# UNIVERSITY OF CALIFORNIA, IRVINE

Identification of a Novel Mechanism for Regulation of F-box Proteins

**DISSERTATION**

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biomedical Sciences

by

Radhika Mathur

Dissertation Committee:
Professor Peter Kaiser, Chair
Professor Haoping Liu
Professor Suzanne Sandmeyer
Professor Marian Waterman

2014

# DEDICATION

To

My Parents, without whom none of this would have been possible.

# TABLE OF CONTENTS


| | Page |
|---|---|
| LIST OF FIGURES | iv |
| LIST OF TABLES | vi |
| ACKNOWLEDGMENTS | vii |
| CURRICULUM VITAE | viii |
| ABSTRACT OF THE DISSERTATION | x |
| CHAPTER 1: General Introduction | 1 |
| CHAPTER 2: Materials and Methods | 11 |
| CHAPTER 3: Skp1 Independent Function of Cdc53 in Maintenance of Met30 Homeostasis | 20 |
| Abstract | 20 |
| Introduction | 21 |
| Results | 23 |
| Discussion | 35 |
| CHAPTER 4: Cdc48 ATPase and Cic1 are essential for 'Skp1-free' Met30 Degradation | 62 |
| Abstract | 62 |
| Introduction | 62 |
| Results | 65 |
| Discussion | 70 |
| CHAPTER 5: Concluding Remarks | 81 |
| REFERENCES | 84 |

# LIST OF FIGURES


| | | Page |
|---|---|---|
| Figure 1-1 | Enzymes of the ubiquitylation pathway | 8 |
| Figure 1-2 | Cullin- RING ligases | 9 |
| Figure 1-3 | Model for substrate receptor exchange mediated by neddylation/Cand1 cycles | 10 |
| Figure 3-1 | $SCF^{Met30}$ regulates Met4 activity | 38 |
| Figure 3-2 | Mutations in Met30 that disrupt Met30-Skp1 binding, destabilize Met30 | 39 |
| Figure 3-3 | Dissociation of Met30 from Skp1 induces its degradation | 40 |
| Figure 3-4 | Degradation of 'Skp1-Free' Met30 is dependent on ubiquitin proteasome system | 41 |
| Figure 3-5 | Degradation of 'Skp1-Free' Met30 is dependent on Cdc53, Cdc34 and Rbx1 | 42 |
| Figure 3-6 | Ubiquitylation and degradation of 'Skp1-Free' Met30 is dependent on Cdc53 but is independent of Skp1 | 43 |
| Figure 3-7 | Degradation of 'Skp1-Free' Met30 is not dependent on Met4 | 44 |
| Figure 3-8 | Skp1 independent degradation of Met30 | 45 |
| Figure 3-9 | Cdc53 mutant unable to bind Skp1 can degrade Met30 | 46 |
| Figure 3-10 | Identification of degron for 'Skp1-free' Met30 degradation pathway | 47,48 |
| Figure 3-11 | Mutation of methionine 178 and isoleucine 179 in Met30 can abolish 'Skp1-free' Met30 degradation | 49 |
| Figure 3-12 | Degradation of 'Skp1-Free' Met30 is not dependent on Lag2 | 50 |
| Figure 3-13 | Cdc53 can bind Met30 in absence of Skp1 | 51 |
| Figure 3-14 | Cdc53/Rbx1 binds Met30 *in vitro* | 52 |

| | | |
|---|---|---|
| Figure 3-15 | Cdc53/Rbx1 can ubiquitylate Met30 *in vitro* | 53 |
| Figure 3-16 | Cdc53/Rbx1 ubiquitylation is specific for 'Skp1-free' Met30 degradation | 54 |
| Figure 3-17 | Skp1- independent degradation of Ctf13 and Cdc4 | 55 |
| Figure 3-18 | Grr1 is not regulated by 'Skp1-free' F-box protein degradation pathway | 56 |
| Figure 3-19 | Characterization of $Met30^{M178E/I179E}$ mutant | 57 |
| Figure 3-20 | 'Skp1-free' Met30 degradation is important for cellular function | 58 |
| Figure 3-21 | Loss of repression of Met4 target genes in $Met30^{M178E/I179E}$ mutant | 59 |
| Figure 3-22 | $Met30^{M178E/I179E}$ mutant is dominant | 60 |
| Figure 3-23 | $Met30^{M178E/I179E}$ mutant exhibits a slight growth delay | 61 |
| Figure 4-1 | Cdc48 plays a role in regulation of F-box proteins | 72 |
| Figure 4-2 | Cdc48 is involved in 'Free' F-box protein degradation pathway | 73 |
| Figure 4-3 | Mitotic arrest does not induce $Met30^{\Delta Fbox}$ stabilization | 74 |
| Figure 4-4 | Cdc48 cofactors Npl4 and Ufd1 are involved in 'Skp1-free' Met30 degradation | 75 |
| Figure 4-5 | Cic1 is involved in 'Skp1-free' Met30 degradation pathway | 76 |
| Figure 4-6 | Cdc48, Npl4, and Cic1 are not required for ubiquitylating $Met30^{\Delta Fbox}$ | 77 |
| Figure 4-7 | Cic1 binds Met30 *in vivo* | 78 |
| Figure 4-8 | Cic1 interaction with Cdc48 depends on Npl4 and Ufd1 | 79 |
| Figure 4-9 | Model for Cic1 as a F-box protein specificity factor for Cdc48/Npl4/Ufd1 | 80 |

# LIST OF TABLES


| | | Page |
|---|---|---|
| Table 1 | Yeast strains used in this study | 18 |

# ACKNOWLEDGMENTS

First and foremost, I would like to express my gratitude to my mentor and advisor, Professor Peter Kaiser for his expert guidance over the course of my graduate studies. His motivation, patience, and continuous support have been crucial to my success in becoming a better scientist.

I would also like to thank members of my thesis committee, Suzanne Sandmeyer, Haoping Liu and Marian Waterman for their insightful comments and helpful discussions on my project.

I would like to sincerely thank past and present members of the Kaiser lab for their dear friendships, companionship and support over the years. I would like to thank Roby, Christie, Karin and Ikram for helping me with experiments and for always being there to answer my questions. Special thanks to Hongwei for the fun morning discussions and Keith, for the late night chats. I would specially like to thank Roby and Linda for always being there to cheer and support me through hard times and for just being great friends.

I gratefully acknowledge Dr. James Yen, whose work was the foundation for my studies presented here. I thank him for allowing me to use some of his figures as part of my dissertation. I would also like to thank Elsevier and Nature publishing Group for permission to include copyrighted figures.

Lastly, I would like to thank my family for all their love and encouragement. To Mummy and Papa, for their unwavering belief in me. To my brother, Raghav, for always being there to make me laugh. To my loving husband, Asheesh, for his remarkable patience and support over the course of my research. To my little girl, Ananya, who is my constant source of joy and smiles. Thank You.

# CURRICULUM VITAE

## RADHIKA MATHUR

## EDUCATION

**University of California, Irvine**
Doctor of Philosophy in Biomedical Sciences, 2014
Dissertation Advisor- Professor Peter Kaiser

**Dartmouth College, Hanover, NH**
Master of Science, 2008
Degree Major: Genetics

**University of Delhi, New Delhi**
Master of Science, 2004
Degree Major: Biomedical Sciences

**University of Delhi, New Delhi**
Bachelor of Science, 2002
Degree Major: Zoology

## RESEARCH EXPERIENCE

**Graduate Student Researcher –2009-Present**
Department of Biological Chemistry, UCI
Advisor: Peter Kaiser

**Graduate Student Researcher –2006-2008**
Department of Genetics, Dartmouth College
Advisor: Jay Dunlap

## PUBLICATIONS

Yen J, Flick K, Papagiannis CV, **Mathur R**, Tyrrell A, Ouni I, Kaake R, Huang L, Kaiser P. Signal-induced disassembly of the SCF ubiquitin ligase complex by Cdc48/p97. *Molecular Cell* (2012) 48:288-297.

**Mathur R**, Kaiser P. PCR-mediated epitope tagging of genes in Yeast. *Methods in Molecular Biology*, Humana Press. In print.

## CONFERENCE PRESENTATION

**Mathur R**, Yen J, Kaiser P (2012) An additional mechanism of F-Box protein degradation. Abstract for poster presentation, FASEB Ubiquitin and Cellular Regulation Meeting at Saxtons River, VT.

**Mathur R**, Yen J, Kaiser P (2011) An additional mechanism of F-Box protein degradation. Abstract for poster presentation, Cell Cycle Meeting at SALK Institute, CA.

**Mathur R**, Yen J, Kaiser P (2011) An additional mechanism of F-Box protein degradation. Abstract for platform presentation, The Ubiquitin Family Meeting at Cold Spring Harbor, NY.

## TEACHING EXPERIENCE

Teaching Assistant, University of California, Irvine- Winter & Spring 2010
Course: D111L

Teaching Assistant, Dartmouth College, Hanover, NH- Winter 2007
Course: BIO13; Gene expression and Inheritance

## HONORS & FELLOWSHIPS

John Wasmuth Graduate Student Research Seminar Award (2011) University of California, Irvine

# ABSTRACT OF THE DISSERTATION

Identification of a Novel Mechanism for Regulation of F-box Proteins

By

Radhika Mathur

Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2014

Professor Peter Kaiser, Chair

Post-translational modification of proteins with ubiquitin plays a role in most biological processes. The key players of this system are the E3 ubiquitin ligases, which mediate substrate specific covalent attachment of ubiquitin. The SCF ubiquitin ligases are amongst best-understood E3 complexes. They are composed of Skp1, Cdc53/Cullin, Rbx1, and one of the multiple F-box proteins, which bind substrates and confer specificity to this complex. As these modular ligases share the same core components but differ in the identity of F-box proteins, the repertoire of SCF ligases present in a cell at any given time is thus governed by the abundance of different F-box proteins. Regulating F-box protein abundance is thus essential for normal cellular function and misregulation causes human diseases. In the studies presented here, we describe a novel mechanism governing maintenance of F-box protein homeostasis.

In the absence of substrates, the core SCF ligase ubiquitylates its F-box protein and targets it for degradation. Using the yeast F-box protein Met30 as a model system, we demonstrate presence of an additional mechanism for F-box protein degradation apart from autoubiquitylation. This pathway targets F-box proteins that are dissociated from Skp1, and plays

a crucial role in limiting substrate shielding effects caused by association of excess F-box proteins with their substrates thereby inhibiting substrate recognition by fully assembled ligases. Characterization of this pathway shows that it is governed by the ubiquitin proteasome system and is mediated by a ligase dependent on Cdc53 but independent of Skp1 and for the first time illustrates uncoupling of Cdc53 and Skp1 function in yeast.

Furthermore, we show that Cic1 and the $AAA^+$ ATPase Cdc48, along with its substrate recruiting factors Npl4/Ufd1 are involved in this mechanism of F-box protein degradation. Preliminary studies conducted to ascribe a role for these proteins in this process, suggest Cic1 as a unique factor that recruits ubiquitylated F-box proteins to Cdc48/Npl4/Ufd1 for targeting to proteasome for degradation.

Together, our findings shed light on the complexity of the ubiquitin system. They uncover a novel degradation pathway, suggest existence of yet unknown types of ligases, and introduce the concept of *protein specificity factors* for Cdc48.

# CHAPTER 1

## General Introduction

### *Ubiquitylation*

The process of covalent modification of proteins with the small 76 amino acid protein ubiquitin is known as ubiquitylation. This process is highly conserved through eukaryotes and is most often associated with its function in the ubiquitin-proteasome system, where poly-ubiquitin chains label substrates for recognition and degradation by the 26S proteasome (Pickart, 2001; Varshavsky, 2012). Modification of proteins via monoubiquitylation, multiubiquitylation, or polyubiquitylation with K63 ubiquitin chains can also serve as nonproteolytic signal such as in DNA repair, mRNA export and trafficking of membrane proteins (Bergink and Jentsch, 2009; Finley et al., 2012). Ubiquitylation regulates a myriad of biological processes including immunity, cell division, signaling, and transcription and defects in this key mechanism of posttranslational modification of proteins have been implicated in numerous human diseases such as cancer, neurodegenerative disorders and developmental defects underscoring the importance of this integral system in normal functioning of the eukaryotic cell (Ciechanover, 2003; Jiang and Beaudet, 2004; Sakamoto, 2002).

### *Enzymes of the Ubiquitin system*

Ubiquitylation of proteins is achieved through sequential action of 3 classes of enzymes (Hershko and Ciechanover, 1998; Varshavsky, 2012) (Fig. 1-1). The E1 ubiquitin activating enzyme, activates ubiquitin by mediating formation of a high-energy thioester bond with the carboxyl group of the C-terminal glycine residue of ubiquitin in an ATP dependent manner.

Next, via transesterification, the activated ubiquitin is transferred from the E1 enzyme to an E2-ubiquitin-conjugating enzyme. Finally, an E3 ubiquitin ligase, which interacts with the substrate, catalyzes formation of isopeptide bond between ε–amino group of acceptor lysine residue in substrate protein and the activated carboxyl group of ubiquitin. This enzymatic cascade is repeated, where additional ubiquitin moieties are attached sequentially to one another via one of the internal lysine residues of ubiquitin, resulting in a polyubiquitin chain.

The enzymes of the ubiquitin system exhibit hierarchical organization. In *Saccharomyces cerevisiae* there exist only one E1 (Uba1), 11 E2s, and around 60-100 E3s (Finley et al., 2012) while in humans currently two E1s, around 50 E2s, and over 600 E3s have been identified (Komander, 2009).

***E3 ubiquitin ligases***

Within the enzymes of the ubiquitin pathway, the E3 ligases (E3s) are key layers of the system as they mediate substrate specific covalent attachment of ubiquitin and confer specificity to this process. These enzymes comprise the largest group of proteins involved in ubiquitylation and can be broadly classified based on the mechanism of ubiquitin transfer, into 2 major groups, HECT domain and RING domain ligases (Deshaies and Joazeiro, 2009).

HECT (**h**omologus to **E**6-AP **c**arboxy **t**erminus) domain E3s contain a 350 amino acid conserved domain that consists of an N-terminal lobe that binds E2 enzyme and a C-terminal region that harbors the active site cysteine residue, which forms thioester with ubiquitin before it is transferred onto the substrate (Huang et al., 1999). There are 5 known HECT domain E3s in yeast and 28 in humans. Apart from ubiquitylating proteins for proteasomal degradation,

these enzymes play role in diverse processes such as regulation of endocytosis and sorting of transmembrane proteins and receptors (Metzger et al., 2012; Rotin and Kumar, 2009).

RING (**R**eally **I**nteresting **N**ew **G**ene) domain E3s include majority of ubiquitin ligases. These enzymes bear a RING domain consisting of conserved cysteine and histidine residues coordinating with two zinc atoms, which yields a rigid globular platform for protein-protein interactions. These enzymes, with the exception of RING-in-between-RING E3s discussed below, do not form a direct thioster intermediate with ubiquitin, instead via the RING domain, they bind to charged E2~Ub and place it in proximity to the substrate acceptor lysine to facilitate effective ubiquitin transfer (Pickart, 2001; Zheng et al., 2000). A subclass of this group is the U-box domain ligases, which are structurally related to RING domain E3s. In these ligases, polar residues replace the zinc binding sites and engage in hydrogen bonding networks to maintain structure and activity. They are also denoted as E4 ligases as they function by extending ubiquitin chains on previously ubiquitylated substrates. (Deshaies and Joazeiro, 2009; Koegl et al., 1999).

RING-in-between-RING E3s are a subclass of RING domain E3s that function as HECT/RING hybrids. These enzymes are defined by presence of a canonical RING domain (RING1), and in-between RING domain (IBR) and another RING domain (RING2). RING1 domain binds E2~Ub and stimulates the transfer of ubiquitin onto a conserved cysteine in the RING2 domain, forming an E3 thioester before conjugation to the substrate (Eisenhaber et al., 2007). Thirteen RBR ligases are known in humans and amongst them, Parkin is most studied RBR E3 because of its association with Parkinson's disease. Yeast has two putative RBR E3s whose function as ligases remains to be demonstrated (Wenzel and Klevit, 2012).

Substrate recruitment in RING domain E3s can be achieved by presence of substrate binding domains, which can reside within the same polypeptide as the RING domain or in a different protein that associates with the RING domain containing protein to form a multi-subunit complex (Deshaies and Joazeiro, 2009) . Examples of single subunit RING E3s are San1, which regulates nuclear protein quality control (Gardner et al., 2005) and Rad18 which catalyzes ubiquitylation of PCNA (Hoege et al., 2002). Eminent members of RING E3s, which employ specific set of substrate adaptors, are anaphase promoting complex or cyclosome (APC/C) and cullin-RING ligases (CRLs).

(APC/C) is a multi-subunit E3 ubiquitin ligase that triggers degradation of multiple substrates crucial for cell cycle progression (Manchado et al., 2010). It consists of 13 subunits, amongst which Apc11 is the RING domain component and Cdh1, Cdc20, and Ama1 are the substrate binding activators. These proteins recognize APC substrates by interacting with distinct degradation elements called- D-boxes and KEN-boxes (Pesin and Orr-Weaver, 2008; Peters, 2006).

***Cullin- RING ligases***

Cullin-RING ligases comprise the biggest class of ubiquitin ligases in all eukaryotes. These multi-subunit ligases consist of four components — one cullin, a RING domain protein that binds E2 enzyme, a substrate recognition subunit, and an adaptor that links the substrate recognition subunit to the cullin core (Deshaies et al., 2010; Duda et al., 2011). Cullin serves as the central subunit for assembly of CRLs. The rigidity of cullin structure juxtaposes E2 and the substrate for favorable ubiquitin transfer and mutations that increase flexibility in structure can destroy CRL activity *in vitro* (Zheng et al., 2002).

Yeast contains three different cullin genes while human cells express 7 distinct cullins (Finley et al., 2012; Zimmerman et al., 2010). Different cullins have their own dedicated set of adaptors (Skp1, Elongin B/C, DDB1) and substrate recognition subunits (F-box, SOCS-box, DCAF) and nucleate distinct classes of CRL complexes (Fig. 1-2). The modular design of CRLs provides a platform for interaction with an array of substrate recognition subunits to target a large number of diverse proteins for regulated ubiquitylation (Duda et al., 2011).

***SCF ubiquitin ligases***

CRLs assembled by the archetypical cullin Cul1 are called SCF (**S**kp1/**C**ullin/**F**-box) ubiquitin ligases. They were the first identified CRLs and are the best understood E3s. They were discovered in yeast with the finding of $SCF^{Cdc4}$ and are most thoroughly characterized in this organism (Feldman et al., 1997; Skowyra et al., 1997). In these ligases, Cul1/Cdc53 serves as a scaffold for facilitating protein-protein interactions. The globular C-terminus of Cdc53 interacts with RING domain protein, Rbx1 that associates with Cdc34, the most common E2 enzyme for yeast SCF ligases. Its helical N-terminal domain binds the adaptor protein Skp1 that associates with one of several F-box proteins, which in turn interact with substrates (Cardozo and Pagano, 2004; Deshaies, 1999).

Distinct SCF ligases share the same core components but differ in the identity of their F-box subunit. Each F-box protein associates with a unique set of targets and confers specificity to the ligase complex. This protein family is defined by the presence of a conserved 40 amino acid N-terminal domain called the F-box domain via which they interact with Skp1 (Bai et al., 1996). Most often, these proteins also harbor protein-protein interaction domains such as WD-40 repeats and leucine rich repeats for substrate association (Petroski and Deshaies, 2005). There

are 22 known F-box proteins in yeast and around 70 in humans (Finley et al., 2012; Flick and Kaiser, 2013). They all contain the F-box domain, but some do not assemble in functional SCF complexes even though they bind Skp1. Ctf13 and Rcy1 are two such examples in yeast. Ctf13 interacts with Skp1 in CBF3 complex, which is involved in kinetochore function (Kaplan et al., 1997) while Rcy1-Skp1 complex is involved in recycling of SNARE proteins (Galan et al., 2001). Substrate and SCF ligase interaction is often controlled by phosphorylation, which marks substrates for recognition by F-box proteins to initiate their ubiquitylation (Skowyra et al., 1997).

***Regulation of SCF ubiquitin ligases***

As ubiquitin ligases dictate the specificity of ubiquitylation reactions, regulating their activity primarily controls protein degradation *in vivo*. One of the best-documented mechanisms of SCF ubiquitin ligase regulation is the covalent modification of Cdc53/Cul1 with ubiquitin like protein Nedd8 (yeast Rub1). Neddylation induces a structural change in Cdc53, which activates it and stimulates ubiquitylation (Duda et al., 2008). In yeast, the Rub1 pathway is dispensable under normal growth conditions (Lammer et al., 1998). In the active orientation, Skp1/F-box protein complex and E2 enzyme can associate with Cdc53/Rbx1 core and bring about substrate ubiquitylation. Once substrates are depleted, Nedd8 is deconjugated by COP9 signalosome, which promotes interaction of Cdc53 with Skp1/F-box protein competing factor, CAND1 (yeast Lag2) (Flick and Kaiser, 2013). CAND1 acts as a negative regulator of SCF ligase activity *in vitro* and promotes Skp1/F-box protein release following SCF deneddylation (Bosu and Kipreos, 2008). New Skp1/F-box protein complexes can dissociate tightly bound CAND1 from Cdc53 resulting in formation of different SCF ligase complexes (Lydeard et al., 2013). Cycles of neddylation/CAND1 association ensure that abundance of different SCF ligases is adjusted in

response to cell cycle and environmental cues to dynamically match substrate levels (Pierce et al., 2013; Wu et al., 2013; Zemla et al., 2013) (Fig. 1-3).

A relatively unexplored area of SCF ligase regulation is understanding how degradation of ligase subunits is coordinated. Amongst the SCF core components, F-box proteins are short lived while the other components are relatively stable. These proteins are ubiquitylated and targeted for degradation and their ubiquitylation is incumbent upon their assembly into an intact SCF complex (Galan and Peter, 1999; Zhou and Howley, 1998). This autocatalytic mode of degradation can be reconstituted *in vitro* using purified SCF subunits (Kus et al., 2004). Substrate binding is known to inhibit this mechanism of F-box protein regulation (Li et al., 2004). WD-40 repeats in F-box proteins contain an ubiquitin binding domain and binding of substrate and ubiquitin is mutually exclusive (Pashkova et al., 2010). This helps to ensure that F-box protein levels are maintained in accordance to the amount of substrate present in the cell and allows for quick reassembly of SCF machinery to adapt to cellular and environmental changes.

In this dissertation, using yeast $SCF^{Met30}$ as a model, we report an additional pathway for F-box protein regulation that specifically targets F-box proteins that are detached from Skp1 for degradation. We identify key components of this pathway and show that it plays an important role in limiting excess Met30 from shielding its substrate from recognition by fully assembled $SCF^{Met30}$ ligase. Furthermore, our results introduce a novel subtype of cullin-RING ligase in *Saccharomyces cerevesiae* that comprises Cdc53/Rbx1/Cdc34 but not Skp1.

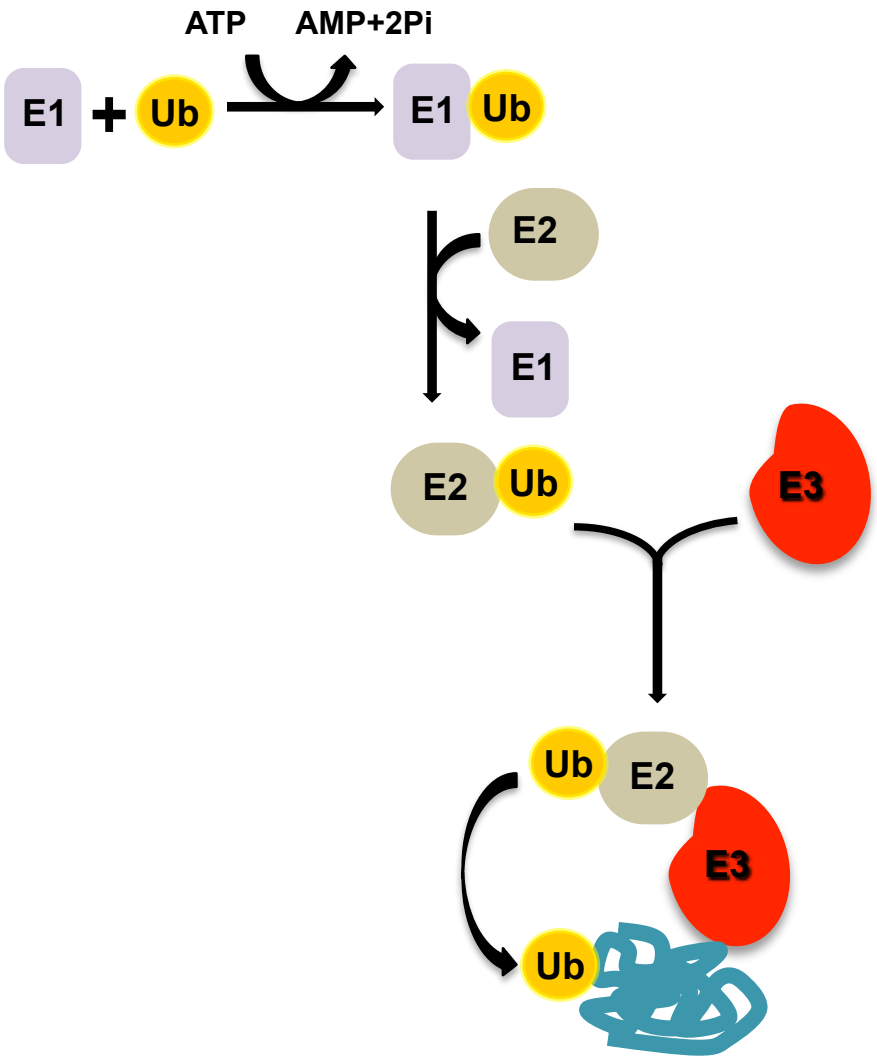


**Figure 1-1. Enzymes of the ubiquitylation pathway.** Ubiquitin is activated by E1 activating enzyme in an ATP-dependent manner and then transferred to the active site cysteine in the ubiquitin-conjugating enzyme (E2). E3 ligases bind substrates and mediate transfer of ubiquitin from the E2 enzyme onto the substrate.

