and Varshavsky, A. (1991) The short-lived MAT α2 transcriptional regulator is ubiquitinated *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 4606–4610
35. Tomko, R. J., Jr., and Hochstrasser, M. (2011) Incorporation of the Rpn12 subunit couples completion of proteasome regulatory particle lid assembly to lid-base joining. *Mol. Cell* **44,** 907–917
36. Chen, P., Johnson, P., Sommer, T., Jentsch, S., and Hochstrasser, M. (1993) Multiple ubiquitin-conjugating enzymes participate in the *in vivo* degradation of the yeast MAT α2 repressor. *Cell* **74,** 357–369
37. Mannhaupt, G., Schnall, R., Karpov, V., Vetter, I., and Feldmann, H. (1999) Rpn4p acts as a transcription factor by binding to PACE, a nonamer box found upstream of 26S proteasomal and other genes in yeast. *FEBS Lett.* **450,** 27–34
38. Xie, Y., and Varshavsky, A. (2001) RPN4 is a ligand, substrate, and transcriptional regulator of the 26S proteasome. A negative feedback circuit. *Proc. Natl. Acad. Sci. U.S.A.* **98,** 3056–3061
39. Burroughs, A. M., Iyer, L. M., and Aravind, L. (2011) Functional diversification of the RING finger and other binuclear treble clef domains in prokaryotes and the early evolution of the ubiquitin system. *Mol. Biosyst.* **7,** 2261–2277
40. Yun, C., Stanhill, A., Yang, Y., Zhang, Y., Haynes, C. M., Xu, C. F., Neubert, T. A., Mor, A., Philips, M. R., and Ron, D. (2008) Proteasomal adaptation to environmental stress links resistance to proteotoxicity with longevity in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. U.S.A.* **105,** 7094–7099
41. Stanhill, A., Haynes, C. M., Zhang, Y., Min, G., Steele, M. C., Kalinina, J., Martinez, E., Pickart, C. M., Kong, X. P., and Ron, D. (2006) An arsenite-inducible 19S regulatory particle-associated protein adapts proteasomes to proteotoxicity. *Mol. Cell* **23,** 875–885
42. Pharoah, P. D., Tsai, Y. Y., Ramus, S. J., Phelan, C. M., Goode, E. L., Lawrenson, K., Buckley, M., Fridley, B. L., Tyrer, J. P., Shen, H., Weber, R., Karevan, R., Larson, M. C., Song, H., Tessier, D. C., Bacot, F., Vincent, D., Cunningham, J. M., Dennis, J., Dicks, E., Australian Cancer Study, Australian Ovarian Cancer Study Group, Aben, K. K., Anton-Culver, H., Antonenkova, N., Armasu, S. M., Baglietto, L., Bandera, E. V., Beckmann, M. W., Birrer, M. J., Bloom, G., Bogdanova, N., Brenton, J. D., Brinton, L. A., Brooks-Wilson, A., Brown, R., Butzow, R., Campbell, I., Carney, M. E., Carvalho, R. S., Chang-Claude, J., Chen, Y. A., Chen, Z., Chow, W. H., Cicek, M. S., Coetzee, G., Cook, L. S., Cramer, D. W., Cybulski, C., Dansonka-Mieszkowska, A., Despierre, E., *et al.* (2013) GWAS meta-analysis and replication identifies three new susceptibility loci for ovarian cancer. *Nat. Genet.* **45,** 362–370, 370e1–2
43. Kelley, L. A., and Sternberg, M. J. (2009) Protein structure prediction on the Web. A case study using the Phyre server. *Nat. Protoc.* **4,** 363–371
44. Wolf, D. H., and Stolz, A. (2012) The Cdc48 machine in endoplasmic reticulum associated protein degradation. *Biochim. Biophys. Acta* **1823,** 117–124
45. Stolz, A., Hilt, W., Buchberger, A., and Wolf, D. H. (2011) Cdc48. A power machine in protein degradation. *Trends Biochem. Sci.* **36,** 515–523
46. Johnson, E. S., Ma, P. C., Ota, I. M., and Varshavsky, A. (1995) A proteo-

lytic pathway that recognizes ubiquitin as a degradation signal. *J. Biol. Chem.* **270,** 17442–17456
47. Hiller, M. M., Finger, A., Schweiger, M., and Wolf, D. H. (1996) ER degradation of a misfolded luminal protein by the cytosolic ubiquitin-proteasome pathway. *Science* **273,** 1725–1728
48. Buchberger, A. (2010) Control of ubiquitin conjugation by cdc48 and its cofactors. *Subcell. Biochem.* **54,** 17–30
49. Guerrero, C., Milenkovic, T., Przulj, N., Kaiser, P., and Huang, L. (2008) Characterization of the proteasome interaction network using a QTAX-based tag-team strategy and protein interaction network analysis. *Proc. Natl. Acad. Sci. U.S.A.* **105,** 13333–13338
50. Verma, R., Chen, S., Feldman, R., Schieltz, D., Yates, J., Dohmen, J., and Deshaies, R. J. (2000) Proteasomal proteomics. Identification of nucleotide-sensitive proteasome-interacting proteins by mass spectrometric analysis of affinity-purified proteasomes. *Mol. Biol. Cell* **11,** 3425–3439
51. Russell, S. J., Steger, K. A., and Johnston, S. A. (1999) Subcellular localization, stoichiometry, and protein levels of 26 S proteasome subunits in yeast. *J. Biol. Chem.* **274,** 21943–21952
52. Ghaemmaghami, S., Huh, W. K., Bower, K., Howson, R. W., Belle, A., Dephoure, N., O'Shea, E. K., and Weissman, J. S. (2003) Global analysis of protein expression in yeast. *Nature* **425,** 737–741
53. Kirkpatrick, D. S., Dale, K. V., Catania, J. M., and Gandolfi, A. J. (2003) Low-level arsenite causes accumulation of ubiquitinated proteins in rabbit renal cortical slices and HEK293 cells. *Toxicol. Appl. Pharmacol.* **186,** 101–109
54. Haugen, A. C., Kelley, R., Collins, J. B., Tucker, C. J., Deng, C., Afshari, C. A., Brown, J. M., Ideker, T., and Van Houten, B. (2004) Integrating phenotypic and expression profiles to map arsenic-response networks. *Genome Biol.* **5,** R95
55. Isakov, E., and Stanhill, A. (2011) Stalled proteasomes are directly relieved by P97 recruitment. *J. Biol. Chem.* **286,** 30274–30283
56. Freeman, M. (2008) Rhomboid proteases and their biological functions. *Annu. Rev. Genet.* **42,** 191–210
57. Zettl, M., Adrain, C., Strisovsky, K., Lastun, V., and Freeman, M. (2011) Rhomboid family pseudoproteases use the ER quality control machinery to regulate intercellular signaling. *Cell* **145,** 79–91
58. Greenblatt, E. J., Olzmann, J. A., and Kopito, R. R. (2011) Derlin-1 is a rhomboid pseudoprotease required for the dislocation of mutant α-1 antitrypsin from the endoplasmic reticulum. *Nat. Struct. Mol. Biol.* **18,** 1147–1152
59. Vij, S., and Tyagi, A. K. (2008) A20/AN1 zinc-finger domain-containing proteins in plants and animals represent common elements in stress response. *Funct. Integr. Genomics* **8,** 301–307
60. Mukhopadhyay, A., Vij, S., and Tyagi, A. K. (2004) Overexpression of a zinc-finger protein gene from rice confers tolerance to cold, dehydration, and salt stress in transgenic tobacco. *Proc. Natl. Acad. Sci. U.S.A.* **101,** 6309–6314
61. Heyninck, K., and Beyaert, R. (2005) A20 inhibits NF-κB activation by dual ubiquitin-editing functions. *Trends Biochem. Sci.* **30,** 1–4
62. Huang, J., Teng, L., Li, L., Liu, T., Li, L., Chen, D., Xu, L. G., Zhai, Z., and Shu, H. B. (2004) ZNF216 is an A20-like and IκB kinase γ-interacting inhibitor of NFκB activation. *J. Biol. Chem.* **279,** 16847–16853
63. Alberts, S. M., Sonntag, C., Schäfer, A., and Wolf, D. H. (2009) Ubx4 modulates cdc48 activity and influences degradation of misfolded proteins of the endoplasmic reticulum. *J. Biol. Chem.* **284,** 16082–16089
64. Schuberth, C., and Buchberger, A. (2005) Membrane-bound Ubx2 recruits Cdc48 to ubiquitin ligases and their substrates to ensure efficient ER-associated protein degradation. *Nat. Cell Biol.* **7,** 999–1006
65. Rumpf, S., and Jentsch, S. (2006) Functional division of substrate processing cofactors of the ubiquitin-selective Cdc48 chaperone. *Mol. Cell* **21,** 261–269
66. Huh, W. K., Falvo, J. V., Gerke, L. C., Carroll, A. S., Howson, R. W., Weissman, J. S., and O'Shea, E. K. (2003) Global analysis of protein localization in budding yeast. *Nature* **425,** 686–691
67. Chia, W. S., Chia, D. X., Rao, F., Bar Nun, S., and Geifman Shochat, S. (2012) ATP binding to p97/VCP D1 domain regulates selective recruitment of adaptors to its proximal N-domain. *PLoS One* **7,** e50490
68. Baek, G. H., Kim, I., and Rao, H. (2011) The Cdc48 ATPase modulates the interaction between two proteolytic factors Ufd2 and Rad23. *Proc. Natl. Acad. Sci. U.S.A.* **108,** 13558–13563
69. Wilson, J. D., Liu, Y., Bentivoglio, C. M., and Barlowe, C. (2006) Sel1p/Ubx2p participates in a distinct Cdc48p-dependent endoplasmic reticulum-associated degradation pathway. *Traffic* **7,** 1213–1223
70. Barthelme, D., and Sauer, R. T. (2013) Bipartite determinants mediate an evolutionarily conserved interaction between Cdc48 and the 20S peptidase. *Proc. Natl. Acad. Sci. U.S.A.* **110,** 3327–3332
71. Schuberth, C., Richly, H., Rumpf, S., and Buchberger, A. (2004) Shp1 and Ubx2 are adaptors of Cdc48 involved in ubiquitin-dependent protein degradation. *EMBO Rep.* **5,** 818–824
72. Wang, B., Alam, S. L., Meyer, H. H., Payne, M., Stemmler, T. L., Davis, D. R., and Sundquist, W. I. (2003) Structure and ubiquitin interactions of the conserved zinc finger domain of Npl4. *J. Biol. Chem.* **278,** 20225–20234
73. Ye, Y., Meyer, H. H., and Rapoport, T. A. (2003) Function of the p97-Ufd1-Npl4 complex in retrotranslocation from the ER to the cytosol. Dual recognition of nonubiquitinated polypeptide segments and polyubiquitin chains. *J. Cell Biol.* **162,** 71–84