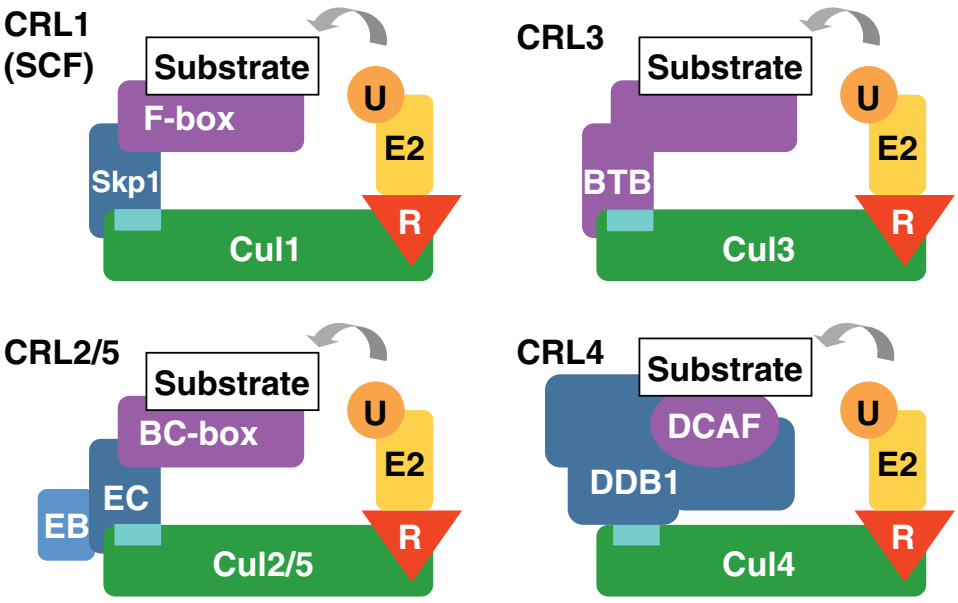


**Figure 1-2. Cullin- RING ligases.** Cullin scaffold proteins (Cul1-5, green) in complex with the RING finger protein (Rbx1/2, R, red) recruit the ubiquitin (U) charged E2 enzymes to form the catalytic core of CRLs. Skp1, Elongin C, DDB1 serve as the adaptor (light blue) to CRL1 (SCF), CRL2/5, and CRL4 (A&B) respectively. F-box proteins, BC-box proteins, BTB domain proteins, and DCAF proteins function as the substrate receptors (magenta) of CRL1(SCF), CRL2/5, CRL3 and CRL4 (A&B), respectively. Adaptor binding regions of all cullins are colored in cyan. Figure is adapted from " Structural assembly of cullin-RING ubiquitin ligase complexes", by Erik S Zimmerman, Brenda A Schulman and Ning Zheng, 2010 *Current Opinion in Structural Biology*, 20, p. 714, copyright 2010 by Elsevier. Adapted with permission.

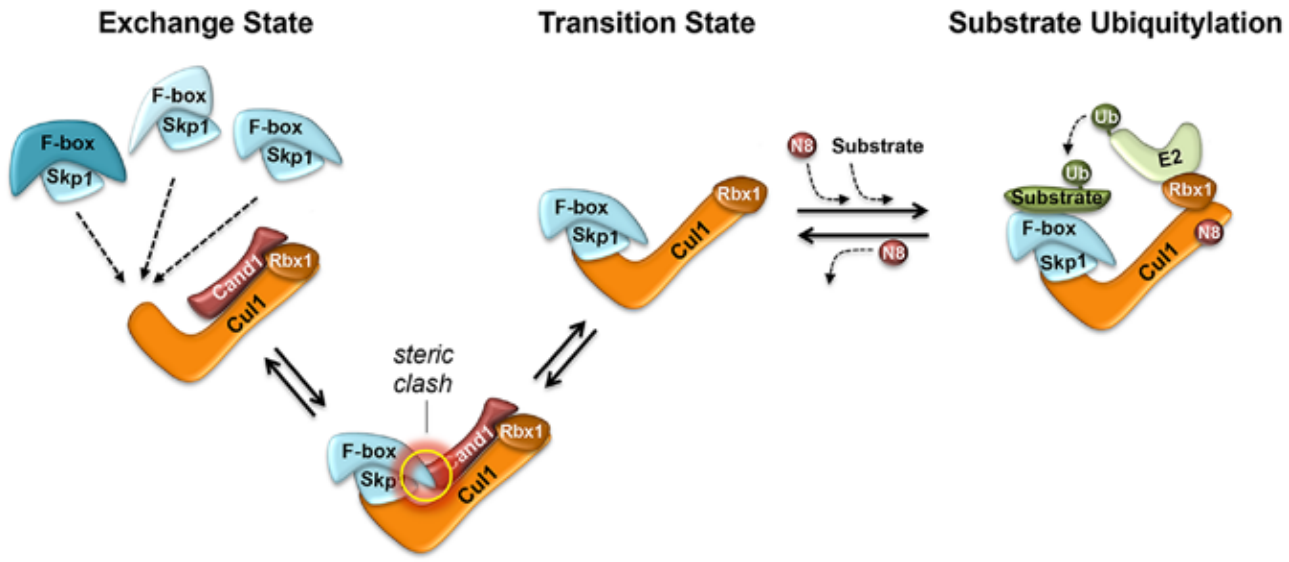


**Figure 1-3. Model for substrate receptor exchange mediated by neddylation/Cand1 cycles.** Substrate availability protects the stable *substrate ubiquitylation state* (right). Depletion of substrates enhances COP9 signalosome mediated deneddylation, shifting the SCF complex into a *transition state* that either finds new substrates and becomes reactivated by Nedd8 conjugation, or forms a transient complex with Cand1. The transient complex is highly unstable because of steric interference between F-box protein and Cand1 causing cycles of Cand1 and Skp1-F-box protein eviction. The *exchange state* allows the repertoire of formed SCF complexes to sample for substrates and, upon engagement, transit into the stable *substrate ubiquitylation state*. Figure is adapted from "Set them free: F-box protein exchange by CAND1", by Karin Flick and Peter Kaiser, 2013 *Cell Research*, 23, p. 870, copyright 2013 by Nature Publishing Group. Adapted with permission.

# CHAPTER 2

## Materials & Methods

### Yeast Strains and Growth Conditions

Yeast strains used in this study are isogenic to 15DaubΔ, a *bar1*Δ *ura3*Δns; a derivative of BF264-15D (Reed et al., 1985) and are listed in Table 1. Standard culture media and yeast genetic techniques were employed (Guthrie, 1991).

### Yeast Spotting Assay

Yeast strains were grown to logarithmic phase and were counted using hemocytometer or cell number was estimated based on optical density ($A_{600}$), assuming one optical density unit at 600 nm equals $10^7$ cells. Serial dilutions in 5-fold increments were spotted onto indicated agar plates by use of a pin replicator (V&P Scientific, Dan Diego, CA). Plates were incubated for indicated times at temperatures as indicated.

### Protein analysis

For analyzing whole cell extracts, protein lysates were prepared in 8 M Urea Buffer (8 M Urea, 200 mM NaCl, 100 mM Tris pH 7.5, 0.2% SDS, 10 mM Na-pyrophosphate, 5 mM EDTA, 5 mM EGTA, 50 mM NaF, 0.1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 μg/ml each aprotenin, leupeptin and pepstatin). Cells were lysed using glass beads three times for 40 seconds at setting 4.5 in FastPrep FP120 (Qbiogene, Carlsbad, CA) and lysates were cleared by centrifugation at room temperature for 10 minutes at 13,000 rpm. Samples were diluted to a final concentration of 4 M urea using 2x SDS loading buffer (4% SDS, 125 mM Tris

pH 8, 20% glycerol, 200 mM DTT, 0.002% bromophenol blue). Proteins were separated by SDS-PAGE, transferred to polyvinylidene difuoride membrane (Immobilon-P) and probed with the antibodies indicated.

For yeast immunopurifications, samples were collected from ~ 25-50 $OD_{600}$ cell pellets and lysed in Triton Buffer (50 mM HEPES pH 7.5, 0.2% Triton-X100, 100 mM NaCl, 10% Glycerol, 10 mM Na-pyrophosphate, 5 mM EDTA, 5 mM EGTA, 50 mM NAF, 0.1 mM orthovanadate, 1 mM PMSF, 1 μg/ml each aprotenin, leupeptin and pepstatin) using FastPrep FP120 (same program as above). Lysates were cleared by centrifugation for 10 min at 13,000 rpm at 4°C and 3 mg of total protein lysates was incubated with 3-μl anti-myc antibody (#sc-789-G, Santa Cruz) for 3 hours at 4°C. Lysate antibody mix was incubated with 20 μl prewashed Protein-G Agarose beads (Pierce) for 1 hour at 4°C, and beads were washed three times with Triton buffer. Bound proteins were eluted by boiling beads in 2x SDS loading buffer and were analyzed by Western blotting.

For HA-immunopurifications, protein lysates were incubated with 20 μl prewashed anti HA agarose beads (Sigma) for 3 hours at 4°C. Beads were then washed thrice in Triton buffer and eluted by boiling in 2x SDS loading buffer.

**Antibodies**

For immunoblot analyses, proteins were detected with the following primary antibodies: anti-Met4 (1:10000; a gift from M. Tyers), anti-Skp1 (1:5000; a gift from R. Deshaies), anti-myc and anti-HA (1:2000; Covance, Princeton, NJ), anti-RGS6H (1:2000; QIAGEN, Germantown, MD),

anti-ubiquitin (1:2000; P4G7, #sc-53509, Santa Cruz), anti-Cdc53 (1:1000; yN-18, #sc-6716, Santa Cruz) and anti-MBP (1:2000; N-17, #sc-809, Santa Cruz).

**Determination of protein degradation rates**

Strains carrying plasmids expressing tagged genes of interest placed under their endogenous promoter were cultured to logarithmic phase in minimal media containing 2% dextrose. Cultures were then transferred to rich media (YEPD) for 1 hour. Cycloheximide (100 μg/ml) was added and cells were collected at time points as indicated.

Yeast strains expressing genes of interest under the control of *GAL1* promoter were cultured in 2% sucrose minimal media until logarithmic phase. Cultures were then transferred to rich media containing 2% galactose (YEPG) for 2 hours or as otherwise indicated. Cultures were then transferred to pre-warmed YEPD, swirled and cells at time point zero was collected. Cells for the remaining time points were collected as indicated.

For protein degradation experiments that required proteasome inhibition, DMSO or MG132 (50 μM) were added 45 minutes prior to the collection of cells for the first time point. All strains used in experiments with the proteasome inhibitor MG132 carried a deletion of *PDR5* to increase drug permeability (Emter et al., 2002).

For temperature sensitive mutants, strains were grown at permissive temperature (25°C) to early logarithmic phase. Cultures were shifted to non- permissive temperature (37°C) for 1.5 hours before protein expression was blocked and cells at the indicated time intervals were collected.

**Purification of ubiquitylated proteins on $Ni^{+2}$ resin**

For purification of HBTH-tagged Met30$^{\Delta Fbox}$, cells were lysed and purified under denaturing conditions in binding buffer (8 M urea, 300 mM NaCl, 0.5% NP-40, 50 mM sodium-phosphate buffer pH 8, 50 mM Tris-HCl pH 8, 20 mM imidazole). 1 mg of total protein lysates was used for binding to $Ni^{2+}$-sepharose (GE Healthcare). Binding was performed overnight, beads were then washed thrice in binding buffer (without imidazole and pH adjusted to 6.3), and eluted in 150 µl elution buffer (8 M urea, 200 mM NaCl, 50 mM sodium-phosphate buffer, 2% SDS, 10 mM EDTA, 100 mM Tris-HCl, pH 4.3). 150 µl 2X SDS loading buffer was added to the eluate and samples were analyzed by Western blotting using anti-ubiquitin antibodies to detect Met30$^{\Delta Fbox}$ ubiquitylation profile.

**Purification of recombinant proteins**

Plasmids expressing MBP- Met30$^{(1-186)}$ and MBP- Met30$^{(1-186)M178E/I179E}$ were cloned by standard techniques in pET28 vector and were transformed into Rosetta cells. Cells were grown at 37°C in the presence of 50 µg/ml kanamycin, 25 µg/ml chloramphenicol and 0.2% dextrose. At $OD_{600}$~ 0.5, cultures were shifted to 30°C and induced with 0.5 mM IPTG for 3 hours. Cells were collected, washed once with cold water, pelleted and frozen. Pellets were later suspended in recombinant protein buffer (0.05% Triton-X100, 0.05% NP-40, 150 mM NaCl, 1 mM PMSF, 1 µg/ml each aprotenin, leupeptin and pepstatin), sonicated and cleared by centrifugation at 13,000 rpm at 4°C for 10 minutes. Lysates were bound to prewashed amylose beads (New England Biolabs) for 3 hours at 4°C. Beads were then washed twice with lysis buffer and thrice with buffer U (50 mM Tris pH 8, 50 mM NaCl, 5 mM ATP, 10 mM $MgCl_2$, 0.2 mM DTT). MBP-tagged proteins were eluted in buffer U supplemented with 10 mM maltose. Purified protein was concentrated using Amicon Ultra 50 kDa centrifugal filter and flash frozen.

For purification of Cdc53/$^{GST}$Rbx1, a polycistronic vector expressing both the proteins (Scott et al., 2010) was transformed into Rosetta cells. Cells were grown at 37°C to $OD_{600}$~ 0.5, shifted to 25°C for 2 hours and then to 16°C. Cultures were induced with 0.2 mM IPTG overnight at 16°C. Cells were collected, washed once with cold water, pelleted and frozen. Pellets were later suspended in recombinant protein buffer, sonicated and cleared by centrifugation at 13,000 rpm at 4°C for 10 minutes. Lysates were bound to prewashed glutathione sepharose beads (GE Life Sciences) for 4 hours at 4°C. Beads were then washed twice with lysis buffer and thrice with buffer U. Cdc53/$^{GST}$Rbx1 was then eluted in buffer U supplemented with 50 mM reduced glutathione pH= 7 and used right away for in vitro ubiquitylation experiments.

**In vitro binding assay**

For the experiment performed with yeast lysates, Cdc53-TAP tagged yeast strain with *GAL1* inducible Skp1 was grown in YEP galactose overnight, washed and then transferred to YEP dextrose media for 12 hours to deplete Skp1 protein levels. MBP, (MBP)-Met30$^{(1-186)}$ and (MBP)-Met30$^{(1-186)M178E/I179E}$ were lysed and bound to amylose resin as described above. Yeast cells pellets were also lysed in recombinant protein buffer and 3 mg of lysate was incubated with MBP tagged proteins bound to amylose resin, for 2 hours at 4°C. Beads were washed thrice with recombinant protein buffer and bound proteins were eluted by boiling beads in 2x SDS loading buffer.

For the experiment performed with Cdc53/Rbx1 expressed from bacteria, bacterial cell pellets were lysed in recombinant protein buffer, sonicated and 3 mg of lysate was incubated with MBP tagged proteins conjugated to amylose resin, for 2 hours at 4°C. Beads were washed thrice with

recombinant protein buffer and bound proteins were eluted by boiling beads in 2x SDS loading buffer.

**In vitro ubiquitylation assay**

In vitro ubiquitylation reaction consisted of 250 nM E1 (Boston Biochem, #E-304), 0.8 μM E2 (Boston Biochem, # E-610), 112 μM ubiquitin and 5 mM ATP. Purified MBP-tagged substrates were mixed with purified Cdc53/Rbx1 ligase and the in vitro ubiquitylation mix and incubated at 30°C for the indicated time. Reaction was terminated by adding 2X SDS loading buffer.

**RNA analysis**

RNA was isolated with the RNeasy kit (QIAGEN, Germantown, MD) following the manufacturer's protocol with the following modification. Cell pellets were lysed with glass beads in buffer RLT four times for 40 seconds at setting 4.5 in a FastPrep FP120 with 1-minute breaks between the runs. Cell debris was removed by centrifugation for 2 minutes at 13,000 rpm. For RNA analysis by real-time reverse transcription-polymerase chain reaction (RT PCR), first strand cDNA synthesis was performed with SuperScriptII following the manufacturer's recommendations, with the exception that 1.5 μg of RNA was used in a 10-μl reaction with 0.3 μl of SuperScriptII. 1 μl of the prepared cDNA was used in real-time PCRs which was done with CFX96 Real Time thermal cycler (Bio-Rad, CA) using iQ SybrGreen Supermix (Bio-Rad). Primers for real-time PCR analyses were designed to amplify 100- to 150-bp fragments by using Beacon Designer 2.1 software (Biosoft International, Palo Alto, CA). For each experiment, a standard curve was generated using fivefold dilutions of cDNA. The first dilution in the series was set arbitrarily to copy number 1,000,000. Only when PCR products were falling within the

range of the standard curve, the amount of cDNA was calculated relative to the standard curve and normalized to the control (*ACT1*) samples. Samples were run in triplicates in a PCR program with an initial 3-minutes at 95°C step, followed by 40 cycles of 10 seconds at 95°C and 45 seconds at 50°C. After each run a melting curve was run to ensure that no primer dimers or secondary products were formed.

**Table1:** Yeast strains used in this study

| Strain | Relevant Genotype |
|---|---|
| 15Daub | a *bar1Δ ura3Δns, ade1 his2 leu2-3112 trp1-1* |
| PY1694 | a *bar1 YCpLeu-13Myc-Met30* |
| PY1695 | a *bar1 YCpLeu-13Myc-Met30(Δ187-227)* |
| PY1673 | a *bar1 YCpLeu-GAL1-RGS6His-Met30(Δ187-227)* |
| PY1682 | a *bar1 cdc34-3 YCpLeu-GAL1-RGS6His-Met30(Δ187-227)* |
| PY1683 | a *bar1 cdc53-1 YCpLeu-GAL1-RGS6His-Met30(Δ187-227)* |
| PY1715 | a *bar1 Rbx1-13Myc::KAN YCpLeu-13Myc-Met30(Δ187-227)* |
| PY1758 | a *bar1 skp1::KAN YCpTrp-skp1-25 YCpLeu-13Myc-Met30(Δ187-227)* |
| PY1759 | a *bar1 cdc53-1 skp1::KAN YCpTrp-skp1-25 YCpLeu-13Myc-Met30(Δ187-227)* |
| PY1938 | a *bar1 YCpTrp-GAL1-HBTH-Met30(Δ187-227)* |
| PY2196 | a *bar1 skp1::KAN YCpTrp-skp1-25 YCpTrp-GAL1-HBTH-Met30(Δ187-227)* |
| PY1941 | a *bar1 cdc53-1 YCpTrp-GAL1-HBTH-Met30(Δ187-227)* |
| PY1556 | a *bar1 CUP1-td-3HA-skp1::URA3* |
| PY1705 | a *bar1 CUP1-td-3HA-skp1::URA3 YCpLeu-13Myc-Met30(Δ187-227)* |
| PY1742 | a *bar1 CUP1-td-3HA-skp1::URA3 YCpLeu-GAL1-13Myc-Ctf13* |
| PY1798 | a *bar1 CUP1-td-3HA-skp1::URA3 YCpLeu-GAL1-3Myc-Cdc4* |
| PY2146 | a *bar1 met30::HYG met32::KAN YCpLeu-13Myc-Met30(Δ137-277)* |
| PY2144 | a *bar1 met30::HYG met32::KAN YCpLeu-13Myc-Met30(Δ187-277)* |
| PY2157 | a *bar1 met30::HYG met32::KAN YCpLeu-13Myc-Met30(Δ137-227)* |
| PY2210 | a *bar1 met30::HYG met32::KAN YCpLeu-13Myc-Met30(Δ187-227)$^{M178E/I179E}$* |
| PY2164 | a *bar1 met30::HYG met32::KAN YCpLeu-13Myc-Met30(Δ167-227)* |
| PY2163 | a *bar1 met30::HYG met32::KAN YCpLeu-13Myc-Met30(Δ137-157)(Δ187-227)* |
| PY2165 | a *bar1 met30::HYG met32::KAN YCpLeu-13Myc-Met30(Δ150-170)(Δ187-227)* |
| PY2209 | a *bar1 met30::HYG met32::KAN YCpLeu-13Myc-Met30$^{M178E/I179E}$* |
| PY2014 | a *bar1 YCpLeu-GAL1-RGS6His-Met30$^{L187D}$* |
| PY1933 | a *bar1 san1::KAN YCpLeu-GAL1-RGS6His-Met30(Δ187-227)* |
| PY2204 | a *bar1 met4::KAN YCpLeu-13Myc-Met30(Δ187-227)* |
| PY2234 | a *bar1 cdc53-1 YCpLeu-13Myc-Met30(Δ187-227) YCp-URA-GAL1* |
| PY2247 | a *bar1 cdc53-1 YCpLeu-13Myc-Met30(Δ187-227) YCp-URA-GAL1-Cdc53* |
| PY2249 | a *bar1 cdc53-1 YCpLeu-13Myc-Met30(Δ187-227) YCp-URA-GAL1-Cdc53$^{Y133R}$* |
| PY1990 | a *bar1 skp1::KAN pep4::URA YIpG2-Skp1 Cdc53-TAP::KAN* |
| PY2240 | a *bar1 met30::HYG YCpLeu-13Myc-Met30* |
| PY2241 | a *bar1 met30::HYG YCpLeu-13Myc-Met30$^{M178E/I179E}$* |
| PY1699 | a *bar1 cdc48-3 YCpLeu-13Myc-Met30(Δ187-227)* |
| PY1697 | a *bar1 npl4-1 YCpLeu-13Myc-Met30(Δ187-227)* |
| PY2051 | a *bar1 ufd1-2 YCpLeu-13Myc-Met30(Δ187-227)* |
| PY1939 | a *bar1 cdc48-3 YCpTrp-GAL1-HBTH-Met30(Δ187-227)* |
| PY1942 | a *bar1 npl4-1 YCpTrp-GAL1-HBTH-Met30(Δ187-227)* |
| PY2132 | a *bar1 cic1::kan cic1-2 (pSJ23) YCpTrp-ADH1-HBTH-Met30(Δ187-227)* |

PY1760 a *bar1 Grr1-13Myc::KAN*
PY2127 a *bar1 Grr1-13Myc::KAN cdc48-3*
PY1656 a *bar1 Cdc4-3HA::KAN*
PY2126 a *bar1 Cdc4-3HA::KAN cdc48-3*
PY1643 a *bar1 Cdc48-RGS6H::HYG pep4::URA*
PY2162 a *bar1 Cdc48-RGS6H::HYG pep4::URA YCpLeu-3HA-Cic1*
PY2167 a *bar1 Cdc48-RGS6H::HYG pep4::URA ufd1-2 YCpLeu-3HA-Cic1*
PY2166 a *bar1 Cdc48-RGS6H::HYG pep4::URA npl4-1 YCpLeu-3HA-Cic1*

# CHAPTER 3

## Skp1 independent function of Cdc53 in maintenance of Met30 homeostasis


**ABSTRACT**

The F-box protein component of SCF ubiquitin ligases plays a pivotal role in providing specificity of substrate recognition to ubiquitin dependent degradation. Tightly controlled F-box protein abundance is critical for proper cellular function and alterations in levels of these proteins has been associated with numerous diseases, demonstrating a need for detailed understanding of their regulation. An autocatalytic pathway has been described for F-box proteins, by which the core ligase catalyzes ubiquitylation of the bound F-box protein and triggers its degradation. Here we describe an additional pathway for regulation of F-box protein Met30, that signals Met30 that is dissociated from Skp1 for degradation. We show that this pathway requires Cdc53 but is independent of Skp1 and plays an important role in limiting abundance of Met30. This pathway thereby prevents excess Met30 from sequestering its substrate, Met4, which likely would shield the substrate from recognition by fully assembled $SCF^{Met30}$ ligase. More importantly, our results suggest existence of a novel subtype of cullin-RING ligase in *Saccharomyces cerevesiae* that comprises Cdc53/Rbx1/Cdc34, and possibly includes a yet unidentified adaptor instead of Skp1. We believe that this pathway may be functional for other F-box proteins as well, as two other F-box proteins were also found to be unstable on Skp1 depletion.

## INTRODUCTION

Ubiquitin dependent proteolysis controls many cellular processes like proliferation, signal transduction and cell cycle progression. The transfer of ubiquitin to substrate is a multistep process and requires coordinated action of 3 classes of enzymes- E1 ubiquitin activating enzyme, E2 ubiquitin conjugating enzyme and E3 ubiquitin ligase (Deshaies and Joazeiro, 2009; Hershko and Ciechanover, 1998). Amongst these, the E3 ubiquitin ligases are the key players of this system as they mediate substrate specific covalent attachment of ubiquitin. Within the E3 ligase family, cullin-RING ligases (CRLs) comprise the largest class and in this group, the SCF ubiquitin ligases are one of the best-understood complexes (Duda et al., 2011). They are composed of Cdc53 (cullin-1), Skp1, Rbx1, ubiquitin conjugating enzyme Cdc34 and one of the multiple F-box proteins, which bind substrates and confer specificity to the complex (Komander, 2009; Zimmerman et al., 2010)

Amongst the SCF components, F-box proteins are relatively unstable in nature, which is believed to be important in maintaining a critical level of unoccupied Cdc53/Skp1 complexes available for association with the numerous F-box proteins within the cell. As these proteins function as substrate adaptors, it is also important to restrict abundance of unbound F-box proteins to prevent substrate shielding effects that would compete with substrate recognition by fully assembled ligases. The SCF core subunits occur in limiting concentration within the cell, and over expression of a single F-box protein in yeast can sequester most of the cullin and Skp1 molecules and thus block formation of functional SCF complexes with other F-box proteins (Galan and Peter, 1999; Mathias et al., 1999; Zhou and Howley, 1998). Many F-box proteins control degradation of critical oncogenes and tumor suppressors and variation in their abundance has been strongly linked to cancer (Frescas and Pagano, 2008; Welcker and Clurman, 2008).

Thus, it is important to understand how F-box protein homeostasis is maintained in nature. F-box proteins are known to be regulated by autoubiquitylation where their degradation is dependent upon their association within a functional SCF complex (Galan and Peter, 1999; Zhou and Howley, 1998).

In *Saccharomyces cerevisiae*, Met30 is one of the best-studied F-box proteins and it functions as a substrate-recruiting factor in the ubiquitin ligase complex- $SCF^{Met30}$. $SCF^{Met30}$ coordinates the metabolic pathway of sulfur containing amino acids with cell cycle progression. It ubiquitylates transcription factor Met4 which renders it inactive. Low levels of the key methyl donor, S-adenosylmethionine cause a block in $SCF^{Met30}$ dependent ubiquitylation of Met4, activating it and results in transcription of methionine response genes (Kaiser et al., 2006). $SCF^{Met30}$ also represses expression of enzymes responsible for glutathione synthesis and is thus a key factor in response to heavy metal stress. Cadmium exposure inhibits $SCF^{Met30}$ thereby inducing glutathione production and cell cycle arrest, which together protect cellular integrity. Depletion of methionine blocks $SCF^{Met30}$ by preventing binding of Met30 to Met4 while cadmium stress results in dissociation of Met30 from Skp1 (Rouillon et al., 2000; Yen et al., 2012; Yen et al., 2005) (Fig. 3-1).

Met30 itself is an unstable protein and is targeted for degradation by autoubiquitylation (Galan and Peter, 1999). Our studies have elucidated a novel mechanism for Met30 regulation, additional to autoubiquitylation. This degradation pathway targets Met30 that is detached from Skp1 and involves the SCF core components- Cdc53, Cdc34 and Rbx1. Importantly, this pathway is independent of Skp1 and suggests existence of a new subtype of cullin-RING ligase that comprises Cdc53/Rbx1/Cdc34, without Skp1, but possibly interacts with other components to regulate F-box protein abundance.

## RESULTS

### *Dissociation of the F-box protein Met30 from Skp1 targets it for degradation*

F-box protein degradation is thought to be primarily controlled through an autocatalytic mechanism (Galan and Peter, 1999; Zhou and Howley, 1998). Surprisingly, a mutant form of Met30 lacking its F-box domain (residues 187-277), and thus cannot associate with the SCF core complex (Brunson et al., 2004; Yen et al., 2012) was constitutively unstable (Fig. 3-2A). Rapid degradation of $Met30^{\Delta Fbox}$ was observed both in cycloheximide chase and promoter shut-off experiments, indicating that degradation was not an indirect consequence of inhibition of general translation (Fig. 3-2A&B). Disruption of the Met30-Skp1 interaction by the single amino acid change L187D (Brunson et al., 2004), instead of complete deletion of the F-box domain also induced Met30 degradation (Fig. 3-2C) suggesting that Met30 that is not bound to Skp1 (referred to as unbound or 'Skp1-free' Met30) is degraded.

To further test this idea and exclude that mutating the F-box region results in conformational changes that target Met30 to a non-physiological degradation pathway, we assayed Met30 stability in other conditions where Met30 is dissociated from Skp1. First, inactivation of Skp1 using a temperature sensitive mutant which blocks essential SCF ligases (Aghajan et al., 2010) was sufficient to induce Met30 degradation (Fig. 3-3A adapted from (Yen, 2008)). Second, addition of the heavy metal cadmium results in selective dissociation of Met30 from the SCF core complex (Barbey et al., 2005; Kaiser et al., 2006; Yen et al., 2012; Yen et al., 2005). Consistent with the notion that Met30 not bound to Skp1 is degraded, cadmium stress resulted in rapid Met30 degradation (Fig. 3-3B adapted from (Yen, 2008)). Cadmium exposure did not further destabilize Met30 when Skp1 was inactivated using a temperature sensitive allele

(data not shown). Collectively these results demonstrate that disruption of the Met30-Skp1 interaction by mutations in Met30 or Skp1, or by signal induced dissociation results in rapid degradation of the F-box subunit Met30. These results were surprising because degradation of Met30, like degradation of other F-box proteins, is thought to follow an autocatalytic pathway (Galan and Peter, 1999; Zhou and Howley, 1998). Interestingly, Met30 degradation in response to methionine depletion was suggested to be Skp1 independent (Smothers et al., 2000).

***Degradation of 'Skp1-free' Met30 requires Cdc53, Cdc34, and Rbx1 but not Skp1***

We sought to further characterize degradation of unbound or 'Skp1-free' Met30 and asked whether it was dependent upon the ubiquitin-proteasome system. In wild-type cells Met30 may exist both unbound or bound to the SCF core. In order to specifically study the degradation pathway targeting 'Skp1-free' Met30, we used $Met30^{\Delta Fbox}$ as a tool to generate a homogenous population of unbound Met30. Inhibition of proteasomes with MG-132 led to stabilization of $Met30^{\Delta Fbox}$, suggesting that 'Skp1-free' Met30 degradation depended on proteasome function (Fig. 3-4). We then asked whether any of the SCF core components or its cognate E2, Cdc34, are required for degradation of unbound Met30. Since all SCF core components are essential for cell viability, we utilized their temperature sensitive mutant alleles. $Met30^{\Delta Fbox}$ was rapidly degraded in wild type cells but substantially stabilized in *cdc34-3* and *cdc53-1* mutants (Fig. 3-5 adapted from (Yen, 2008)). In addition, $Met30^{\Delta Fbox}$ degradation was greatly reduced in a strain expressing a 13Myc-tagged version of the RING finger component Rbx1, which has previously been shown to be a hypomorph allele that compromises SCF function at high temperature (Seol et al., 1999) (Fig. 3-5 adapted from (Yen, 2008)). Results using temperature sensitive *skp1-25* allele suggested that degradation of 'Skp1-free' Met30 is independent of Skp1 (Fig. 3-2A).

Consequently, these experiments not only indicate a degradation pathway for Met30 that is independent of the canonical autoubiquitylation mechanism, but more surprisingly, suggested that the cullin-1 (Cdc53) based ligase complex might have functions independent of its adaptor component Skp1. To further probe this hypothesis, we tested $Met30^{\Delta Fbox}$ half-life in *skp1* single and *skp1 cdc53* double mutants. Unbound Met30 was rapidly degraded in *skp1-25* mutants but stabilized upon inactivation of Cdc53 (Fig. 3-6A adapted from (Yen, 2008)) demonstrating that degradation was dependent on Cdc53 but not Skp1. In agreement with the $Met30^{\Delta Fbox}$ degradation data, ubiquitylated $Met30^{\Delta Fbox}$ was readily detectable in *skp1* mutants but absent in *cdc53* mutants further supporting the hypothesis of an Skp1- independent degradation function for Cdc53 (Fig. 3-6B). Transcription factor Met4 has been shown to function as a substrate receptor in the context of $SCF^{Met30/Met4}$ to coordinate degradation of its co-factors (Ouni et al., 2010). Analysis of $Met30^{\Delta Fbox}$ degradation in *met4Δ* mutant showed that it was not involved in degradation of 'Skp1-free' Met30 (Fig. 3-7). The results from these experiments suggest that this novel mechanism of Met30 regulation targeting specifically Met30 that is displaced from Skp1 is dependent on the ubiquitin proteasome system and requires the function of Cdc53, Rbx1 and Cdc34 but not Skp1.

### *$Met30^{\Delta Fbox}$ degradation is independent of Skp1*

Temperature sensitive *skp1* alleles have been previously shown to differentially affect SCF ligases depending on the identity of F-box subunit (Bai et al., 1996; Galan and Peter, 1999). Thus a possibility existed that the *skp1-25* allele, although inactive in the context of $SCF^{Cdc4}$ and $SCF^{Met30}$ and likely defective in binding to most F-box proteins may form an intact SCF ligase that could ubiquitylate $Met30^{\Delta Fbox}$ in trans. To address this issue we employed the temperature inducible degron tag (*td*) strategy to deplete Skp1 from cells (Dohmen et al., 1994), rather than

rely on inactivation of temperature sensitive alleles. An *skp1-td* strain was constructed by expressing Skp1 fused to the temperature inducible degron under control of the inducible *CUP1* promoter. Combined *CUP1* promoter repression and temperature induced Skp1-td degradation efficiently ablated Skp1 protein level (Fig. 3-8A&B adapted from (Yen, 2008)). Attenuation of Skp1 induced the expected biological response such as cell cycle arrest with elongated multi-budded cell morphology indicative of $SCF^{Cdc4}$ inactivation (Schwob et al., 1994), and block of $SCF^{Met30}$ function as assayed by loss of ubiquitylated forms of Met4 (Fig. 3-8B adapted from (Yen, 2008)). Consistent with results using temperature sensitive alleles of *skp1*, $Met30^{\Delta Fbox}$ degradation was unaffected when Skp1 function was blocked using the *skp1-td* strategy (Fig. 3-8C adapted from (Yen, 2008)). These results strongly supported our hypothesis of Skp1-independent degradation of 'free' Met30.

***Cdc53 mutant unable to bind Skp1 can degrade Met30***

The experiments with *skp1* single and *skp1 cdc53* double mutants strongly suggested that the 'Skp1-free' degradation pathway for Met30 was dependent on Cdc53 and independent of Skp1. We reasoned that in such a scenario, a Cdc53 mutant defective in its ability to interact with Skp1 should be capable to degrade $Met30^{\Delta Fbox}$. To examine this, a *GAL1* inducible $Cdc53^{Y133R}$ mutant was constructed. Mutation of tyrosine in position 133 to arginine has been previously shown to disrupt Cdc53-Skp1 interaction (Deshaies, 1999). Accordingly, expression of $Cdc53^{Y133R}$ was unable to rescue the *cdc53-1* growth arrest phenotype at restrictive temperature (Fig. 3-9A). Congruous with our hypothesis, $Cdc53^{Y133R}$ supported degradation of $Met30^{\Delta Fbox}$ to the same extent as wild type Cdc53 (Fig. 3-9B). This result strongly suggests existence of a

novel cullin-1 (Cdc53) based ligase complex independent of its adaptor component Skp1 functioning in regulating levels of Met30.

***Hydrophobic region proximal to the F-box domain is important for degradation of 'Skp1-free' Met30***

Instability of Met30 in *skp1-25* temperature sensitive mutant led us to hypothesize that the ubiquitin ligase responsible for degrading 'Skp1-free' Met30 may bind close to the F-box domain. In accordance with this idea, a so-called R-motif has been described in Cdc4, which is adjacent to its F-box domain. Also F-box - Skp1 interaction acts to suppress R-motif mediated Cdc4 degradation (Mathias et al., 1999). Thus, to identify the degradation sequences (degron) in Met30 recognized by the 'Skp1-free' degradation pathway, we generated deletions in $Met30^{\Delta Fbox}$ near the F-box domain and compared their stability to that of $Met30^{\Delta Fbox}$. Deletion of 100 amino acids proximal and distal to the F-box domain (residues 137-277) completely stabilized $Met30^{\Delta Fbox}$ (Fig. 3-10A). To further narrow down the degron, shorter deletions encompassing either the C terminal or the N terminal 50 amino acids were constructed. Deletion of the N terminal amino acids adjacent to the F-box motif (residues 137-187) stabilized $Met30^{\Delta Fbox}$ (Fig. 3-10A). Smaller deletions within the N terminal 50 amino acids contiguous to the F-box domain suggested that 170-187 amino acids of Met30 (Fig. 3-10B), corresponding to a region rich in hydrophobic residues were important for ligase binding.

***Mutation of methionine 178 and isoleucine 179 of Met30 can abolish 'Skp1-free' Met30 degradation***

A 45 amino acid stretch immediately N-terminal to the F-box domain has been defined for WD-40 repeat containing F-box proteins, which mediates dimerization and is conserved from yeast to humans (Tang et al., 2007). Overlap of the degron region and the dimerization domain raised the possibility that the ligase for 'Skp1-free' Met30 degradation recognized the dimerization motif in Met30. Mutation of isoleucine 159 and leucine 160- amino acids crucial for Met30 dimerization (Tang et al., 2007), in $Met30^{\Delta Fbox}$ failed to stabilize the protein (Fig. 3-11A), suggesting that different residues within this stretch were being recognized by the ligase. $Met30^{\Delta Fbox}$ deletion analysis identified 170-187 amino acids of Met30 to be important for the 'Skp1-free' degradation pathway. To identify key residues in the degron, we generated mutations in methionine 178 and isoleucine 179, a hydrophobic patch close to the F-box domain and conserved amongst WD-40 repeat containing F-box proteins. The $Met30^{\Delta Fbox(M178E/I179E)}$ mutant was able to effectively block 'Skp1-free' Met30 degradation and stabilized $Met30^{\Delta Fbox}$ (Fig. 3-11B). Same mutations were also able to strongly arrest degradation of full length Met30 (Fig. 3-11C) suggesting that majority of Met30 in the cell was being targeted for proteolysis via the 'Skp1-free' degradation pathway.

***Lag2 is not required for 'Skp1-free' Met30 degradation***

Cullins are regulated by covalent modification with ubiquitin like protein Nedd8 (Rub1 in yeast) which induces a conformational rearrangement of cullin and stimulates ubiquitin transfer by the SCF-bound E2 (Duda et al., 2011). Deneddylated cullins interact with Cand1 (Lag2 in yeast), which inhibits binding of Skp1- F-box protein complex and prevents SCF ligase function

(Siergiejuk et al., 2009; Zemla et al., 2013). As rubylation is dispensable in yeast (Lammer et al., 1998), we posit that probably Cdc53 might bind Lag2 and that Lag2 may serve as a adaptor for regulating F-box proteins dissociated from Skp1. Deletion of *LAG2* failed to stabilize $Met30^{\Delta Fbox}$ (Fig. 3-12), suggesting that Lag2 did not function as an adaptor for the Cdc53 dependent ligase responsible for 'Skp1-free' Met30 degradation.

***Cdc53/Rbx1 can bind and ubiquitylate Met30 in absence of Skp1***

To determine the composition of the ligase and identify unknown adaptor components we performed mass spectrometry with purified $Met30^{\Delta Fbox}$ to analyze its binding partners. In addition, we used the same strategy to profile Cdc53 interacting proteins, with the hope to identify adaptor proteins that function as Skp1 alternatives by searching for commonalities between these two mass spectrometry datasets. We failed to identify any proteins that fit these criteria. Although negative result can never be conclusive, this result suggested to us that perhaps Cdc53/Rbx1 without any adaptor might bind to Met30 and be sufficient to ubiquitylate 'Skp1-free' Met30. To examine binding in absence of Skp1, we tagged Cdc53 in a yeast strain with a *GAL1* inducible Skp1 allele such that Skp1 depletion could be achieved by growing the strain in media containing dextrose (Fig 3-13A). Also, maltose-binding protein (MBP) tagged Met30 constructs containing the N terminal region of Met30, proximal to the F-box domain were designed, which could be expressed in bacteria and used as substrate. *In vitro*-binding assay was performed with immobilized (MBP)-$Met30^{(1-186)}$ and Skp1 depleted yeast lysates. In the absence of Skp1, Cdc53 bound effectively to (MBP)-$Met30^{(1-186)}$ and binding was significantly reduced when (MBP)-$Met30^{(1-186)M178E/I179E}$ was used as the bait (Fig. 3-13B). Therefore, the same mutations, M178E and I179E that prevent degradation of Met30 by the 'Skp1-free' pathway *in*

*vivo* also block Skp1-independent binding of Cdc53 to Met30 *in vitro*. Because this binding assay contained total yeast lysates, albeit lacking Skp1, it was possible that an unknown yeast protein mediated the interaction between (MBP)-Met30$^{(1-186)}$ and Cdc53. To rule out the involvement of any other factor apart from Cdc53/Rbx1 in binding, we utilized $^{6XHis}$Cdc53/$^{GST}$Rbx1 expressed in bacteria (Scott et al., 2010) for *in vitro* binding experiment. Immobilized (MBP)-Met30$^{(1-186)}$ was incubated with Cdc53/Rbx1 bacterial lysate and binding was analyzed by immunoblotting with anti-Cdc53 antibody. Similar to the result with yeast lysate, Cdc53/Rbx1 could specifically interact with (MBP)-Met30$^{(1-186)}$ and binding was decreased in the degron mutant (Fig. 3-14). Cdc53 expressed in *E.coli* spontaneously cleaves at the N-terminal and produces a truncated version (residues 267-851) lacking the region required for Skp1 binding (Scott et al., 2010). This demonstrates that Met30 can directly bind Cdc53/Rbx1 at a site different from Skp1.

As binding studies strongly suggested that Cdc53/Rbx1 could directly interact with Met30 without an adaptor protein, we tested whether Cdc53/Rbx1 could ubiquitylate 'Skp1-free' Met30 and was sufficient to function as a ligase. (MBP)-Met30$^{(1-186)}$ was immobilized to amylose resin and incubated with bacterial lysate expressing Cdc53/Rbx1. The substrate-ligase complex was eluted and the ubiquitylation reaction was initiated by addition of the reaction mix containing E1 enzyme, E2 conjugating enzyme, ubiquitin and ATP. (MBP)-Met30$^{(1-186)}$ was ubiquitylated *in vitro* and the reaction was dependent on the ubiquitin activating enzyme E1 (Fig. 3-15). The MBP tag shows some ubiquitylation in the presence of E1 activating enzyme but its ubiquitylation profile is different than that of (MBP)-Met30$^{(1-186)}$. To test whether this ubiquitylation seen was specific for the 'Skp1-free' degradation pathway of Met30, we compared ubiquitylation in (MBP)-Met30$^{(1-186)}$ and (MBP)-Met30$^{(1-186)M178E/I179E}$ mutant. Cdc53/Rbx1 were

purified from *E.coli* and eluted from glutathione sepharose beads. Only a relatively small fraction of Cdc53/Rbx1 could be purified (Fig. 3-16A). Purified (MBP)-Met30$^{(1\text{-}186)}$ or (MBP)-Met30$^{(1\text{-}186)M178E/I179E}$ and the ubiquitylation reaction mix were combined with Cdc53/Rbx1 and substrate ubiquitylation was analyzed (Fig. 3-16B). (MBP)-Met30$^{(1\text{-}186)}$ was ubiquitylated while ubiquitylation of the degron mutant was substantially reduced. Although these experiments demonstrate ubiquitylation of Met30 by a minimal cullin-1 (Cdc53) complex lacking Skp1 the observed activity is not very robust and requires a long reaction time (~ 16 hours) and yet only a relatively small fraction of the substrate is ubiquitylated. This suggests possible involvement of another factor or a post-translational modification, which is likely not required for binding Cdc53/Rbx1 to Met30 but is required for effective ubiquitylation of the substrate. Alternatively, Cdc53 expressed in *E. coli* may not efficiently fold into its active structure and thus be only partially active.

***Degradation of F-box proteins Ctf13 & Cdc4 is also independent of Skp1***

Our results strongly supported the hypothesis of Skp1-independent degradation of 'free' Met30. We next asked whether this Skp1-independent degradation pathway was unique for Met30 or may be a more general mechanism to limit F-box protein abundance. Such a mechanism for F-box protein homeostasis could be important not only to limit the overall abundance, but also to limit competition for substrates between assembled SCF ligases and the 'free' F-box subunits, which could lead to substrate shielding and thus prevent substrate ubiquitylation. Although degradation of the F-box protein Cdc4 is thought to follow the autoubiquitylation pathway (Galan and Peter, 1999; Zhou and Howley, 1998) evidence has also been reported that Cdc4 is degraded in *skp1-3* temperature sensitive mutant and shows a general

stabilization when Skp1 is overexpressed (Mathias et al., 1999). In addition, the kinetochore component and F-box protein Ctf13 was shown to be rapidly degraded in a Cdc34 dependent mechanism when Skp1 was inactivated (Kaplan et al., 1997). These examples are consistent with 'Skp1-free' Cdc4 and Ctf13 degradation pathways as we suggest here for Met30. To further test this idea, we measured degradation of the F-box proteins Ctf13 and Cdc4 in *skp1-td* mutant (Fig. 3-17A&B). Consistent with a previous report (Kaplan et al., 1997) Ctf13 degradation was accelerated in *skp1-td* mutant (Fig. 3-17A). Cdc4 is mainly regulated via autoubiquitylation. In *skp1-td* mutant, Cdc4 was slightly more stable compared to wild type cells, but still degraded rapidly even though Skp1 function was blocked as evident by deubiquitylated Met4 (Fig. 3-17B) and elongated, multibudded cells (data not shown) indicating inactivation of $SCF^{Cdc4}$. It has also been reported that deletion of F-box region in Cdc4 significantly stabilizes the protein (Zhou and Howley, 1998), which contradicts our result. However, in this mutant the region corresponding to the degron region in Met30 is deleted along with the F-box domain (Johnson, 1991) explaining the stabilization seen in this Cdc4 mutant. We constructed a $Cdc4^{\Delta Fbox}$ mutant lacking just the F-box domain (residues 278-319). Similar to $Met30^{\Delta Fbox}$, $Cdc4^{\Delta Fbox}$ was rapidly degraded and inactivation of Cdc53 resulted in stabilization of $Cdc4^{\Delta Fbox}$ (data not shown).

If the autocatalytic pathway was the sole mode of degradation responsible for maintaining F-box protein homeostasis then reduction in Skp1 levels should induce complete stabilization of these proteins. Thus, these results provide evidence for existence of an additional, Skp1-independent, degradation pathway for several F-box proteins, which appear to target F-box proteins that are not bound to the SCF core complex. We denoted this degradation pathway as **'Skp1-Free' F-box protein degradation pathway** to demarcate it from the autoubiquitylation mode of F-box protein regulation.

We also tested whether Grr1- another well-characterized F-box protein in yeast, was regulated via this mechanism. Differing from $Met30^{\Delta Fbox}$, deletion of the F-box region in Grr1 completely stabilized the protein (Fig. 3-18A). Grr1 was also stabilized upon Skp1 inactivation in *skp1-25* temperature sensitive mutant (Fig. 3-18B) suggesting that this pathway is not universal for all F-box proteins and is probably functional for only those F-box proteins which harbor the hydrophobic region paramount for ligase binding.

***'Skp1-free' F-box degradation is important for cellular function***

To explore the significance of the 'Skp1-free' F-box degradation pathway in normal cellular dynamics, we generated yeast strains bearing either wild type Met30 or the $Met30^{M178E/I179E}$ mutant, which abrogates binding of the Cdc53/Rbx1 ligase and is deficient in the pathway. As expected, Met30 displayed increased abundance in the cells expressing the point mutant in comparison to those expressing the wild type allele (Fig. 3-19). $SCF^{Met30}$ ubiquitylates transcription factor Met4 (Kaiser et al., 2006) and thus activity of the $Met30^{M178E/I179E}$ was assessed by studying Met4 ubiquitylation profile in this mutant. A decrease in the fraction of ubiquitylated species of Met4 was observed in the degron mutant of Met30 indicating a possible defect in function (Fig. 3-19).

Met30 is a low abundant protein in cells (Yen et al., 2012) and a block in one of its regulation pathways might not have very strong effects during optimal growth conditions. However, during heavy metal stress the Met30-Skp1 interaction is disrupted (Barbey et al., 2005; Yen et al., 2012; Yen et al., 2005) generating a burst of 'Skp1-free' Met30. We reasoned that cells rely on 'Skp1-free' F-box protein degradation pathway to counteract the generation of 'free' Met30 during heavy metal stress. We therefore, tested sensitivity of wild type Met30 and

Met30$^{M178E/I179E}$ mutant to cadmium exposure. Cells carrying Met30$^{M178E/I179E}$ were significantly more sensitive and exhibited a growth arrest in response to cadmium stress (Fig. 3-20). This result demonstrates the critical physiological role of 'Skp1-free' F-box degradation pathway for cellular function.

We were curious to determine the mechanism behind this observed growth defect. Our initial hypothesis was that this was a result of sequestration of Skp1 by excess Met30. However, Met30 is a relatively low abundant F-box protein and even the increased steady state levels due to block in one of the mechanisms for its proteolysis, may not be sufficient to sequester Skp1 and negatively affect formation of other SCF complexes. Indeed, Met30$^{M178E/I179E}$ mutant cells do not display elongated, multibudded phenotype (data not shown) typical of SCF$^{Cdc4}$ inactivation, but we cannot exclude effects on other Skp1 dependent complexes like CBF3 or RAVE complex (Connelly and Hieter, 1996; Seol et al., 2001). Interestingly, we observed reduced chromosome stability in Met30$^{M178E/I179E}$ mutant cells suggestive of defect of Skp1 function in kinetochore assembly (data not shown). To test whether Skp1 sequestration might be involved in cadmium hypersensitivity of Met30$^{M178E/I179E}$ mutant cells we overexpressed Skp1 in Met30$^{M178E/I179E}$ mutants to compensate for such a defect. However, increasing Skp1 had no effect on cadmium sensitivity (data not shown).

This result pointed us towards an alternative hypothesis. Likely the biological importance of unbound F-box protein degradation is to limit substrate protection. Excess Met30 may associate with Met4 and prevent its ubiquitylation by fully assembled SCF$^{Met30}$ ligase. This hypothesis is supported by the observation that the fraction of ubiquitylated species of Met4 is decreased in cells expressing the degron mutant of Met30 (Fig. 3-19). In wild type cells, SCF$^{Met30}$ ubiquitylates transcription factor Met4, which inactivates it and results in repression of

downstream target genes. Consistent with the idea of substrate protection, derepression of Met4 target genes was seen in Met30$^{M178E/I179E}$ mutant (Fig. 3-21). The effect on Met4 target repression was dominant as expression of wild type Met30 could not compensate for the effect mediated by Met30$^{M178E/I179E}$ mutant (Fig. 3-22). *MET30* is an essential gene in yeast. Also, the yeast strain expressing the degron mutant was viable and exhibited only a slight growth delay in comparison to that expressing wild type Met30 (Fig. 3-23). These results suggest that SCF$^{Met30}$ ligase activity in the degron mutant is not limiting and access to Met4 may be restricted due to substrate shielding effects.

## DISCUSSION

The architectural theme of SCF ubiquitin ligases employs common core components and multiple F-box domain containing substrate receptors that complete for Cdc53/Rbx1 core. Cycles of cullin neddylation /CAND1 association maintain a critical level of unoccupied core while Skp1- F-box heterodimers bind substrates and recruit them to the Cdc53/Rbx1 complex (Flick and Kaiser, 2013). To ensure that activity of SCF ligases is dictated by the amount and identity of F-box proteins available at any given time in the cell. It is thus necessary to remove competition by 'free' F-box proteins.

Our data provides experimental evidence for existence of a novel pathway for F-box protein regulation additional to autoubiquitylation, that specifically targets F-box proteins that are not bound to Skp1. Using the F-box protein- Met30 as a model, we show that this pathway is constitutively active. Also, *in vivo* data suggests that this mechanism of F-box protein regulation is dependent upon functional Cdc53 but not Skp1. Some groups have reported stabilization of F-box protein Cdc4 in certain temperature sensitive *skp1* alleles (Galan and Peter,

1999; Zhou and Howley, 1998), which is conflicting with our results. However we have tested protein levels in several different *skp1*-temperature sensitive mutants and found that none of them showed reduced Skp1 protein levels at the restrictive temperature, suggesting that these proteins may be structurally intact and not compromised in their ability to bind F-box protein. To circumvent this issue, we utilized *skp1-td* mutant in which Skp1 levels are reduced substantially in comparison to levels at permissive temperature thereby abolishing Skp1 cellular function. 'Skp1-free' Met30 was destabilized in both *skp1-ts* and *skp1-td* mutants suggesting no involvement of Skp1 in this pathway. This was further supported by the observation that Cdc53 mutant incapable of binding to Skp1 can degrade 'Skp1-free' Met30, unequivocally demonstrating presence of an Skp1 independent pathway for F-box protein regulation. We also show that Cdc53/Rbx1 can bind and ubiquitylate Met30 *in vitro*. Cdc53/Rbx1 dependent ubiquitylation was not very robust and required a long reaction time suggesting possible involvement of another factor, inefficient protein folding in *E. coli*, or a post-translational modification lacking in this expression system. Further studies need to be conducted to explore these options in detail.

Our study also provides mechanistic insight for the importance of regulating unbound F-box proteins suggesting that it is crucial in limiting substrate-shielding effects. $Met30^{M178E/I179E}$ is resistant to 'Skp1-free' F-box degradation and cells expressing this mutant show decreased ubiquitylation of substrate Met4 and lose repression of Met4 target genes. This mutant is dominant in function suggesting that the effects observed in Met4 are unlikely due to deficiency in ligase activity. Furthermore, Met30 is an essential gene and cells relying on $Met30^{M178E/I179E}$ showed only slightly reduced proliferation rate. It remains to be checked whether $Met30^{M178E/I179E}$ shows increased binding to Met4 as compared to wild type Met30.

This study, to the best of our knowledge is the first example illustrating uncoupling of Cdc53 (cullin-1) and Skp1 function in yeast. We identify a new type of cullin-1 based ubiquitin ligase that does not rely on Skp1. A Skp1 independent cullin-1 based ubiquitylation event has been suggested previously for humans where Rictor, a component of mTORC2 complex associates with Cullin-1 instead of Skp1, to form a functional E3 ubiquitin ligase and promotes ubiquitylation of SGK1 (Gao et al., 2010). Our findings shed light on the complexity of E3 ligases and suggests that there might exist yet unknown types of ligases playing key roles in diverse biological processes.

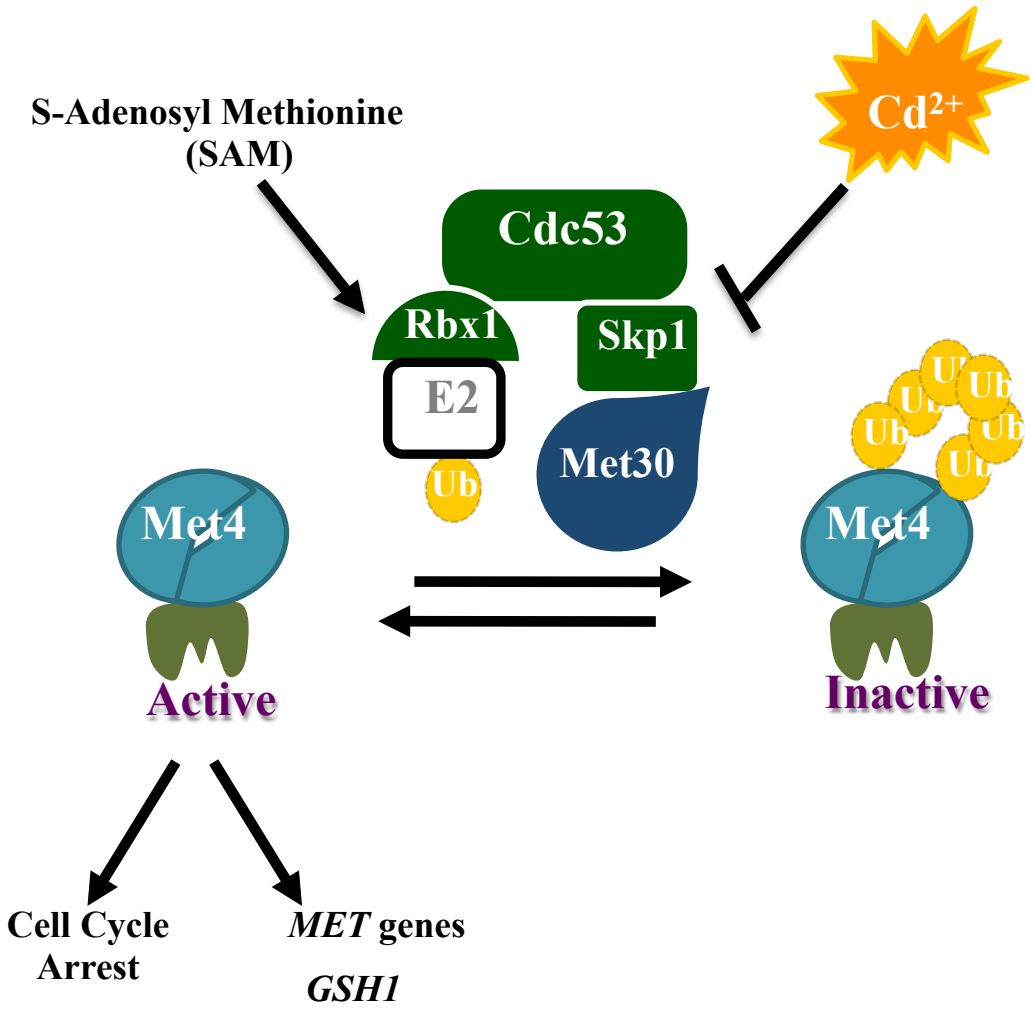


**Figure 3-1. SCF$^{Met30}$ regulates Met4 activity.** Under normal cellular conditions, transcription factor Met4 is ubiquitylated and maintained in an inactive state by SCF$^{Met30}$. Low levels of S-adnosyl methionine or heavy metal stress inactivate SCF$^{Met30}$. Met4 is deubiquitylated, becomes active and induces expression of a subset of genes involved in the sulfur amino acid pathway (*MET* genes) or in the case of heavy metal stress, *GSH1* which leads to glutathione synthesis. Strong activation of Met4 induces cell cycle arrest.

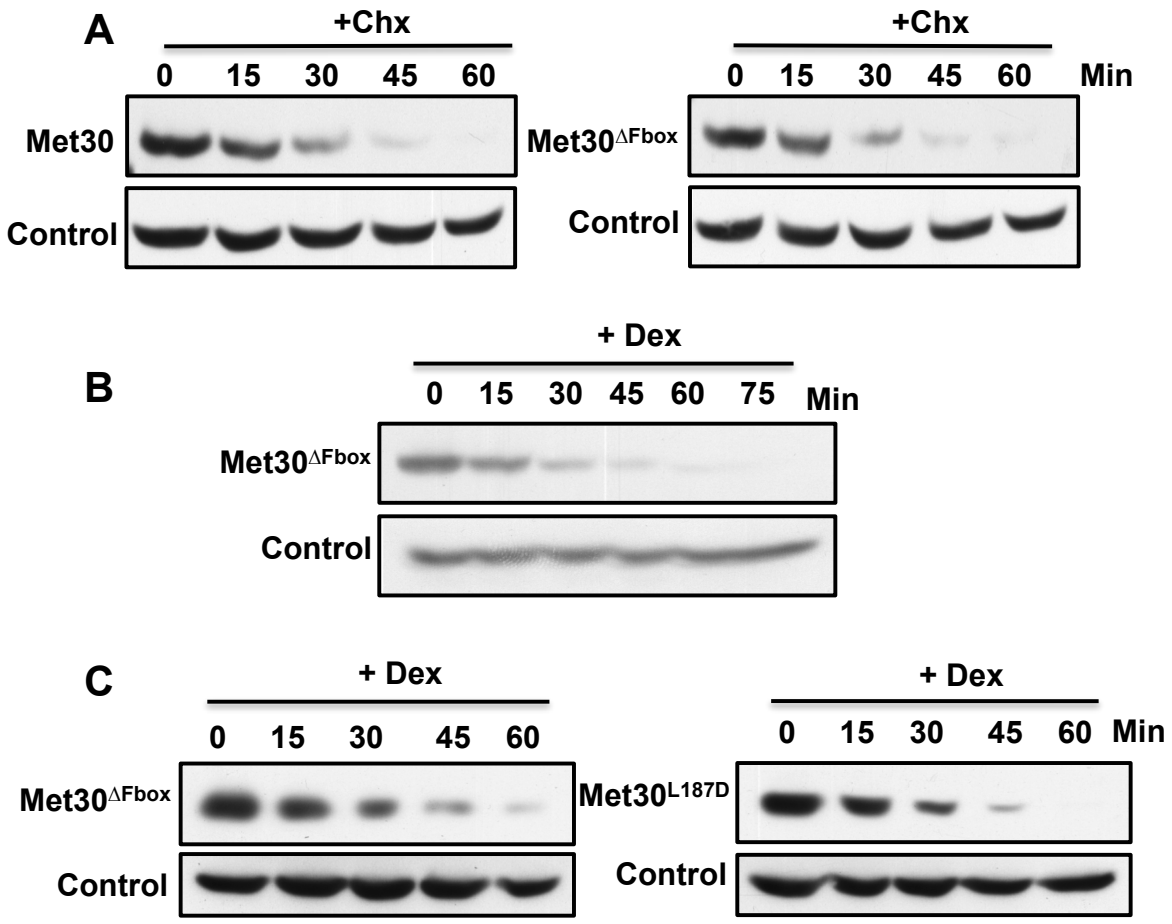


**Figure 3-2. Mutations in Met30 that disrupt Met30-Skp1 binding, destabilize Met30.** (A) Cells expressing either endogenous $^{12Myc}$Met30 or $^{12myc}$Met30$^{\Delta Fbox}$ (residues 187-227 deleted) were grown at 30°C. Protein translation was inhibited by addition of 100µg/ml cycloheximide and samples were collected at the time intervals indicated. Met30 stability was analyzed by immunoblotting with anti-myc antibodies. The proteasome subunit- Cim5 was detected as a loading control. (B) Cells expressing $^{RGS6H}$Met30$^{\Delta Fbox}$ under control of *GAL1* promoter was grown in sucrose medium at permissive temperature at 30°C. Expression of Met30 was induced by addition of 2% galactose for 2.5 h following which 2% dextrose was added to repress *GAL1-MET30* expression. Samples were collected at the time intervals indicated and analyzed by immunoblotting with anti-RGS6H antibodies. (C) Experiment as described for panel B, cells expressing $^{RGS6H}$Met30$^{\Delta Fbox}$ and $^{RGS6H}$Met30$^{L187D}$ under the control of *GAL1* promoter were compared.

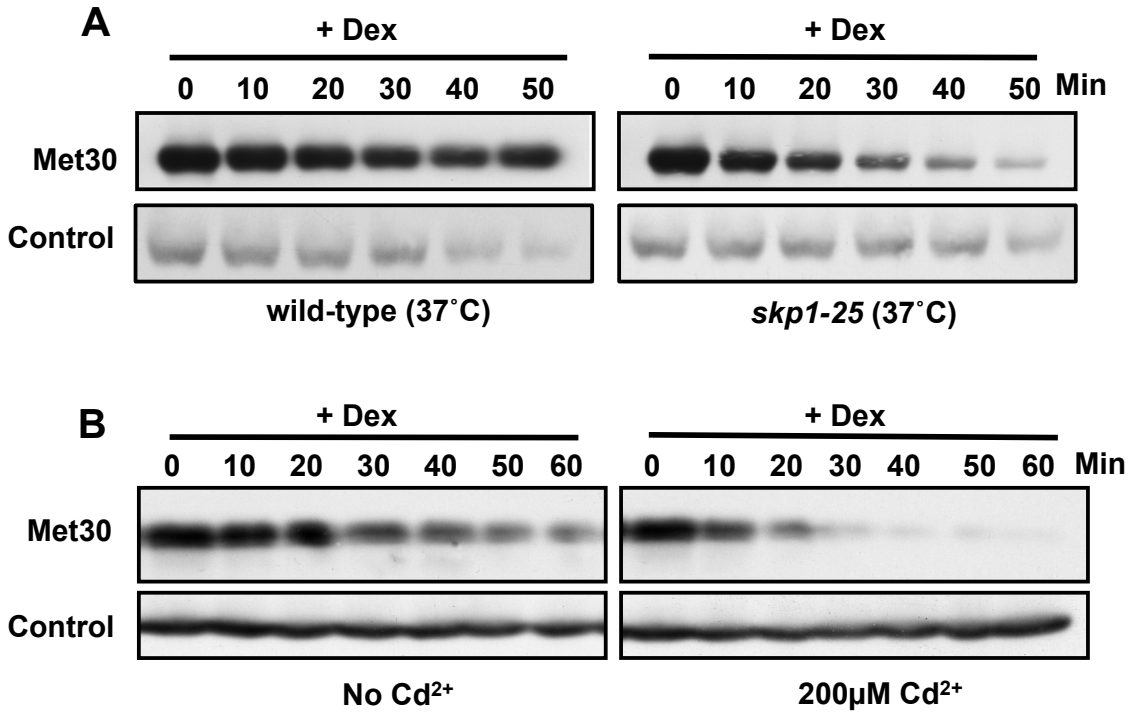


**Figure 3-3. Dissociation of Met30 from Skp1 induces its degradation.** (A) Cells expressing $^{RGS6H}$Met30 under control of *GAL1* promoter in wild type and *skp1-25* mutant, were grown in sucrose medium at permissive temperature (25°C). Expression of Met30 was induced by addition of 2% galactose for 1 h, cells were shifted to 37°C for 1.5 h to inactivate the temperature sensitive allele, and 2% dextrose was added to repress *GAL1-MET30* expression. Samples were collected at the time intervals indicated and analyzed by immunoblotting with anti-RGS6H antibodies. (B) Experiment as described for panel A, but at 30°C, and cadmium was added to a final concentration of 200μM at the time of *GAL1* promoter repression. The proteasome subunit, Cim5 was detected as a loading control.

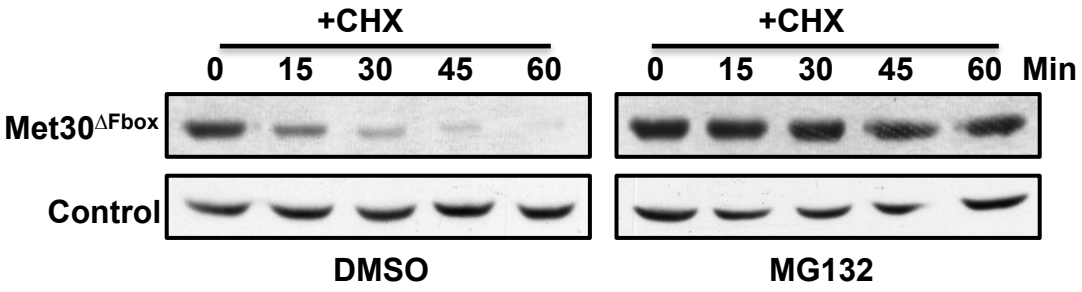


**Figure 3-4. Degradation of 'Skp1-Free' Met30 is dependent on ubiquitin proteasome system.** Cells expressing $^{12myc}Met30^{\Delta Fbox}$ were grown at 30°C. Proteasomes were inhibited with 50μM MG-132 for 45 min before cycloheximide was added to block translation. Cells carried a deletion of *PDR5* to increase MG-132 permeability.

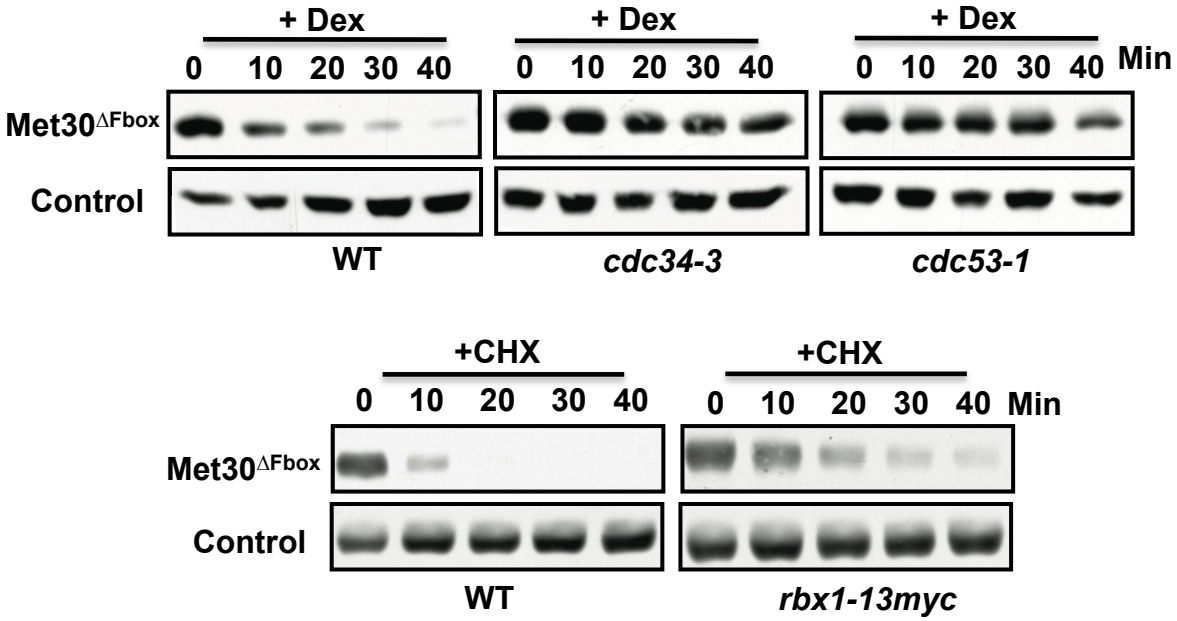


**Figure 3-5. Degradation of 'Skp1-Free' Met30 is dependent on Cdc53, Cdc34 and Rbx1.** $^{RGS6H}$Met30$^{\Delta Fbox}$ stability was analyzed in wild type, *cdc34-3,* and *cdc53-1* temperature sensitive mutants by *GAL1* promoter shut off experiments and immunoblotting with anti-RGS6H antibodies as described for Figure 3-3A. For RBX1, wild type cells and cells carrying the *rbx1-13myc* allele (previously shown to be a hypomorph allele that compromises SCF function at high temperature (Seol et al., 1999)), both expressing $^{12myc}$Met30$^{\Delta Fbox}$ were analyzed. Cells were grown at 25°C, shifted to 37°C for 1.5 h to inactivate Rbx1$^{13myc}$ and $^{12myc}$Met30$^{\Delta Fbox}$ degradation was analyzed.

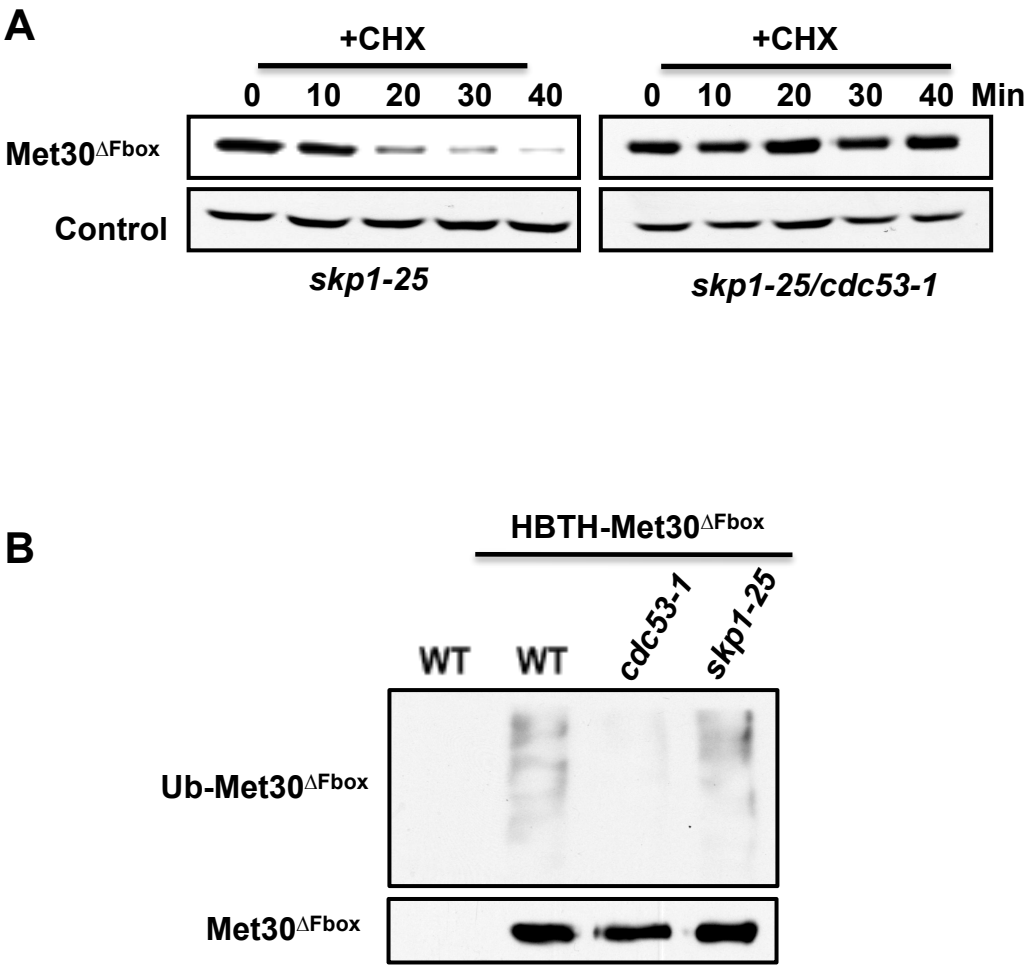


**Figure 3-6. Ubiquitylation and degradation of 'Skp1-Free' Met30 is dependent on Cdc53 but is independent of Skp1.** (A) Cycloheximide chase experiment as described for Fig. 3-2A, but $^{12myc}Met30^{\Delta Fbox}$ stability was analyzed in *skp1-25* single and *skp1-25 cdc53-1* double mutants. (B) Cells expressing $^{HBTH}Met30^{\Delta Fbox}$ under control of the *GAL1* promoter were shifted to 37°C for 1.5 h to inactivate temperature sensitive alleles. $^{HBTH}Met30^{\Delta Fbox}$ was purified on $Ni^{2+}$ - sepharose under denaturing conditions and analyzed by immunoblotting using antibodies directed against ubiquitin (upper panel) or the RGS6H epitope in HBTH tag (lower panel). Cells expressing untagged $Met30^{\Delta Fbox}$ were processed as control.

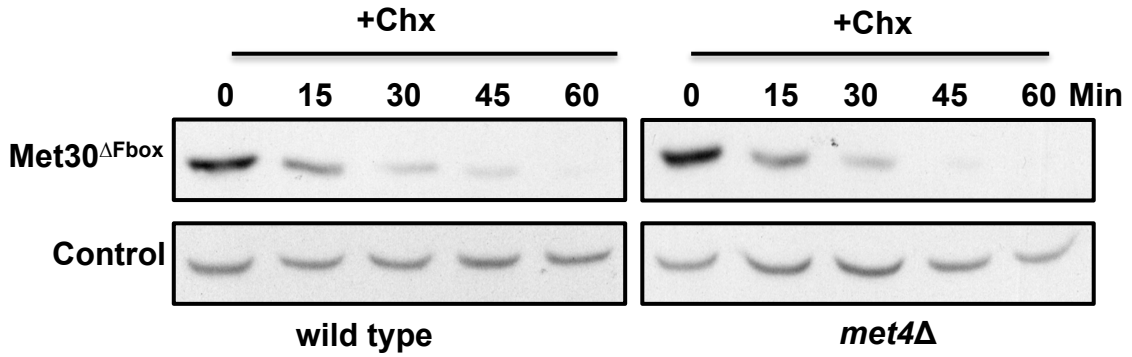


**Figure 3-7. Degradation of 'Skp1-Free' Met30 is not dependent on Met4.** Cycloheximide chase experiment as described for Fig. 3-2A was performed in wild type and *MET4* deleted cells and $^{12myc}Met30^{\Delta Fbox}$ stability was assayed.

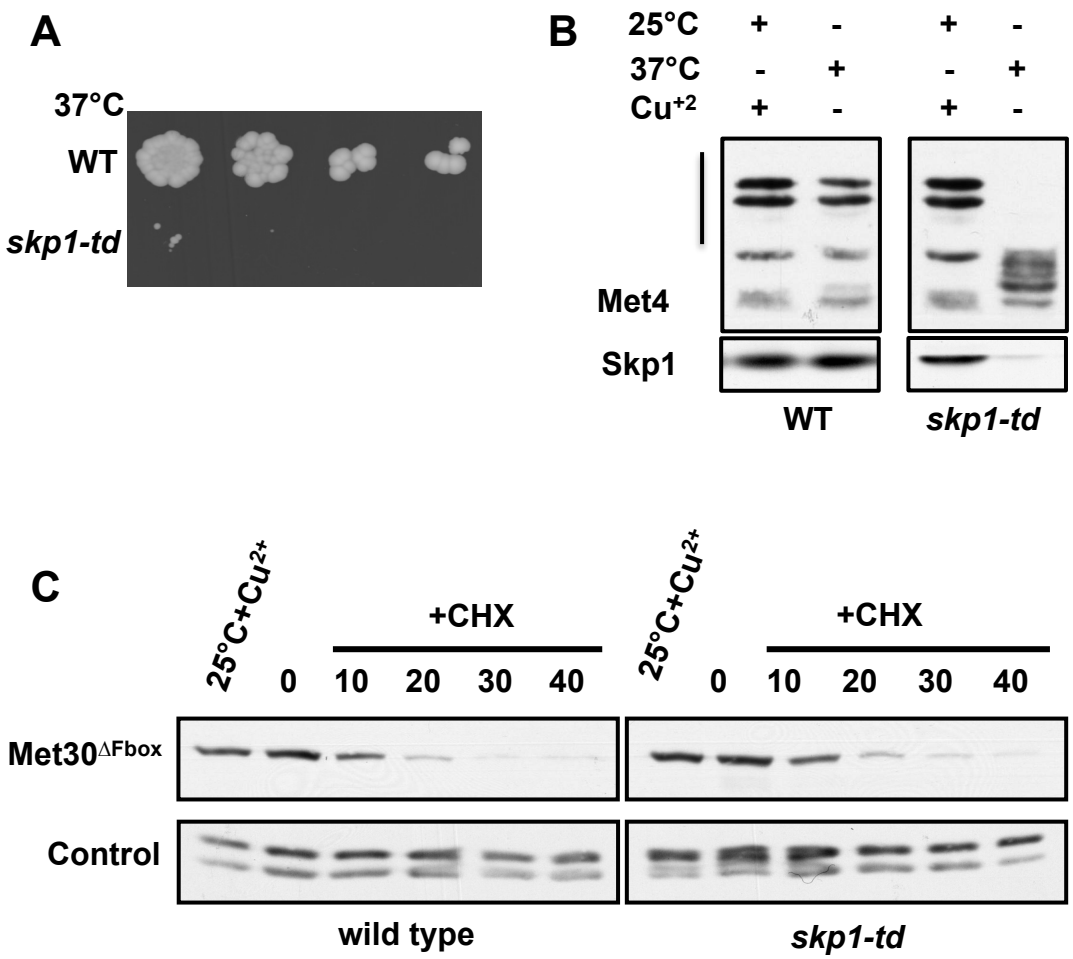


**Figure 3-8. Skp1 independent degradation of Met30.** (A) *skp1-td* mutants express an N-terminal temperature inducible degron (td) fused to Skp1 under the control of the *CUP1* promoter. Wild type and *skp1-td* cells were serially diluted and spotted on YEPD plates without copper at 37°C for 2 days. (B) Wild type and *skp1-td* mutants were cultured at permissive (25°C + $Cu^{2+}$) and non-permissive conditions (37°C for 1 h without $Cu^{2+}$). Immunoblot analysis showed that Skp1 was depleted and that Met4 ubiquitylation was blocked in *skp1-td* mutants indicative of inactivation of $SCF^{Met30}$. (C) Wild type and *skp1-td* strains expressing endogenous $^{12myc}Met30^{\Delta Fbox}$ were cultured under permissive conditions and then shifted to non-permissive conditions for 1 h to deplete *skp1-td*. $Met30^{\Delta Fbox}$ stability was analyzed using cycloheximide to block translation. Anti-PSTAIRE detection of Cdc28 was used as loading control.

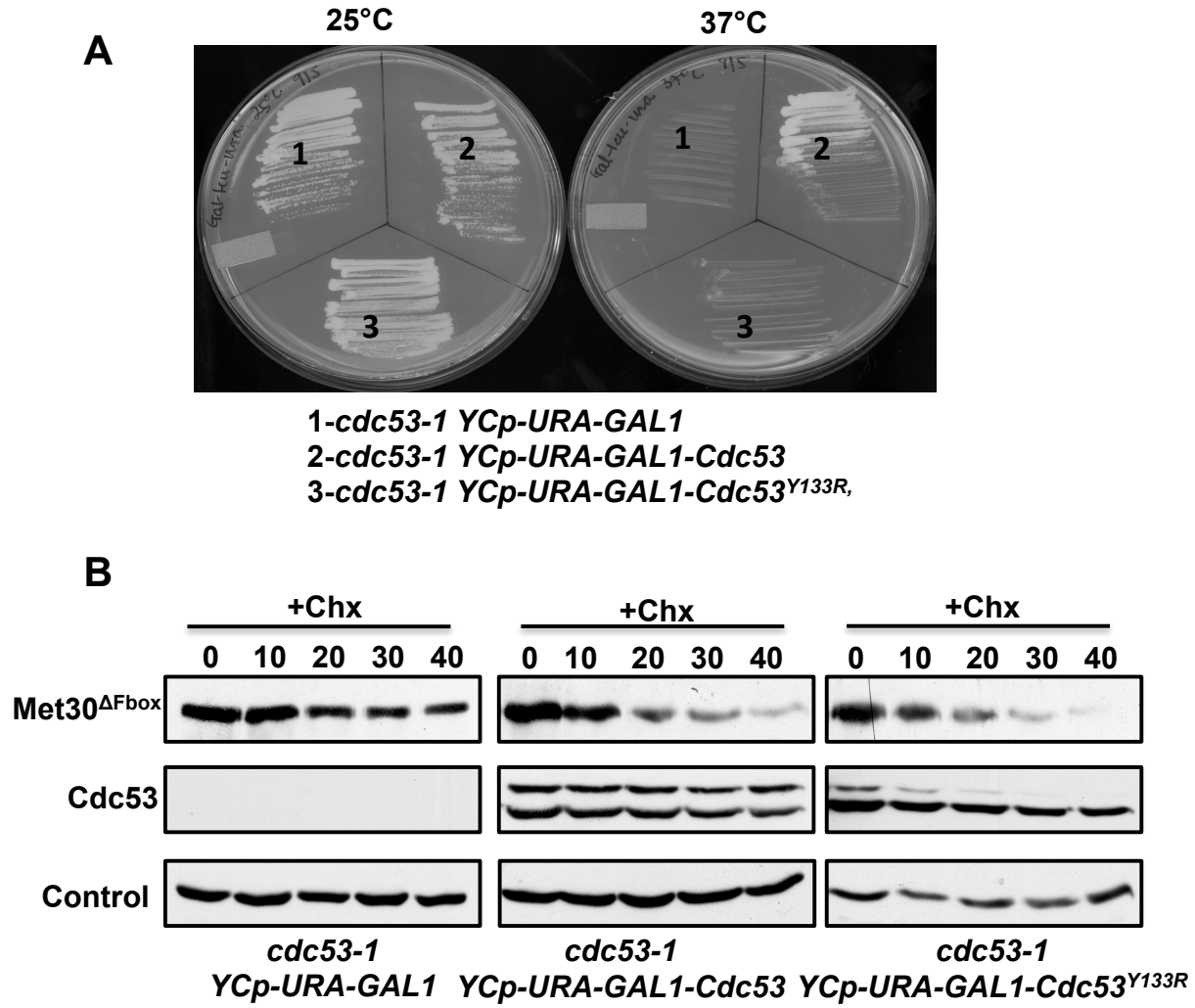


**Figure 3-9. Cdc53 mutant unable to bind Skp1 can degrade Met30.** (A) *cdc53-1* temperature sensitive strain was transformed with different Cdc53 expressing plasmids and plated on Gal-URA minimal media plate. Plate was incubated at 25°C overnight, and then replica plated on another Gal-URA plate. This plate was kept at 37°C for two days. (B) *cdc53-1* temperature sensitive mutant bearing plasmids expressing $^{12myc}Met30^{\Delta Fbox}$ and *GAL1* inducible $^{RGS6H}Cdc53$/ $^{RGS6H}Cdc53^{Y133R}$/ empty vector were grown at 25°C in minimal media containing sucrose. Cells were transferred to minimal media containing 2% galactose and were grown at 25°C for 20 h to induce Cdc53 expression. Cultures were shifted to 37°C for 2 h to inactivate *cdc53-1*. $Met30^{\Delta Fbox}$ stability was analyzed using cycloheximide to block translation.

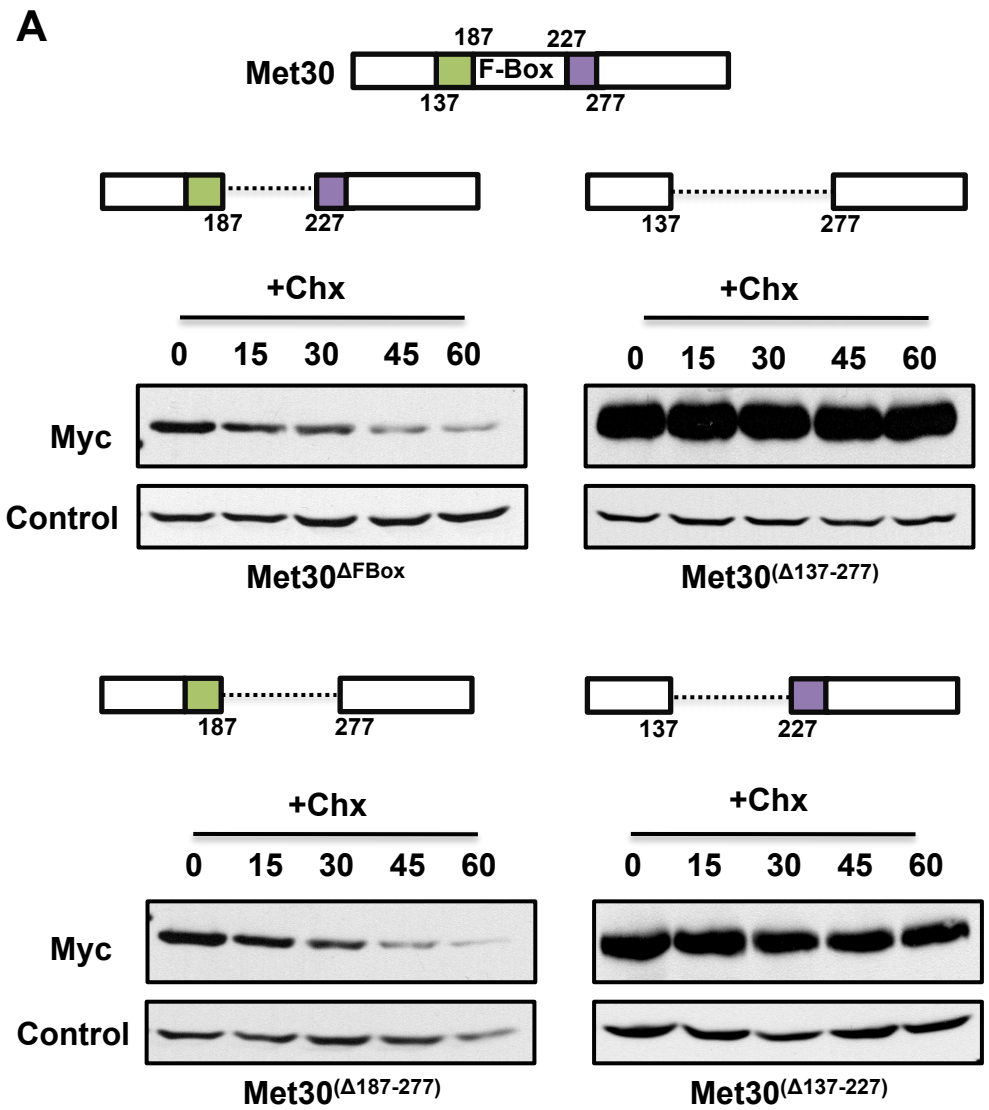


**Figure 3-10. Identification of degron for 'Skp1-free' Met30 degradation pathway.** (A) Cells expressing either endogenous $^{12myc}$Met30$^{\Delta Fbox}$ or different Met30$^{\Delta Fbox}$ deletion mutants were grown at 30°C. Protein translation was inhibited by addition of cycloheximide and cells were collected at the time intervals indicated. Met30$^{\Delta Fbox}$ stability was analyzed by immunoblotting with anti-myc antibodies.

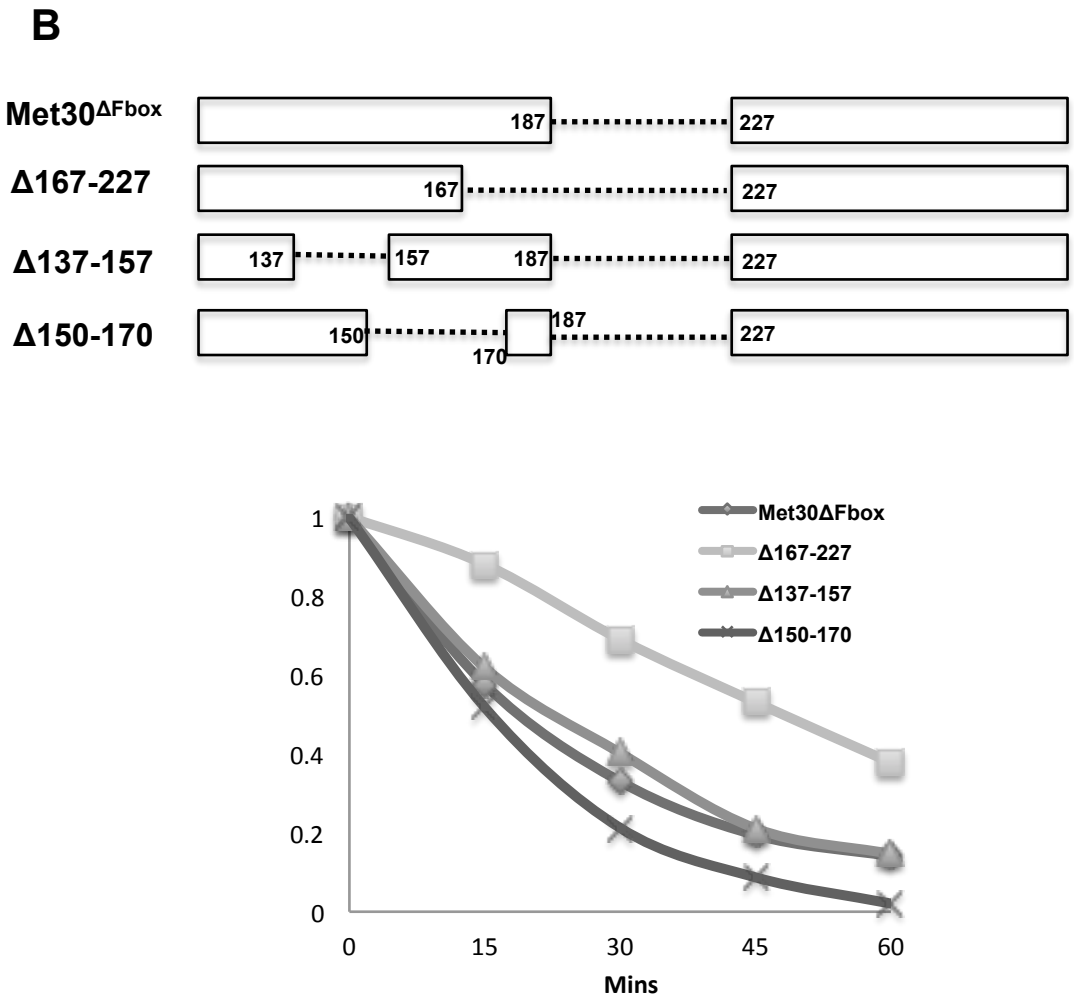


**Figure 3-10. Identification of degron for 'Skp1-free' Met30 degradation pathway.** (B) Cells expressing either endogenous $^{12myc}Met30^{\Delta Fbox}$ or different $Met30^{\Delta Fbox}$ deletion mutants were grown at 30°C. Protein translation was inhibited by addition of cycloheximide and cells were collected at the time intervals indicated. $Met30^{\Delta Fbox}$ stability was analyzed by immunoblotting with anti-myc antibodies.

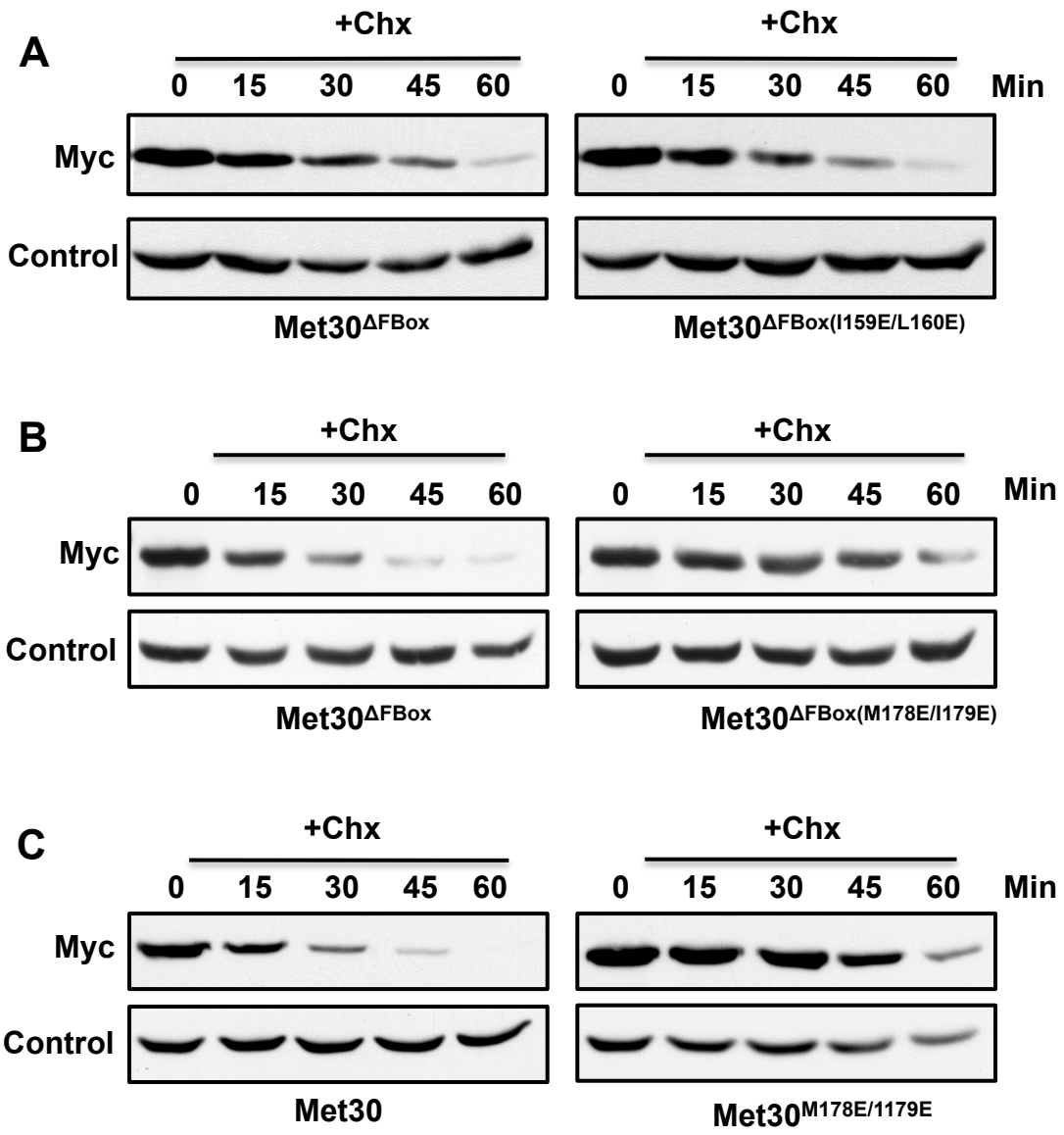


**Figure 3-11. Mutation of methionine 178 and isoleucine 179 in Met30 can abolish 'Skp1-free' Met30 degradation.** (A,B and C) Cells expressing either endogenous $^{12myc}$Met30$^{\lambda Fbox}$ or $^{12myc}$Met30 or different $^{12myc}$Met30 point mutants were grown at 30°C. Protein translation was inhibited by addition of cycloheximide and cells were collected at the time intervals indicated. Met30$^{\lambda Fbox}$/Met30 stability was analyzed by immunoblotting with anti-myc antibodies.

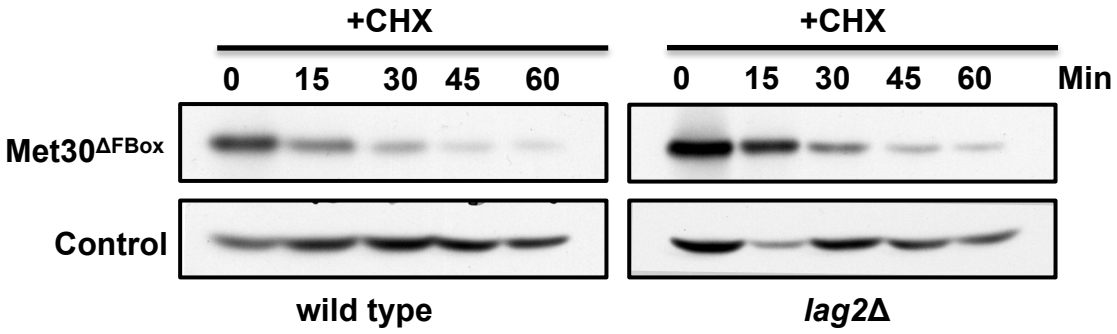


**Figure 3-12. Degradation of 'Skp1-Free' Met30 is not dependent on Lag2.** Cycloheximide chase experiment as described for Fig. 3-2A was performed in wild type and *LAG2* deleted cells and $^{12myc}Met30^{\Delta Fbox}$ stability was assayed.

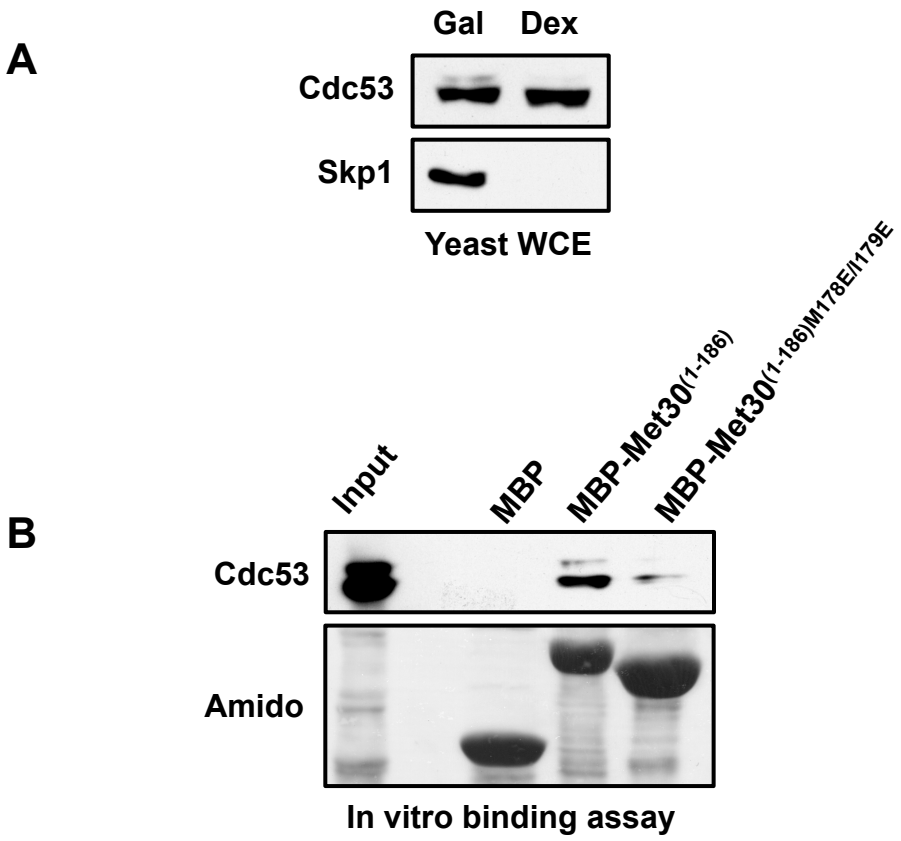


**Figure 3-13. Cdc53 can bind Met30 in absence of Skp1** (A) Yeast strain with $Cdc53^{TAP}$ and *GAL1* inducible *SKP1* was cultured in media containing 2% galactose to express Skp1 and then shifted to media containing 2% dextrose for 12 h to deplete Skp1. Skp1 was efficiently depleted from cells. (B) MBP, (MBP)-$Met30^{(1-186)}$ and (MBP)-$Met30^{(1-186)M178E/I179E}$ were expressed in *E.coli* and bound to amylose resin. They were incubated with Skp1 depleted yeast lysates expressing $Cdc53^{TAP}$. Beads were washed, bound proteins were eluted and analyzed by Western blotting. Cdc53 levels were detected with PAP antibody.

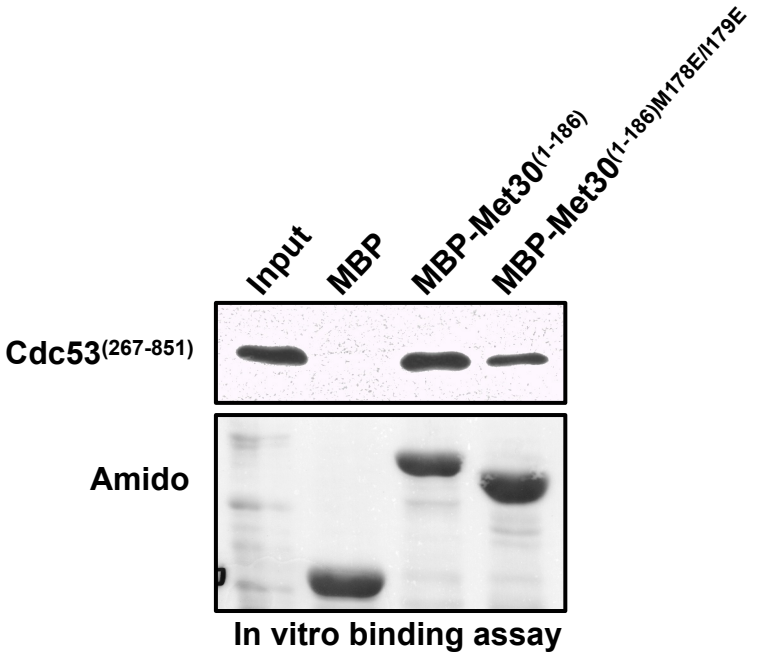


**Figure 3-14. Cdc53/Rbx1 binds Met30 *in vitro*.** Amylose beads were bound with MBP, (MBP)-Met30$^{(1-186)}$ and (MBP)-Met30$^{(1-186)M178E/I179E}$ and were incubated with bacterial lysate expressing Cdc53$^{267-851}$/Rbx1. Beads were washed, bound proteins were eluted and analyzed by Western blotting. Cdc53 levels were detected with anti-Cdc53 antibody.

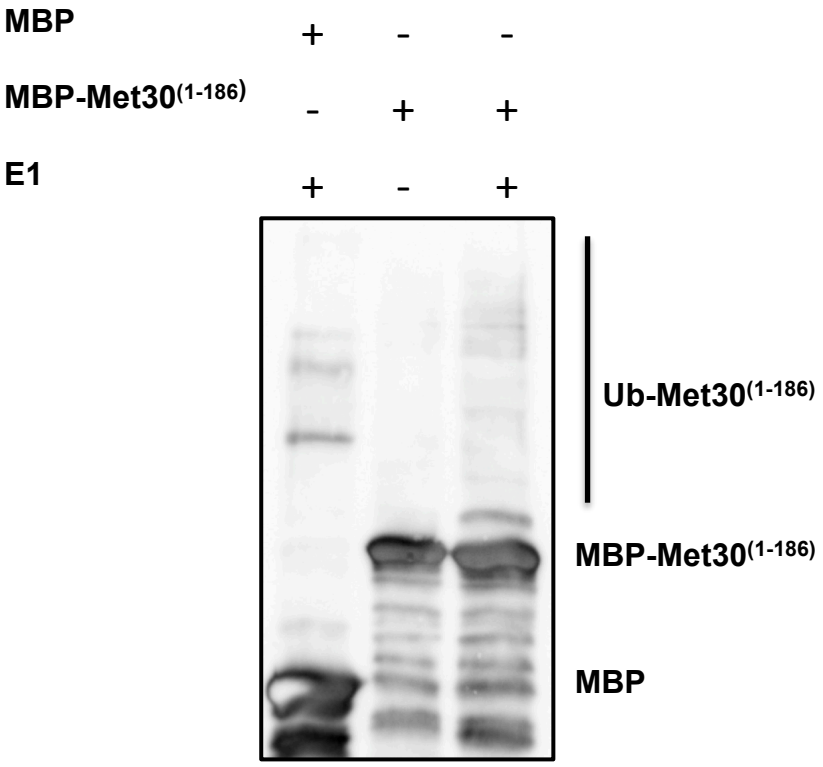


**Figure 3-15. Cdc53/Rbx1 can ubiquitylate Met30 *in vitro*.** MBP and (MBP)-Met30$^{(1-186)}$ were immobilized to amylose resin and incubated with Cdc53$^{267-851}$/Rbx1 expressed in bacteria. Substrate-ligase complex was eluted with 10mM maltose and incubated with ubiquitylation reaction mix for 16 h at 30°C. Ubiquitylation was analyzed by immunoblotting with anti-MBP antibody.

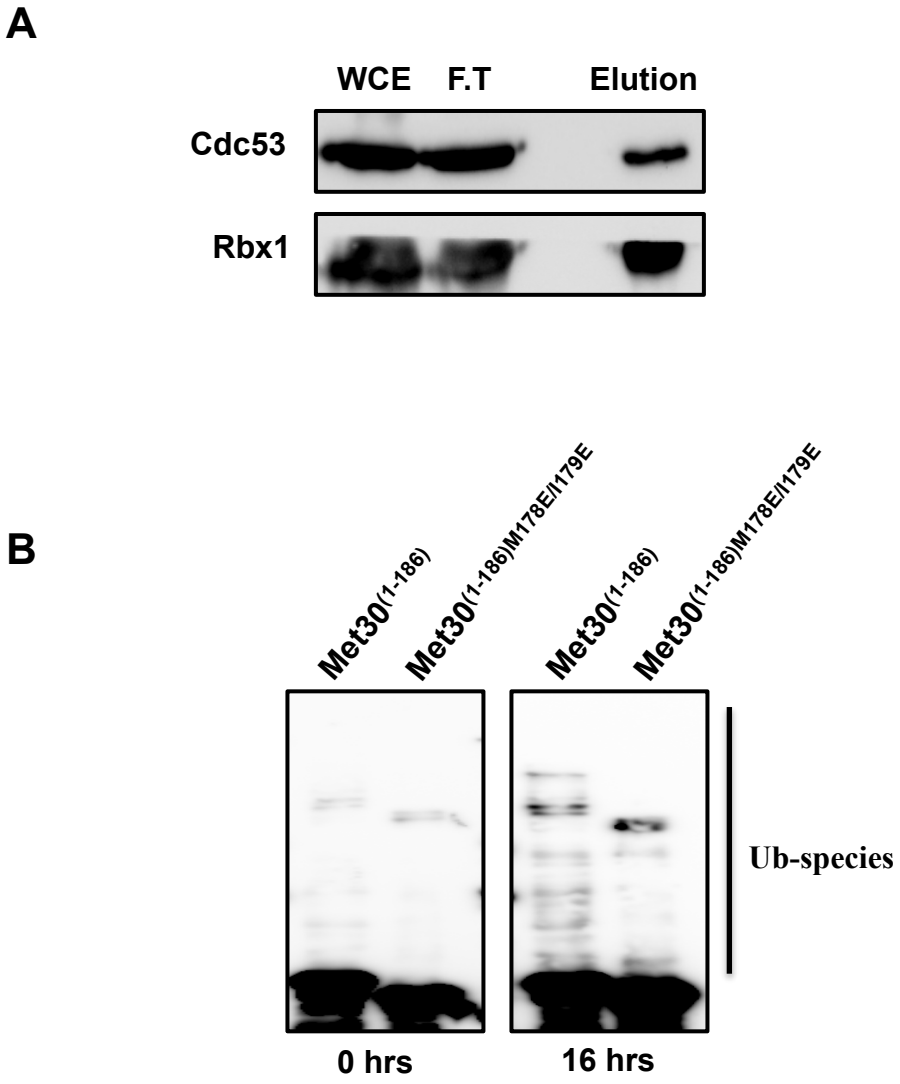


**Figure 3-16. Cdc53/Rbx1 ubiquitylation is specific for 'Skp1-free' Met30 degradation.** (A). $Cdc53^{267-851}$/Rbx1 was purified on glutathione sepharose beads. Efficiency of purification was determined by immunoblotting the eluate with anti-Cdc53 antibody and anti-GST antibody. (B). In vitro ubiquitylation reaction was performed with purified (MBP)-$Met30^{(1-186)}$ and (MBP)-$Met30^{(1-186)M178E/I179E}$ and purified $Cdc53^{267-851}$/Rbx1. '0' and 16 h time points were collected. Ubiquitylation profile was assayed by immunoblotting with anti-MBP antibody.

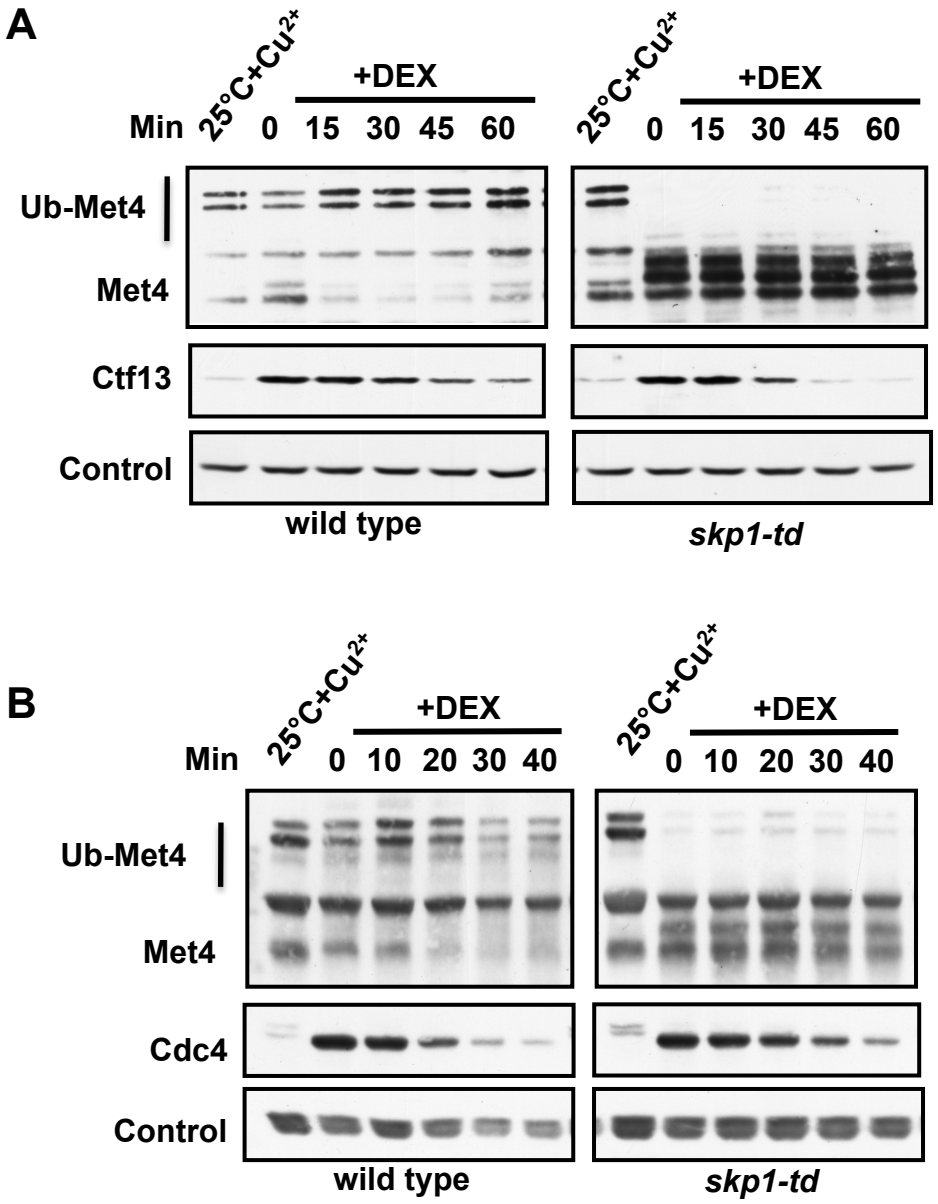


**Figure 3-17. Skp1- independent degradation of Ctf13 and Cdc4.** (A and B) *skp1-td* cells expressing Ctf13 or Cdc4 under control of inducible *GAL1* promoter were depleted of Skp1 as described for Fig. 3-8C. Dextrose was added to terminate transcription from the *GAL1* promoter and degradation of Ctf13 and Cdc4 was analyzed by immunoblotting.

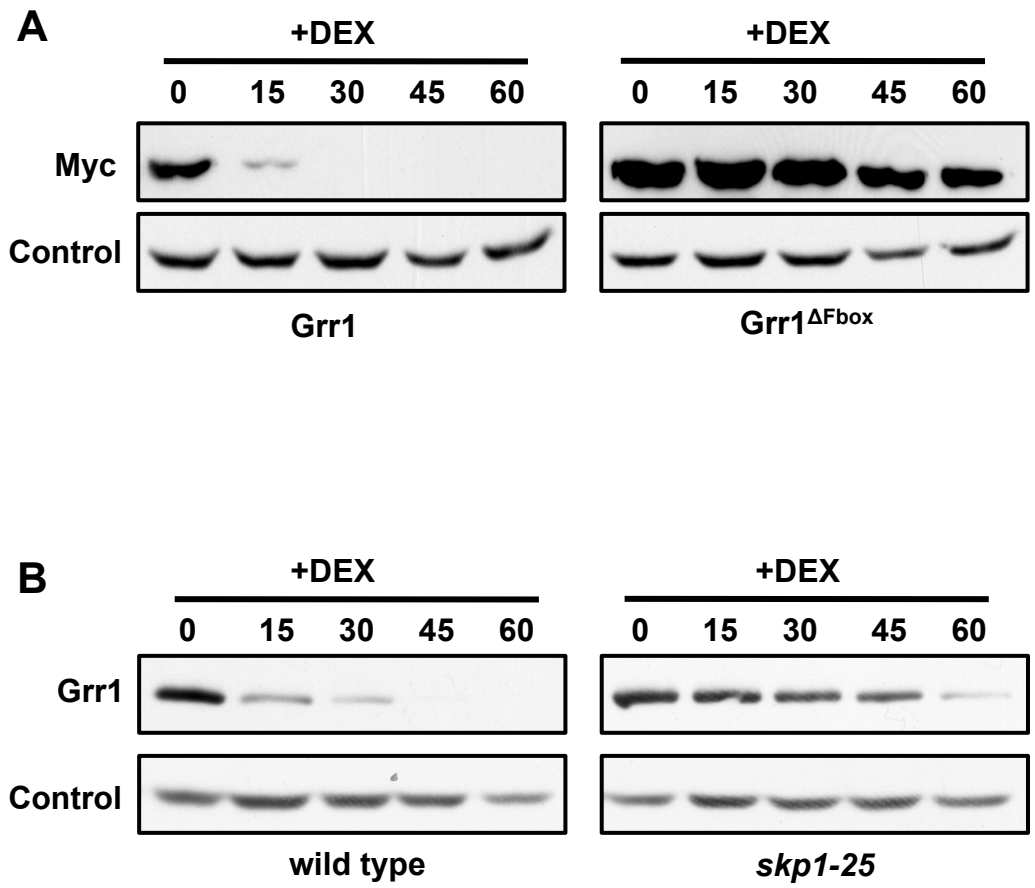


**Figure 3-18. Grr1 is not regulated by 'Skp1-free' F-box protein degradation pathway.** (A) $^{13Myc}$Grr1 and $^{13Myc}$Grr1$^{\Delta Fbox}$ stability was analyzed in wild type cells by performing *GAL1* promoter shut off experiment and immunoblotting with anti-myc antibodies as described for Figure 3-2B. (B) $^{13Myc}$Grr1 stability was analyzed in wild type and *skp1-25* temperature sensitive mutant by performing *GAL1* promoter shut off experiment and immunoblotting with anti-myc antibodies as described for Figure 3-3A.

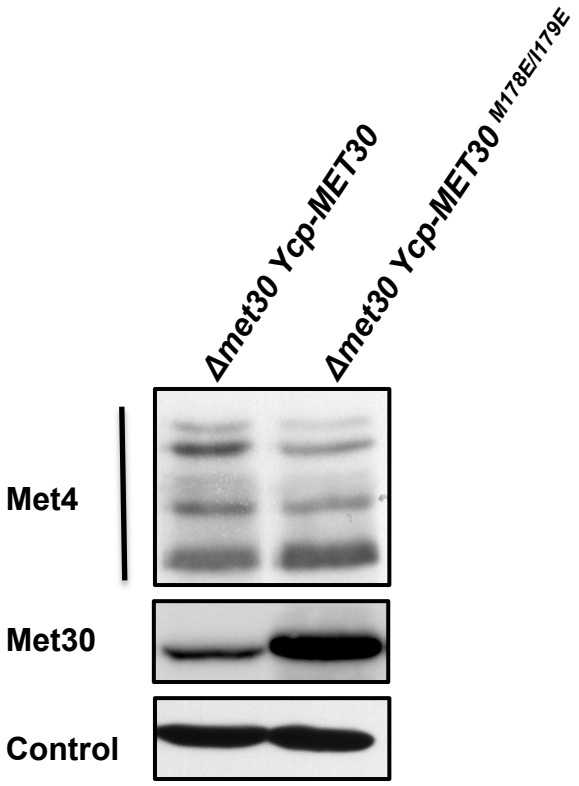


**Figure 3-19. Characterization of Met30$^{M178E/I179E}$ mutant.** Steady state protein levels for Met30 and Met30$^{M178E/I179E}$ strains were compared by immunoblotting. Anti-myc and anti-Met4 antibodies were used for detecting Met30 and Met4 respectively. Cim5 was used as loading control.

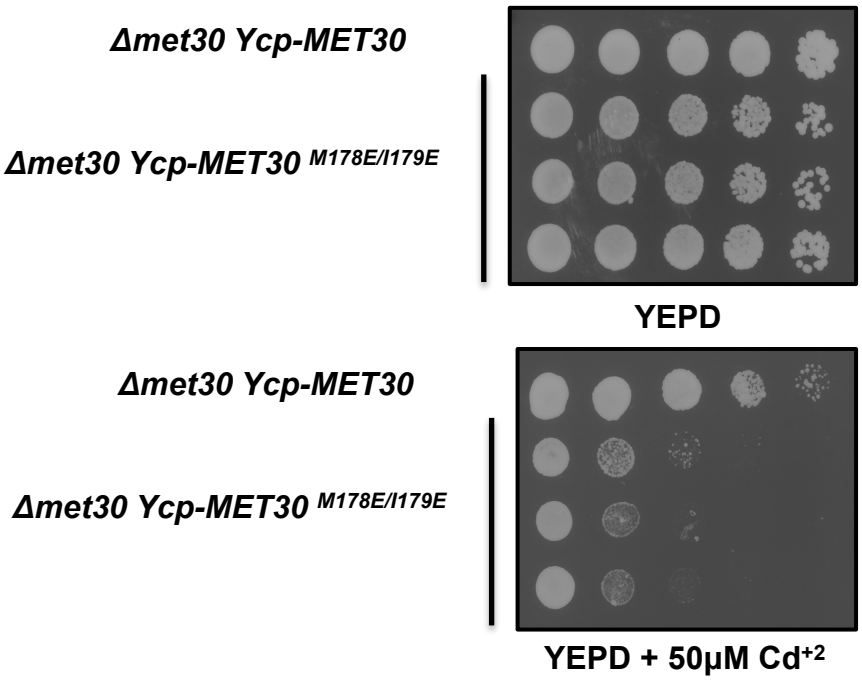


**Figure 3-20. 'Skp1-free' Met30 degradation is important for cellular function.** Met30 and $Met30^{M178E/I179E}$ strains were grown at 30°C to mid-log phase and serial dilutions of cells were spotted on YEPD plates with or without 50μM $CdCl_2$. Plates were incubated at 30°C for 2 days.

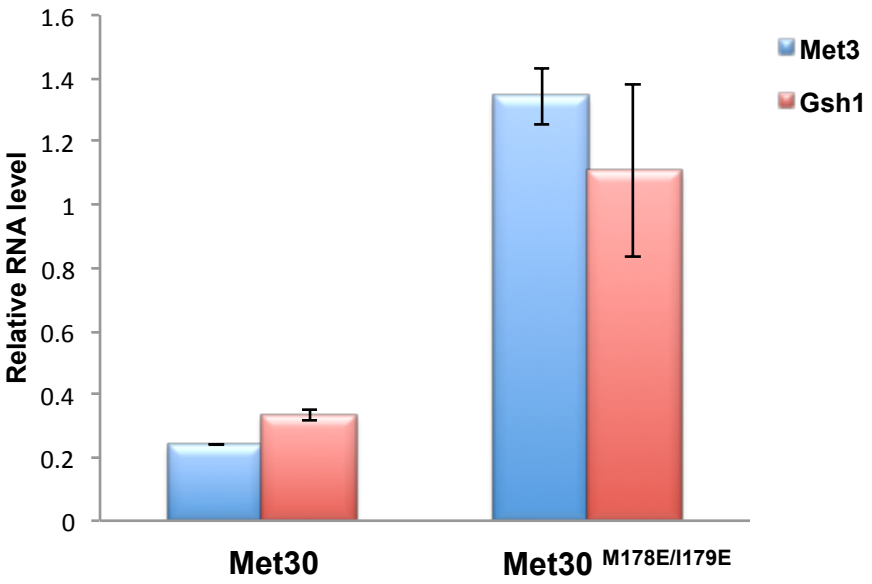


**Figure 3-21. Loss of repression of Met4 target genes in Met30$^{M178E/I179E}$ mutant.** Met30 and Met30$^{M178E/I179E}$ strains were cultured in YEPD. Expression of Met4 target genes, *MET3* and *GSH1* was analyzed by rt-qPCR (n=3). Data are represented as mean ± SD.

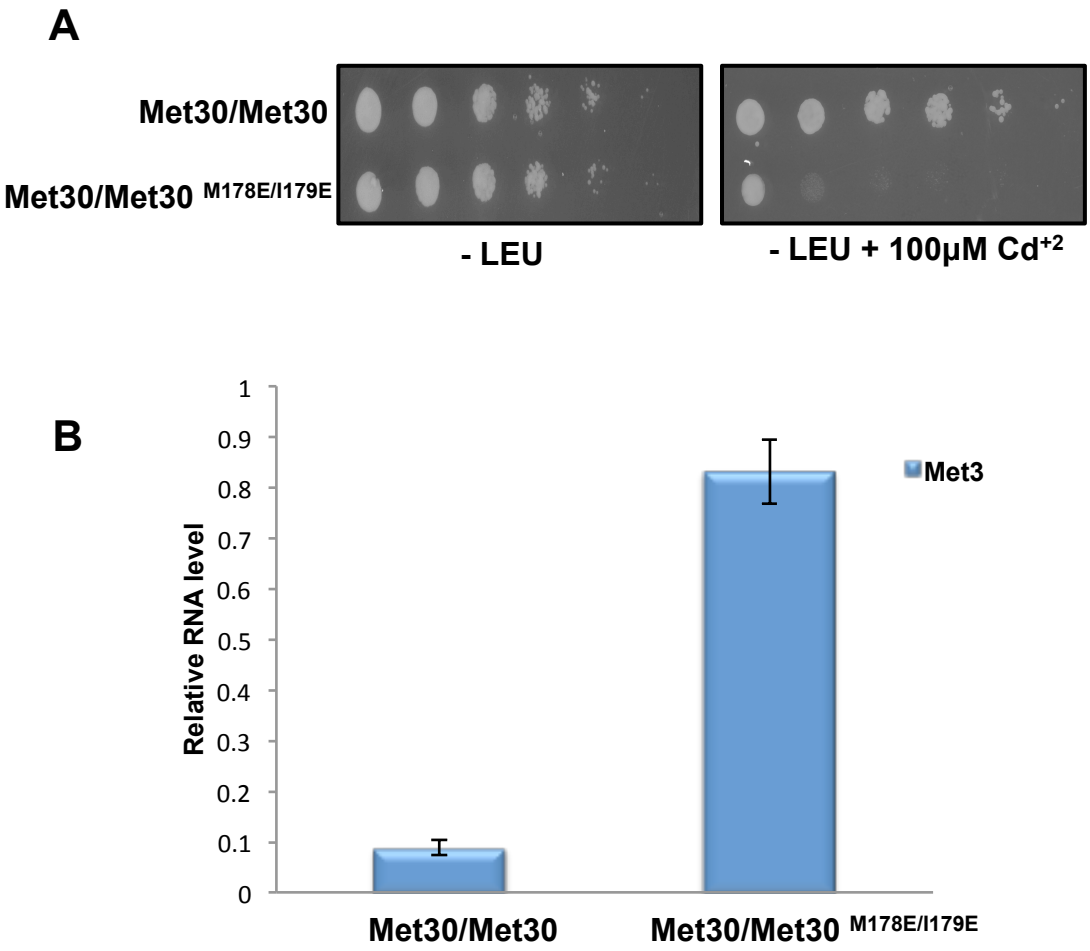


**Figure 3-22. Met30$^{M178E/I179E}$ mutant is dominant.** (A). Strains containing wild type Met30 along with plasmid expressing wild type or Met30$^{M178E/I179E}$ were grown at 30°C to mid-log phase and serial dilutions of cells were spotted on minimal media plates with or without 100μM $CdCl_2$. Higher concentration of $CdCl_2$ was used as cells were spotted on minimal media plates rather than YEPD. Plates were incubated at 30°C for 2 days. (B). Cells as above were grown at 30°C in minimal media to mid log phase and then shifted to YEPD for 2 hours. Expression of *MET3* was analyzed by rt-qPCR (n=3). Data are represented as mean ± SD.

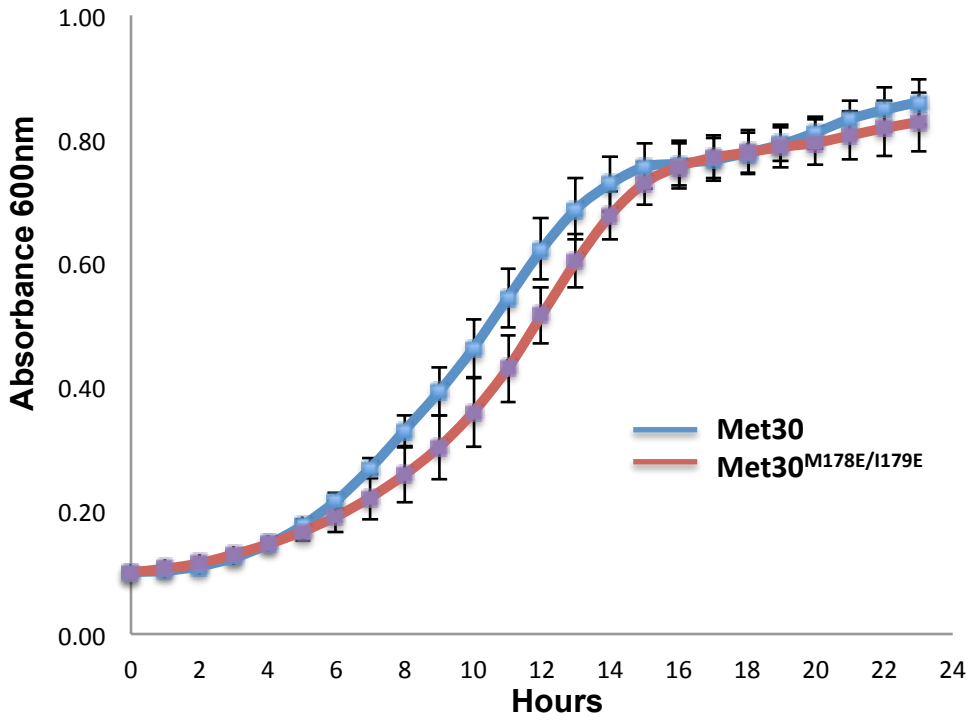


**Figure 3-23. Met30$^{M178E/I179E}$ mutant exhibits a slight growth delay.** Growth curve was plotted for Met30 and Met30$^{M178E/I179E}$ strains by measuring absorbance of cultures at 600 nm. The cultures were grown at 30°C for 24 h. Experiment was performed in triplicate. Data are represented as mean ± SD.

# CHAPTER 4

## Cdc48 ATPase and Cic1 are essential for 'Skp1-free' Met30 degradation


**ABSTRACT**

F-box proteins function as the substrate recognition subunit of the SCF ubiquitin ligase complex. These proteins are unstable in nature and undergo degradation by two mechanisms - autoubiquitylation and 'Skp1-free' mechanism of regulation. The former method targets F-box proteins associated with Skp1/Cdc53 core when substrates are unavailable while the latter degrades F-box proteins that are detached from Skp1. In the previous chapter, we described this 'Skp1-free' mechanism of F-box regulation for F-box protein Met30. Here we introduce two novel components involved in the pathway – the $AAA^+$ ATPase Cdc48 and Cic1. We show that Cdc48 functions along with its cofactors, Npl4 and Ufd1 in this process. Importantly, these proteins do not play a role in ubiquitylation of 'Skp1-free' F-box proteins but are likely associated with a post ubiquitylation step in the pathway. Additional experiments also indicate that these proteins interact with each other and form a complex.


**INTRODUCTION**

Post-translational modification of proteins with ubiquitin regulates a gamut of cellular and developmental processes. Ubiquitylation can lead to changes in protein localization, activity, stability, and protein-protein interactions. These effects are mediated by diverse ubiquitin receptor proteins that recognize ubiquitylated targets and interpret the signal. Two related classes of ubiquitin receptors bring about proteolysis of substrates. Ubiquitin receptors such as Dsk2, Rad23, and Ddi1 escort ubiquitylated substrates to the proteasome for proteolysis. They have

ubiquitin-like domain (UBL) that is recognized by the proteasome and ubiquitin-associated domain (UBA) that binds ubiquitin. Degradation of cyclin dependent kinase inhibitor Sic1 has been shown to be dependent on specific UBA-UBL protein, Rad23 *in vivo* and *in vitro* (Verma et al., 2004). Another family of related ubiquitin receptors recruit ubiquitylated proteins to Cdc48, an ATPase ring complex that can also unfold proteins (Elsasser and Finley, 2005).

Cellular proteins destined for proteolysis are covalently modified with ubiquitin by the action of three classes of enzymes — E1, E2 and E3 enzymes. E3 ubiquitin ligases bind substrates and confer specificity to this process. Amongst the E3 ligases, SCF ubiquitin ligases are the best-understood complexes (Finley et al., 2012). These multi-subunit enzymes comprise of three invariant components, Cdc53 (cullin-1), Skp1, Rbx1, and a changeable substrate recognition adaptor, the F-box protein. Different F-box proteins function in their respective SCF complexes, and recognize a specific subset of substrates (Deshaies and Joazeiro, 2009). In yeast, the best-characterized F-box proteins are Cdc4, Grr1 and Met30 which function in $SCF^{Cdc4}$, $SCF^{Grr1}$ and $SCF^{Met30}$ respectively and regulate ubiquitylation of distinct set of substrates.

The protein levels of invariable SCF components remains unchanged in the cells while the F-box proteins are unstable and readily degraded (Galan and Peter, 1999; Zhou and Howley, 1998). Upon association with a functional SCF complex, F-box proteins are regulated by autoubiquitylation (Galan and Peter, 1999; Zhou and Howley, 1998). When they are dissociated from Skp1 they are targeted by another mechanism called 'Skp1-free' mechanism of F-box protein regulation. This pathway has been demonstrated for F-box protein Met30 and is likely applicable for other F-box proteins as well (Chapter 3). It plays an important role in limiting abundance of Met30 to prevent excess Met30 from sequestering its substrate, Met4, which likely shields the substrate from recognition by fully assembled $SCF^{Met30}$ ligase. The unique feature of

this pathway is that it is mediated by a ligase that comprises of Cdc53 and Rbx1, but is independent of Skp1. We were interested in characterizing this pathway further by identifying new components of this pathway and elucidating their role in this process. In our present study we uncover two novel players of this pathway- Cdc48 and Cic1.

The $AAA^+$ (**A**TPase **A**ssociated with various **A**ctivities) ATPase Cdc48 is a conserved, key component of the ubiquitin system with an extensive breadth of functions. Some activities that it is known to be involved in are endoplasmic reticulum (ER) associated degradation, vesicle fusion and cell cycle regulation (Buchberger, 2013). Most Cdc48 dependent functions seem to be directly linked to its ability to bind ubiquitylated proteins and chaperone them to the proteasome or segregate them from their binding partners. The wide range of Cdc48 actions are attributable to its various cofactors. Most Cdc48 cofactors share either ubiquitin associated (UBA) and/or ubiquitin regulatory (UBX) domains (Meyer et al., 2012). The UBA domain is an ubiquitin-binding motif that facilitates Cdc48 and cofactor recruitment to ubiquitylated substrates. The UBX domain is structurally similar to ubiquitin and is considered a Cdc48 binding motif. Cdc48 cofactors are generally categorized as either substrate processing or substrate recruiting factors. Substrate processing factors are enzymes such as ubiquitin ligases, deubiquitylating enzymes and deacetylases. Substrate recruiting factors regulate Cdc48 by conferring specificity of substrate binding. One of the well-known substrate-recruiting cofactor is the Npl4/Ufd1 complex. Cdc48 along with heterodimeric Npl4/Ufd1 complex is recruited to substrates destined for degradation within the ER (Bays and Hampton, 2002). They extract ubiquitylated proteins from intermemberane pores and then direct these substrates for degradation by handing them off to Rad23, which takes them to the 26S proteasome (Medicherla et al., 2004; Richly et al., 2005).

Cic1 was identified as a protein associating with fully assembled 26S proteasomes. It is an essential nuclear protein whose function is not required for general proteasome-mediated degradation but is necessary for F-box protein degradation (Jager et al., 2001). F-box proteins Cdc4 and Grr1 are stabilized in *cic1* mutant cells and Cic1 has been shown to interact with Cdc4 *in vivo* and *in vitro* (Jager et al., 2001) indicating that Cic1 may be an adaptor between the SCF complex and the 26S proteasome.

Our preliminary studies show that Cic1 and Cdc48, along with its substrate recruiting factors Npl4/Ufd1 are involved in the 'Skp1-free' F-box protein degradation pathway. No direct role of Cdc48 is known in F-box protein proteolysis, thus this study would be the first in establishing this function.

## RESULTS

### *Cdc48 ATPase plays a role in regulation of F-box proteins*

Cdc48 is an established player of the ubiquitin proteasome system and has been shown to physically associate with several CRLs (Alexandru et al., 2008; den Besten et al., 2012). Previous studies done in our lab have shown Cdc48 to regulate $SCF^{Met30}$ by mediating dissociation of Met30 from Skp1 in response to cadmium stress (Yen et al., 2012). We were curious to know whether this multi-functional protein also played a role in degradation of Met30. To investigate this, we ascertained half- life of wild type Met30 in *cdc48-3* temperature sensitive mutant using translation inhibitor cycloheximide. Met30 was stabilized upon inactivation of Cdc48 suggesting involvement of Cdc48 in Met30 proteolysis (Fig. 4-1). Note that Cdc48

mediated $SCF^{Met30}$ disassembly induced during cadmium stress is independent of its function in Met30 proteolysis (Yen et al., 2012).

We were curious whether Cdc48's function was specific for Met30 or whether it was applicable for other F-box proteins as well. Cycloheximide chase experiments were performed to look at stability of two other well-characterized F-box proteins from yeast, Cdc4 and Grr1, in *cdc48-3* mutant. Similar to Met30, Cdc4 and Grr1 were stabilized upon Cdc48 inactivation (Fig. 4-1) suggesting a general role for this ATPase in F-box protein homeostasis.

***Cdc48 is involved in 'Skp1-free' Met30 degradation***

In wild-type cells Met30 may exist in two states- either bound to the SCF core or dissociated from Skp1. When attached to the SCF core, Met30 undergoes autoubiquitylation (Galan and Peter, 1999) and if it is unbound, it is degraded by 'Skp1-free' degradation pathway (Chapter 3). As our interests lay in getting a better understanding of the 'Skp1-free' mechanism of Met30 regulation, we tested whether Cdc48 played a role in this pathway**.** To specifically assay 'Skp1-free' Met30 degradation, we utilized a mutant form of Met30 lacking its F-box domain (residues 187-277) and is thus unable to associate with the SCF core complex (Brunson et al., 2004; Yen et al., 2012). $Met30^{\Delta Fbox}$ served as a convenient tool to generate a homogenous population of unbound Met30**.** $Met30^{\Delta Fbox}$ was highly unstable in wild type cells but was strongly stabilized in *cdc48-3* temperature sensitive mutant (Fig. 4-2) suggesting its involvement in 'Skp1-free' Met30 degradation pathway.

Cdc48 has multiple functions in the cell cycle and the *cdc48-3* temperature sensitive mutant is known to arrest in metaphase (Cheng and Chen, 2010). Analysis of $Met30^{\Delta Fbox}$

degradation in cells treated with mitotic inhibitor nocodazole showed that stabilization of Met30$^{\Delta Fbox}$ in *cdc48-3* mutant was not an indirect effect of cell cycle arrest (Fig. 4-3).

***Cdc48 cofactors- Npl4 and Ufd1 are involved in 'Skp1-free' Met30 degradation***

The numerous cellular functions of Cdc48 are attributed to its diverse family of cofactors (Jentsch and Rumpf, 2007). These cofactors encompass ubiquitin ligases, ubiquitin binding proteins and deubiquitylating enzymes and can thus affect Cdc48 substrates in a variety of ways (Baek et al., 2013). To better understand how Cdc48 is involved in 'Skp1-free' Met30 regulation, we examined whether its function in this pathway was specific for a certain cofactor. Npl4 and Ufd1 are two well-characterized substrate-recruiting cofactors of Cdc48 and are essential in yeast. To test their involvement in 'Skp1-free' Met30 degradation, we took advantage of their temperature sensitive alleles for assaying half-life of Met30$^{\Delta Fbox}$. Met30$^{\Delta Fbox}$ was stabilized in *npl4-1* and *ufd1-2* temperature sensitive mutants (Fig. 4-4) indicating that these two cofactors were involved with Cdc48 in regulation of F-box proteins that are detached from Skp1. Complete stabilization of Met30$^{\Delta Fbox}$ is not seen in *npl4-1* and *ufd1-2* mutants probably because these mutant alleles do not have a very strong defect and may not result in complete inactivation of their respective proteins at restrictive temperature.

***Cic1 is involved in ' Skp1-free' F-box degradation pathway***

Cic1 is an essential protein that has been connected to degradation of F-box proteins Cdc4 and Grr1 (Jager et al., 2001). Also, we identified Cic1 in a mass spectrometry experiment performed to profile Cdc53 interacting proteins in search of an Skp1 alternative adaptor for F-box proteins (data not shown). To evaluate whether Cic1 was associated with 'Skp1-free'

mechanism of Met30 regulation, we assayed Met30$^{\Delta Fbox}$ half-life in *cic1-2* temperature sensitive mutant. Met30$^{\Delta Fbox}$ was stabilized upon Cic1 inactivation (Fig. 4-5) suggesting that Cic1 is a mediator of 'Skp1-free' F-box protein degradation.

***Cdc48/Npl4/Ufd1 and Cic1 are not required for Met30$^{\Delta Fbox}$ ubiquitylating***

The results from Met30$^{\Delta Fbox}$ half-life experiments unraveled new players in the Skp1 independent mechanism of Met30 regulation. As we were interested in identifying candidates that could serve as substrate adaptors for the ligase responsible for regulating 'Skp1-free' Met30, we were curious to know whether Cdc48/Npl4/Ufd1 and Cic1 played a role in Met30$^{\Delta Fbox}$ ubiquitylation. HBTH-tagged Met30$^{\Delta Fbox}$ was expressed under control of the inducible *GAL1* promoter in wild type, *cdc48-3*, *npl4-1*, *cdc53-1* and *cdc34-2* temperature sensitive strains. Nickel affinity purification of Met30$^{\Delta Fbox}$ from these yeast strains was performed under denaturing conditions, which disrupts all protein-protein interactions except covalent associations like ubiquitylation. Ubiquitylation status of Met30$^{\Delta Fbox}$ was then assessed by immunoblotting using anti-ubiquitin antibodies (Fig. 4-6A). Because the Npl4/Ufd1 heterodimer functions together as a cofactor for Cdc48 (Meyer et al., 2000), we assayed Met30$^{\Delta Fbox}$ ubiquitylation only in *npl4-1* temperature sensitive mutants, as we expect the same result for *ufd1* mutants. Cdc53 and Cdc34 are known components of the E3 ligase mediating 'Skp1-free' Met30 degradation (Chapter 3). As expected, ubiquitylation of Met30$^{\Delta Fbox}$ was decreased in mutants abolishing function of Cdc53 and Cdc34. Interestingly, no defect in Met30$^{\Delta Fbox}$ ubiquitylation was detected in *cdc48-3* and *npl4-1* mutants suggesting that Cdc48/Npl4/Ufd1 complex was not involved in the ubiquitylation step (Fig. 4-6A). Similar experiment was also performed to elucidate the role for Cic1 in 'Skp1-free' F-box protein degradation pathway.

Ubiquitylation of Met30$^{\Delta Fbox}$ was unaffected upon Cic1 inactivation (Fig. 4-6B) suggesting that it was not a component of the ligase. In agreement with this finding, it has been shown that ubiquitylation of Cdc4 is independent of Cic1 function (Jager et al., 2001).

***Cic1 can interact with Met30 in vivo***

As the function of Cic1 is specific for F-box proteins, we wanted to test whether it could interact with them. It has been shown that Cic1 can directly bind Cdc4 (Jager et al., 2001). Mass spectrometry based protein interaction studies have shown Cic1 to associate with Cdc53 (data not shown) indicating that this interaction might be mediated by F-box protein. To confirm this, immunoprecipitation was performed with Cic1. Met30 co-purified with Cic1 (Fig. 4-7) indicating that Cic1 binds Met30.

***Cic1 interacts with Cdc48 and binding depends on Npl4/Ufd1***

Cdc48/Npl4/Ufd1 complex is associated with myriad biological processes in the cell. As Cic1 binds F-box proteins, we hypothesized that maybe Cic1 may serve as an F-box protein specificity factor that could recruit Cdc48/Npl4/Ufd1 complex to F-box proteins ubiquitylated via the 'Skp1-free' pathway. To test this premise, we investigated whether Cic1 and Cdc48 formed a complex. Cdc48 co-purified with Cic1 in immunoprecipitation experiments suggesting that the two proteins interacted *in vivo* (Fig. 4-8). Cdc48 cofactors, Npl4 and Ufd1 are also required for 'Skp1-free' F-box protein degradation pathway. We therefore investigated the relationship between Cic1 and Npl4/Ufd1 and questioned whether its interaction with Cdc48 was dependent on them. Inactivation of Npl4 or Ufd1 abolished Cic1-Cdc48 binding *in vivo*, indicating that Cic1-Cdc48 association was mediated via the Cdc48 cofactors (Fig. 4-8). This

result supports our hypothesis that Cic1 functions as a specificity factor for Cdc48/Npl4/Ufd1 complex in F-box protein degradation.

## DISCUSSION

Capability of substrate recognition for ubiquitylation in the SCF ligase complex is conferred by key adaptor moieties - the F-box proteins. Cycles of association and dissociation of core SCF components with various F-box proteins ensures that activity of SCF ligases is dictated by the amount and identity of F-box proteins available at any given time in the cell. To ensure formation of complete repertoire of SCF ligases for normal cellular function, it is thus necessary to regulate the abundance of F-box proteins.

We have uncovered a novel pathway for regulation of F-box proteins that specifically targets F-box proteins that are detached from Skp1 (Chapter 3). This pathway is dependent on the ubiquitin proteasome system and the ligase responsible for the process constitutes of Cdc53, Rbx1 and Cdc34, but not Skp1. In vitro ubiquitylation with Cdc53/Rbx1 in the presence of Cdc34 was not efficient and required a long reaction time suggesting possible involvement of another factor or a post-translational modification, for effective ubiquitylation of F-box protein substrate. Alternatively, Cdc53 expressed in *E. coli* may not efficiently fold into its active structure and thus be only partially active (Chapter 3).

Previous studies indicated Cdc48 and Cic1 to be associated with F-box protein regulation (Jager et al., 2001; Yen et al., 2012). Inactivation of Cdc48, its cofactors- Npl4/Ufd1, or Cic1 abolished degradation of 'Skp1-free' Met30. However, no loss in Met30 ubiquitylation was observed upon inactivation of these mutants suggesting that though they were involved in $Met30^{\Delta Fbox}$ degradation, they were not components of the ligase.

Cdc48 and its cofactors Npl4/Ufd1 target ubiquitylated proteins to the proteasome for degradation. Also, Cic1 associates with the proteasome and is required for F-box protein degradation. We were intrigued to uncover the relationship between Cdc48/Npl4/Ufd1 complex and Cic1 in F-box protein regulation. To gain insight into their function we tested whether these proteins interacted with one another. We observed that Cic1 binds Met30 and Cdc48. Also, Cic1 binding to Cdc48 was dependent on its cofactors. As Cic1 function is distinct for F-box proteins we suggest it acts as a novel factor to specify ubiquitylated F-box proteins to Cdc48/Npl4/Ufd1 for targeting to proteasome for degradation. Our preliminary studies point in this direction but detailed experimentation is required to substantiate this theory.

We observed Cdc48 to be involved in regulation of F-box protein Grr1. Also Cic1 has been associated with degradation of Grr1 (Jager et al., 2001). Our studies also indicate that Grr1 is not regulated by 'Skp1-free' mechanism (Chapter 3) and is targeted via autoubiquitylation (Galan and Peter, 1999). This suggests that Cdc48 and Cic1 may also play a role in the autocatalytic mode of F-box protein regulation. Future studies need to be conducted to explore this possibility.

Our findings uncover novel regulators for the 'Skp1-free' F-box protein degradation pathway. Further studies are necessary to identify the exact molecular mechanism of Cdc48 and Cic1 in this pathway in detail. We also hypothesize that Cic1 may function as a F-box protein specificity factor for Cdc48/Npl4/Ufd1, which introduces the concept of *protein specificity factors* for Cdc48.

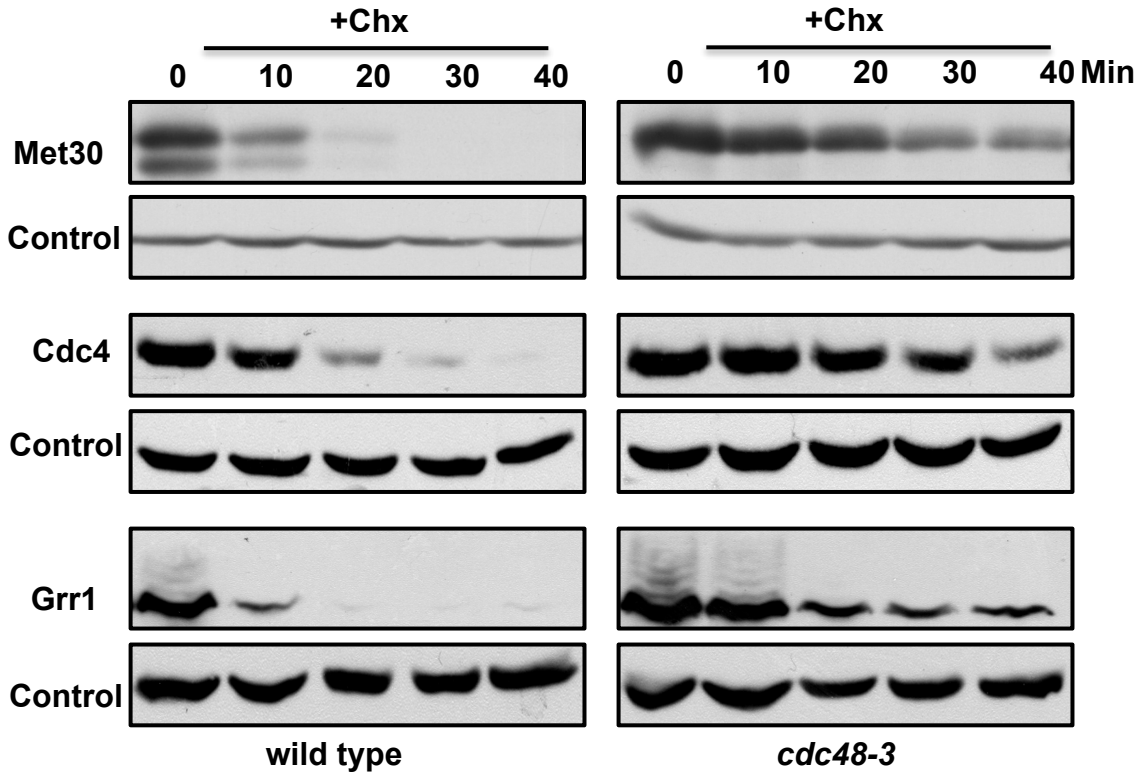


**Figure 4-1. Cdc48 plays a role in regulation of F-box proteins.** Cells expressing endogenous $^{12Myc}$Met30, Cdc4$^{3HA}$, or Grr1$^{13Myc}$ in wild-type or *cdc48-3* mutant were grown at permissive temperature (25°C). Cultures were then shifted to 37°C for 1.5 h following addition of 100µg/ml cycloheximide to inhibit protein translation. Cells were collected at the time intervals indicated. F-box protein stability was analyzed by immunoblotting with anti-myc and anti-HA antibodies. The proteasome subunit, Cim5 was detected as a loading control.

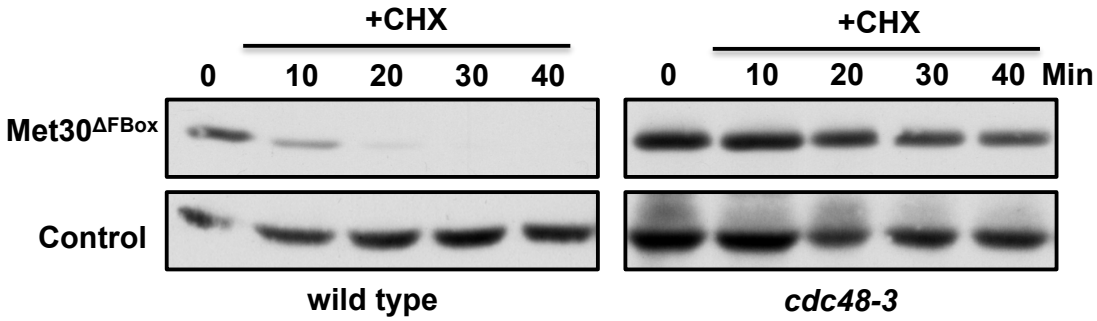


**Figure 4-2. Cdc48 is involved in 'Free' F-box protein degradation pathway**. Wild type and *cdc48-3* temperature sensitive mutants expressing $^{12myc}Met30^{\Delta Fbox}$ were cultured at permissive temperature (25°C) and then shifted to non-permissive temperature (37°C) for 1.5 h. Cycloheximide was added and samples were collected at the time intervals indicated. $Met30^{\Delta Fbox}$ protein stability was analyzed by immunoblotting with anti-myc antibodies.

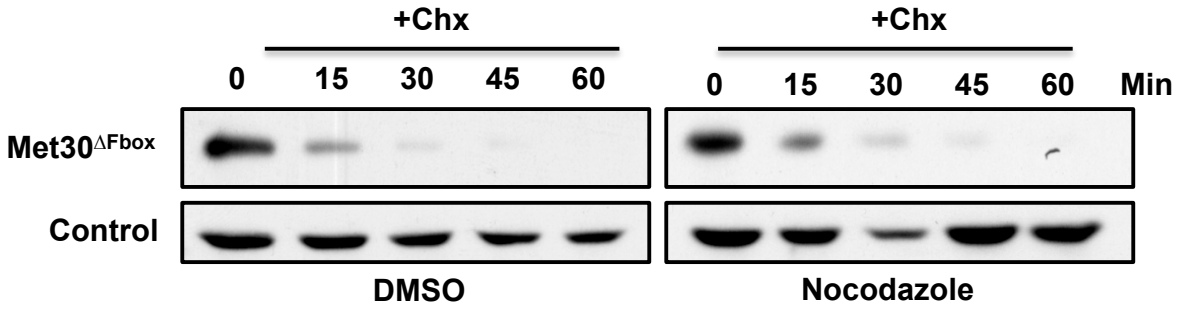


**Figure 4-3. Mitotic arrest does not induce Met30$^{\Delta Fbox}$ stabilization.** Cells expressing $^{12myc}$Met30$^{\Delta Fbox}$ were grown at 30°C. Mitotic arrest was induced by adding 15μg/ml nocodazole for 3 h before cycloheximide was added to block translation. Met30$^{\Delta Fbox}$ protein stability was analyzed by immunoblotting with anti-myc antibodies.

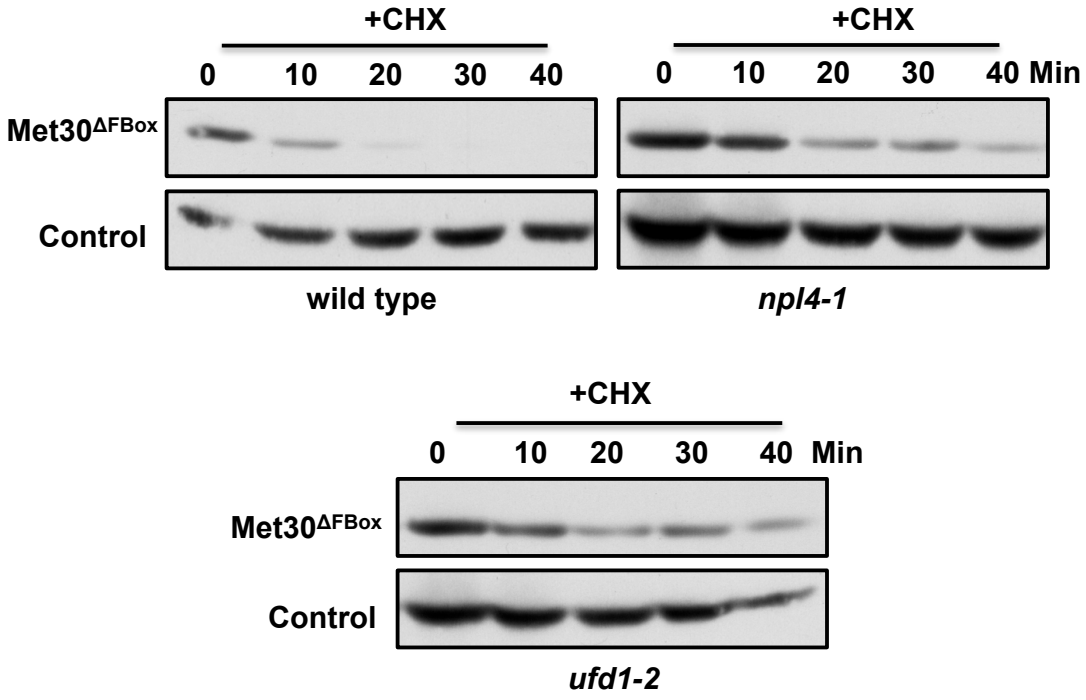


**Figure 4-4. Cdc48 cofactors Npl4 and Ufd1 are involved in 'Skp1-free' Met30 degradation.** Wild type, *npl4-1,* and *ufd1-2* temperature sensitive strains expressing $^{12myc}Met30^{\Delta Fbox}$ were cultured at permissive temperature (25°C) and then shifted to non-permissive temperature (37°C) for 1.5 h. Cycloheximide was added and samples were collected at the time intervals indicated. $Met30^{\Delta Fbox}$ protein stability was analyzed by immunoblotting with anti-myc antibodies.

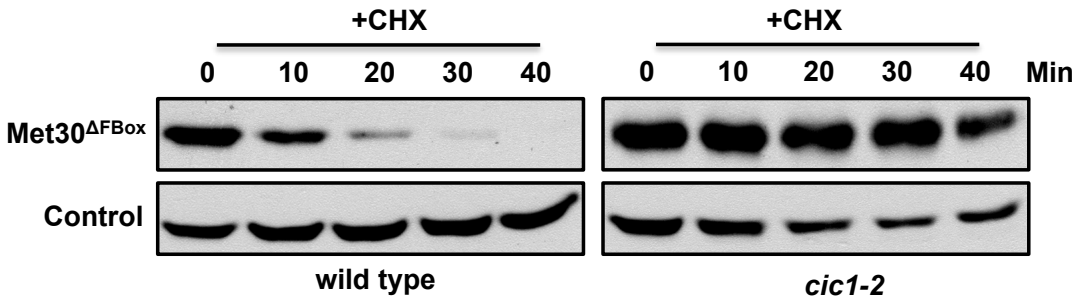


**Figure 4-5. Cic1 is involved in 'Skp1-free' Met30 degradation pathway.** Wild type and *cic1-2* strains expressing $^{12myc}Met30^{\Delta Fbox}$ were cultured at permissive temperature (25°C) and then shifted to non-permissive temperature (37°C) for 8 h. Cycloheximide was added and cells were collected at the time intervals indicated. $Met30^{\Delta Fbox}$ protein stability was analyzed by immunoblotting with anti-myc antibodies.

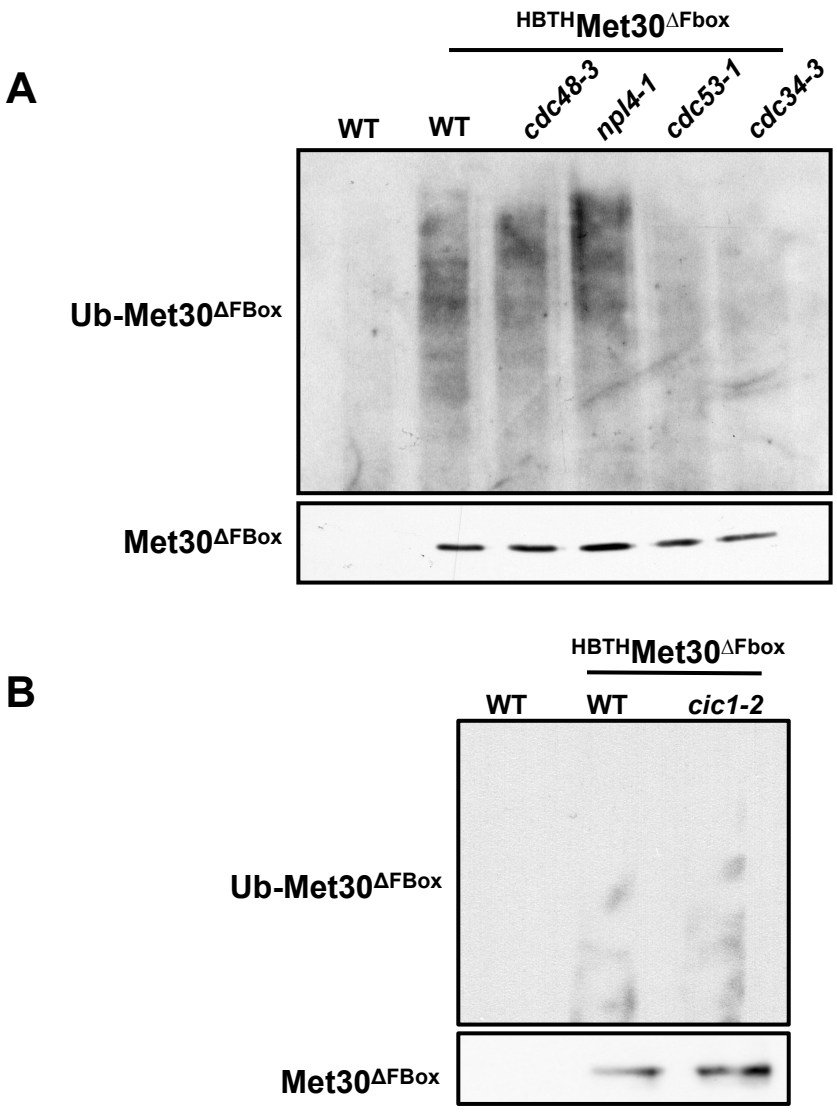


**Figure 4-6. Cdc48, Npl4, and Cic1 are not required for ubiquitylating Met30$^{\Delta Fbox}$** (A) Wild type, *cdc48-3*, *npl4-2*, *cdc53-1*, and *cdc34-2* cells expressing $^{HBTH}$Met30$^{\Delta Fbox}$ under control of the *GAL1* promoter were shifted to 37°C for 1.5 h to inactivate temperature sensitive alleles. $^{HBTH}$Met30$^{\Delta Fbox}$ was purified on $Ni^{2+}$ -sepharose under denaturing conditions and analyzed by immunoblotting using antibodies directed against ubiquitin (upper panel) or the RGS6H epitope in HBTH tag (lower panel). Cells expressing untagged Met30$^{\Delta Fbox}$ were processed as control. (B) Wild type and *cic1-2* mutants expressing $^{HBTH}$Met30$^{\Delta Fbox}$ under control of the *ADH1* promoter were shifted to 37°C for 8 h to inactivate temperature sensitive allele. Cells were processed as described above.

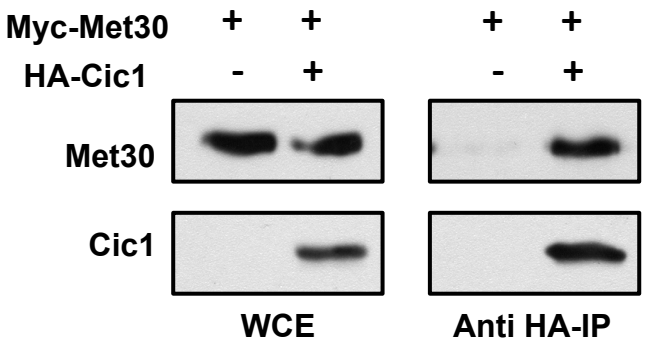


**Figure 4-7. Cic1 binds Met30 *in vivo*.** Cells expressing $^{3HA}$Cic1 and endogenous $^{12Myc}$Met30 were cultured at 30°C. $^{3HA}$Cic1 was immunopurified and co-purified proteins were analyzed by immunoblotting using anti-myc and anti-HA antibodies. Yeast strain expressing untagged Cic1 was used as control.

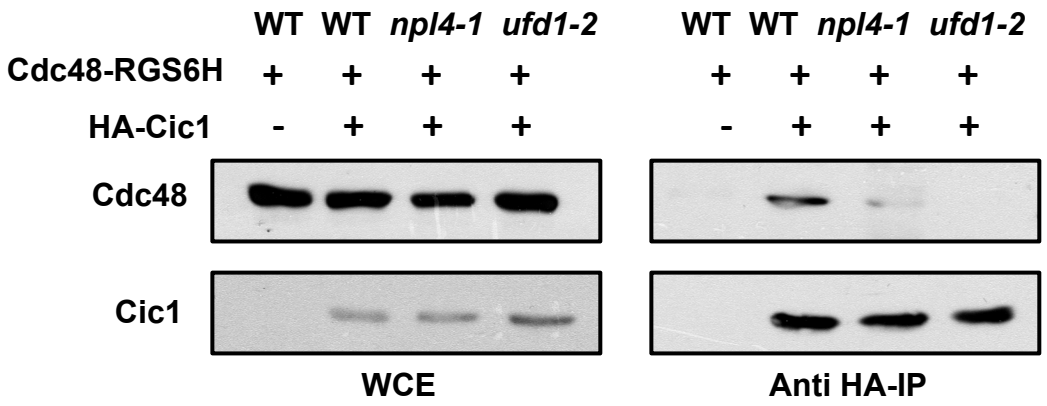


**Figure 4-8. Cic1 interaction with Cdc48 depends on Npl4 and Ufd1.** Cells expressing $^{3HA}$Cic1 and endogenous $^{RGS6H}$Cdc48 in wild type, *npl4-1*, and *ufd1-2* mutants were cultured at permissive temperature (25°C) and then shifted to non-permissive temperature (37°C) for 1.5 h. $^{3HA}$Cic1 was immunopurified, and co-purified proteins were analyzed by immunoblotting using anti-RGS6H and anti-HA antibodies. Yeast strain expressing untagged Cic1 was used as control.

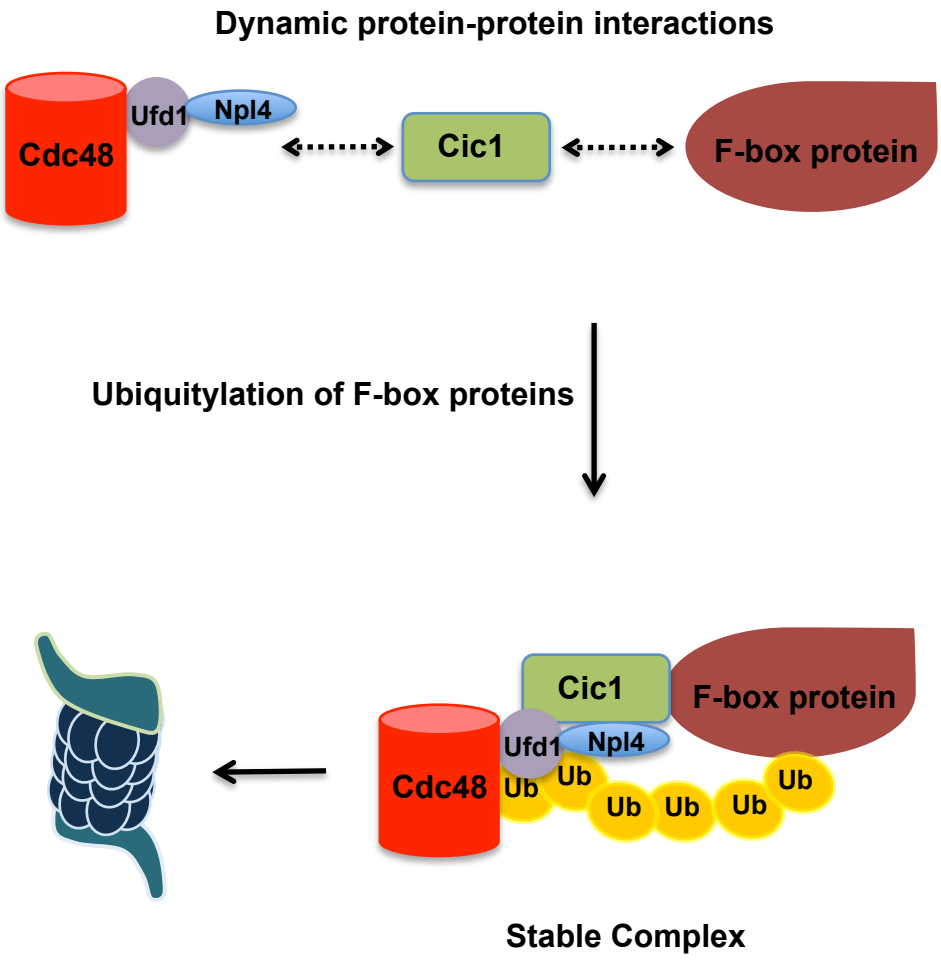


**Figure 4-9. Model for Cic1 as a F-box protein specificity factor for Cdc48/Npl4/Ufd1.** Cic1 forms dynamic interactions with F-box proteins and Cdc48/Npl4/Ufd1 complex. Upon ubiquitylation of F-box proteins, Cdc48 cofactors Ufd1 and Npl4 bind to the ubiquitin chain and stabilize this complex. Cdc48 then directs this complex to the proteasome for degradation of F-box proteins.

# CHAPTER 5

## Concluding Remarks

SCF ubiquitin ligases comprise the biggest subfamily of E3 enzymes within the ubiquitin system. These ligases ubiquitylate their targets and modulate stability, protein-protein interactions, localization, or activity thereby affecting numerous biological and cellular processes in diverse ways. SCF ligases consist of three invariant components - Cdc53 (cullin-1), Skp1, Rbx1, and a changeable substrate recognition adaptor — the F-box protein (Deshaies and Joazeiro, 2009; Duda et al., 2011). Distinct SCF ligases are defined by their F-box proteins, each of which recognizes a specific subset of substrates thereby endowing this system with high specificity of reaction. The results presented in this dissertation shed light on how SCF ligases are regulated by limiting abundance of F-box proteins.

The protein levels of invariable SCF components remains unchanged in the cells while the F-box proteins are unstable and readily degraded. As SCF ligases comprise of the same core components but differ in substrate adaptors, regulation of these ligases by degradation of F-box proteins allows for a quick reassembly of the complex with different F-box proteins to adapt to environmental and cellular changes. In agreement with this mechanism of regulation, F-box proteins assembled into SCF complexes are self-ubiquitylated and targeted for degradation. This autocatalytic degradation is attenuated by substrates ensuring that sufficient levels of F-box proteins are maintained to target their substrates (Galan and Peter, 1999; Zhou and Howley, 1998).

As F-box proteins function as substrate adaptors, it is also important to restrict abundance of unbound F-box proteins. Excess F-box proteins can compete with other F-box proteins for Skp1 binding and can interact with substrates and limit their recognition by fully assembled

ligases. Using F-box protein Met30 as a model, we have uncovered a novel mechanism of F-box protein regulation that targets excess F-box proteins that are 'free' of an SCF complex. This pathway is dependent on the ubiquitin proteasome system and requires functional Cdc53, Cdc34, and Rbx1, but not Skp1. Also, this mechanism of regulation is likely applicable for other F-box proteins, as both Cdc4 and Ctf13 are degraded in the absence of Skp1.

The $SCF^{Met30}$ ubiquitin ligase negatively regulates Met4 by degradation independent ubiquitylation. Met4 has two ubiquitin binding domains which protect polyubiquitylated Met4 from proteasomal recognition (Flick et al., 2006; Tyrrell et al., 2010). Met4 also functions as a substrate receptor in the context of $SCF^{Met30/Met4}$ to coordinate degradation of its cofactor Met32 (Ouni et al., 2010). Thus, under normal cellular conditions, Met30 remains associated with Met4 preventing Met30 degradation by autoubiquitylation. Our results demonstrate that optimal Met30 levels are primarily maintained by 'Skp1-free' F-box protein degradation pathway, which plays an important role in limiting its abundance to prevent any excess Met30 from sequestering Met4.

Upon further characterization of this novel 'Skp1-free' F-box protein degradation pathway, we uncovered two novel regulators of F-box protein degradation, which likely play a role in a post ubiquitylation step in the pathway. Cic1 and Cdc48, along with its cofactors Npl4 and Ufd1, are required for degradation of 'Skp1-free' F-box proteins. Our preliminary studies suggest that Cic1 acts as a novel factor to specify ubiquitylated F-box proteins to Cdc48/Npl4/Ufd1 for targeting to the proteasome for degradation. Further studies will explore the exact molecular mechanism of Cdc48 and Cic1 in this pathway.

Our work suggests a new type of cullin-1 based ubiquitin ligase that does not rely on Skp1. *In vitro* ubiquitylation of Met30 with Cdc53/Rbx1 in the presence of Cdc34 was not efficient and required long reaction times. We have yet not been able to determine whether

another factor or a post-translational modification missing in the recombinant proteins is required for effective ubiquitylation of F-box protein substrate. Alternatively, Cdc53 expressed in *E. coli* may not efficiently fold into its active structure and thus be only partially active. Exploring these options will be a subject of future studies.

In summary, the studies presented here advance our knowledge about regulation of SCF ubiquitin ligases by describing a novel mechanism for regulation of F-box proteins. It will be interesting to explore similar regulatory mechanisms in higher eukaryotes and define their role in human health.

# REFERENCES


Aghajan, M., Jonai, N., Flick, K., Fu, F., Luo, M., Cai, X., Ouni, I., Pierce, N., Tang, X., Lomenick, B.*, et al.* (2010). Chemical genetics screen for enhancers of rapamycin identifies a specific inhibitor of an SCF family E3 ubiquitin ligase. Nature biotechnology *28*, 738-742.

Alexandru, G., Graumann, J., Smith, G.T., Kolawa, N.J., Fang, R., and Deshaies, R.J. (2008). UBXD7 binds multiple ubiquitin ligases and implicates p97 in HIF1alpha turnover. Cell *134*, 804-816.

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.

Bai, C., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J.W., and Elledge, S.J. (1996). SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. Cell *86*, 263-274.

Barbey, R., Baudouin-Cornu, P., Lee, T.A., Rouillon, A., Zarzov, P., Tyers, M., and Thomas, D. (2005). Inducible dissociation of SCF(Met30) ubiquitin ligase mediates a rapid transcriptional response to cadmium. The EMBO journal *24*, 521-532.

Bays, N.W., and Hampton, R.Y. (2002). Cdc48-Ufd1-Npl4: stuck in the middle with Ub. Current biology : CB *12*, R366-371.

Bergink, S., and Jentsch, S. (2009). Principles of ubiquitin and SUMO modifications in DNA repair. Nature *458*, 461-467.

Bosu, D.R., and Kipreos, E.T. (2008). Cullin-RING ubiquitin ligases: global regulation and activation cycles. Cell division *3*, 7.

Brunson, L.E., Dixon, C., Kozubowski, L., and Mathias, N. (2004). The amino-terminal portion of the F-box protein Met30p mediates its nuclear import and assimilation into an SCF complex. The Journal of biological chemistry *279*, 6674-6682.

Buchberger, A. (2013). Roles of cdc48 in regulated protein degradation in yeast. Sub-cellular biochemistry *66*, 195-222.

Cardozo, T., and Pagano, M. (2004). The SCF ubiquitin ligase: insights into a molecular machine. Nature reviews Molecular cell biology *5*, 739-751.

Cheng, Y.L., and Chen, R.H. (2010). The AAA-ATPase Cdc48 and cofactor Shp1 promote chromosome bi-orientation by balancing Aurora B activity. Journal of cell science *123*, 2025-2034.

Ciechanover, A. (2003). The ubiquitin proteolytic system and pathogenesis of human diseases: a novel platform for mechanism-based drug targeting. Biochemical Society transactions *31*, 474-481.

Connelly, C., and Hieter, P. (1996). Budding yeast SKP1 encodes an evolutionarily conserved kinetochore protein required for cell cycle progression. Cell *86*, 275-285.

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.

Deshaies, R.J. (1999). SCF and Cullin/Ring H2-based ubiquitin ligases. Annual review of cell and developmental biology *15*, 435-467.

Deshaies, R.J., Emberley, E.D., and Saha, A. (2010). Control of cullin-ring ubiquitin ligase activity by nedd8. Sub-cellular biochemistry *54*, 41-56.

Deshaies, R.J., and Joazeiro, C.A. (2009). RING domain E3 ubiquitin ligases. Annual review of biochemistry *78*, 399-434.

Duda, D.M., Borg, L.A., Scott, D.C., Hunt, H.W., Hammel, M., and Schulman, B.A. (2008). Structural insights into NEDD8 activation of cullin-RING ligases: conformational control of conjugation. Cell *134*, 995-1006.

Duda, D.M., Scott, D.C., Calabrese, M.F., Zimmerman, E.S., Zheng, N., and Schulman, B.A. (2011). Structural regulation of cullin-RING ubiquitin ligase complexes. Current opinion in structural biology *21*, 257-264.

Eisenhaber, B., Chumak, N., Eisenhaber, F., and Hauser, M.T. (2007). The ring between ring fingers (RBR) protein family. Genome biology *8*, 209.

Elsasser, S., and Finley, D. (2005). Delivery of ubiquitinated substrates to protein-unfolding machines. Nature cell biology *7*, 742-749.

Emter, R., Heese-Peck, A., and Kralli, A. (2002). ERG6 and PDR5 regulate small lipophilic drug accumulation in yeast cells via distinct mechanisms. FEBS letters *521*, 57-61.

Feldman, R.M., Correll, C.C., Kaplan, K.B., and Deshaies, R.J. (1997). A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. Cell *91*, 221-230.

Finley, D., Ulrich, H.D., Sommer, T., and Kaiser, P. (2012). The ubiquitin-proteasome system of Saccharomyces cerevisiae. Genetics *192*, 319-360.

Flick, K., and Kaiser, P. (2013). Set them free: F-box protein exchange by Cand1. Cell research *23*, 870-871.

Flick, K., Raasi, S., Zhang, H., Yen, J.L., and Kaiser, P. (2006). A ubiquitin-interacting motif protects polyubiquitinated Met4 from degradation by the 26S proteasome. Nature cell biology *8*, 509-515.

Frescas, D., and Pagano, M. (2008). Deregulated proteolysis by the F-box proteins SKP2 and beta-TrCP: tipping the scales of cancer. Nature reviews Cancer *8*, 438-449.

Galan, J.M., and Peter, M. (1999). Ubiquitin-dependent degradation of multiple F-box proteins by an autocatalytic mechanism. Proceedings of the National Academy of Sciences of the United States of America *96*, 9124-9129.

Galan, J.M., Wiederkehr, A., Seol, J.H., Haguenauer-Tsapis, R., Deshaies, R.J., Riezman, H., and Peter, M. (2001). Skp1p and the F-box protein Rcy1p form a non-SCF complex involved in recycling of the SNARE Snc1p in yeast. Molecular and cellular biology *21*, 3105-3117.

Gao, D., Wan, L., Inuzuka, H., Berg, A.H., Tseng, A., Zhai, B., Shaik, S., Bennett, E., Tron, A.E., Gasser, J.A.*, et al.* (2010). Rictor forms a complex with Cullin-1 to promote SGK1 ubiquitination and destruction. Molecular cell *39*, 797-808.

Gardner, R.G., Nelson, Z.W., and Gottschling, D.E. (2005). Degradation-mediated protein quality control in the nucleus. Cell *120*, 803-815.

Guthrie, C., and Fink, G.R. (1991). Guide to yeast genetics and molecular biology, Vol 194 (San Diego: Academic Press, Inc.).

Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. Annual review of biochemistry *67*, 425-479.

Hoege, C., Pfander, B., Moldovan, G.L., Pyrowolakis, G., and Jentsch, S. (2002). RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. Nature *419*, 135-141.

Huang, L., Kinnucan, E., Wang, G., Beaudenon, S., Howley, P.M., Huibregtse, J.M., and Pavletich, N.P. (1999). Structure of an E6AP-UbcH7 complex: insights into ubiquitination by the E2-E3 enzyme cascade. Science *286*, 1321-1326.

Jager, S., Strayle, J., Heinemeyer, W., and Wolf, D.H. (2001). Cic1, an adaptor protein specifically linking the 26S proteasome to its substrate, the SCF component Cdc4. The EMBO journal *20*, 4423-4431.

Jentsch, S., and Rumpf, S. (2007). Cdc48 (p97): a "molecular gearbox" in the ubiquitin pathway? Trends Biochem Sci *32*, 6-11.

Jiang, Y.H., and Beaudet, A.L. (2004). Human disorders of ubiquitination and proteasomal degradation. Current opinion in pediatrics *16*, 419-426.

Johnson, S.L. (1991). Structure and function analysis of the yeast Cdc4 gene product (Ph.D. Thesis, University of Washington, Seattle, Washington).

Kaiser, P., Su, N.Y., Yen, J.L., Ouni, I., and Flick, K. (2006). The yeast ubiquitin ligase SCFMet30: connecting environmental and intracellular conditions to cell division. Cell division *1*, 16.

Kaplan, K.B., Hyman, A.A., and Sorger, P.K. (1997). Regulating the yeast kinetochore by ubiquitin-dependent degradation and Skp1p-mediated phosphorylation. Cell *91*, 491-500.

Koegl, M., Hoppe, T., Schlenker, S., Ulrich, H.D., Mayer, T.U., and Jentsch, S. (1999). A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. Cell *96*, 635-644.

Komander, D. (2009). The emerging complexity of protein ubiquitination. Biochemical Society transactions *37*, 937-953.

Kus, B.M., Caldon, C.E., Andorn-Broza, R., and Edwards, A.M. (2004). Functional interaction of 13 yeast SCF complexes with a set of yeast E2 enzymes in vitro. Proteins *54*, 455-467.

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 & development *12*, 914-926.

Li, Y., Gazdoiu, S., Pan, Z.Q., and Fuchs, S.Y. (2004). Stability of homologue of Slimb F-box protein is regulated by availability of its substrate. The Journal of biological chemistry *279*, 11074-11080.

Lydeard, J.R., Schulman, B.A., and Harper, J.W. (2013). Building and remodelling Cullin-RING E3 ubiquitin ligases. EMBO reports *14*, 1050-1061.

Manchado, E., Eguren, M., and Malumbres, M. (2010). The anaphase-promoting complex/cyclosome (APC/C): cell-cycle-dependent and -independent functions. Biochemical Society transactions *38*, 65-71.

Mathias, N., Johnson, S., Byers, B., and Goebl, M. (1999). The abundance of cell cycle regulatory protein Cdc4p is controlled by interactions between its F box and Skp1p. Molecular and cellular biology *19*, 1759-1767.

Medicherla, B., Kostova, Z., Schaefer, A., and Wolf, D.H. (2004). A genomic screen identifies Dsk2p and Rad23p as essential components of ER-associated degradation. EMBO Rep *5*, 692-697.

Metzger, M.B., Hristova, V.A., and Weissman, A.M. (2012). HECT and RING finger families of E3 ubiquitin ligases at a glance. Journal of cell science *125*, 531-537.

Meyer, H., Bug, M., and Bremer, S. (2012). Emerging functions of the VCP/p97 AAA-ATPase in the ubiquitin system. Nature cell biology *14*, 117-123.

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.

Ouni, I., Flick, K., and Kaiser, P. (2010). A transcriptional activator is part of an SCF ubiquitin ligase to control degradation of its cofactors. Molecular cell *40*, 954-964.

Pashkova, N., Gakhar, L., Winistorfer, S.C., Yu, L., Ramaswamy, S., and Piper, R.C. (2010). WD40 repeat propellers define a ubiquitin-binding domain that regulates turnover of F box proteins. Molecular cell *40*, 433-443.

Pesin, J.A., and Orr-Weaver, T.L. (2008). Regulation of APC/C activators in mitosis and meiosis. Annual review of cell and developmental biology *24*, 475-499.

Peters, J.M. (2006). The anaphase promoting complex/cyclosome: a machine designed to destroy. Nature reviews Molecular cell biology *7*, 644-656.

Petroski, M.D., and Deshaies, R.J. (2005). Function and regulation of cullin-RING ubiquitin ligases. Nature reviews Molecular cell biology *6*, 9-20.

Pickart, C.M. (2001). Mechanisms underlying ubiquitination. Annual review of biochemistry *70*, 503-533.

Pierce, N.W., Lee, J.E., Liu, X., Sweredoski, M.J., Graham, R.L., Larimore, E.A., Rome, M., Zheng, N., Clurman, B.E., Hess, S.*, et al.* (2013). Cand1 promotes assembly of new SCF complexes through dynamic exchange of F box proteins. Cell *153*, 206-215.

Reed, S.I., Hadwiger, J.A., and Lorincz, A.T. (1985). Protein kinase activity associated with the product of the yeast cell division cycle gene CDC28. Proceedings of the National Academy of Sciences of the United States of America *82*, 4055-4059.

Richly, H., Rape, M., Braun, S., Rumpf, S., Hoege, C., and Jentsch, S. (2005). A series of ubiquitin binding factors connects CDC48/p97 to substrate multiubiquitylation and proteasomal targeting. Cell *120*, 73-84.

Rotin, D., and Kumar, S. (2009). Physiological functions of the HECT family of ubiquitin ligases. Nature reviews Molecular cell biology *10*, 398-409.

Rouillon, A., Barbey, R., Patton, E.E., Tyers, M., and Thomas, D. (2000). Feedback-regulated degradation of the transcriptional activator Met4 is triggered by the SCF(Met30 )complex. The EMBO journal *19*, 282-294.

Sakamoto, K.M. (2002). Ubiquitin-dependent proteolysis: its role in human diseases and the design of therapeutic strategies. Molecular genetics and metabolism *77*, 44-56.

Schwob, E., Bohm, T., Mendenhall, M.D., and Nasmyth, K. (1994). The B-type cyclin kinase inhibitor p40SIC1 controls the G1 to S transition in S. cerevisiae. Cell *79*, 233-244.

Scott, D.C., Monda, J.K., Grace, C.R., Duda, D.M., Kriwacki, R.W., Kurz, T., and Schulman, B.A. (2010). A dual E3 mechanism for Rub1 ligation to Cdc53. Molecular cell *39*, 784-796.

Seol, J.H., Feldman, R.M., Zachariae, W., Shevchenko, A., Correll, C.C., Lyapina, S., Chi, Y., Galova, M., Claypool, J., Sandmeyer, S.*, et al.* (1999). Cdc53/cullin and the essential Hrt1 RING-H2 subunit of SCF define a ubiquitin ligase module that activates the E2 enzyme Cdc34. Genes & development *13*, 1614-1626.

Seol, J.H., Shevchenko, A., and Deshaies, R.J. (2001). Skp1 forms multiple protein complexes, including RAVE, a regulator of V-ATPase assembly. Nature cell biology *3*, 384-391.

Siergiejuk, E., Scott, D.C., Schulman, B.A., Hofmann, K., Kurz, T., and Peter, M. (2009). Cullin neddylation and substrate-adaptors counteract SCF inhibition by the CAND1-like protein Lag2 in Saccharomyces cerevisiae. The EMBO journal *28*, 3845-3856.

Skowyra, D., Craig, K.L., Tyers, M., Elledge, S.J., and Harper, J.W. (1997). F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. Cell *91*, 209-219.

Smothers, D.B., Kozubowski, L., Dixon, C., Goebl, M.G., and Mathias, N. (2000). The abundance of Met30p limits SCF(Met30p) complex activity and is regulated by methionine availability. Molecular and cellular biology *20*, 7845-7852.

Tang, X., Orlicky, S., Lin, Z., Willems, A., Neculai, D., Ceccarelli, D., Mercurio, F., Shilton, B.H., Sicheri, F., and Tyers, M. (2007). Suprafacial orientation of the SCFCdc4 dimer accommodates multiple geometries for substrate ubiquitination. Cell *129*, 1165-1176.

Tyrrell, A., Flick, K., Kleiger, G., Zhang, H., Deshaies, R.J., and Kaiser, P. (2010). Physiologically relevant and portable tandem ubiquitin-binding domain stabilizes polyubiquitylated proteins. Proceedings of the National Academy of Sciences of the United States of America *107*, 19796-19801.

Varshavsky, A. (2012). The ubiquitin system, an immense realm. Annual review of biochemistry *81*, 167-176.

Verma, R., Oania, R., Graumann, J., and Deshaies, R.J. (2004). Multiubiquitin chain receptors define a layer of substrate selectivity in the ubiquitin-proteasome system. Cell *118*, 99-110.

Welcker, M., and Clurman, B.E. (2008). FBW7 ubiquitin ligase: a tumour suppressor at the crossroads of cell division, growth and differentiation. Nature reviews Cancer *8*, 83-93.

Wenzel, D.M., and Klevit, R.E. (2012). Following Ariadne's thread: a new perspective on RBR ubiquitin ligases. BMC biology *10*, 24.

Wu, S., Zhu, W., Nhan, T., Toth, J.I., Petroski, M.D., and Wolf, D.A. (2013). CAND1 controls in vivo dynamics of the cullin 1-RING ubiquitin ligase repertoire. Nature communications *4*, 1642.

Yen, J.L. (2008). A novel mechanism of regulation of SCF ubiquitin ligases. (Ph.D. Thesis, University of California, Irvine).

Yen, J.L., Flick, K., Papagiannis, C.V., Mathur, R., Tyrrell, A., Ouni, I., Kaake, R.M., Huang, L., and Kaiser, P. (2012). Signal-Induced Disassembly of the SCF Ubiquitin Ligase Complex by Cdc48/p97. Molecular cell *48*, 288-297.

Yen, J.L., Su, N.Y., and Kaiser, P. (2005). The yeast ubiquitin ligase SCFMet30 regulates heavy metal response. Molecular biology of the cell *16*, 1872-1882.

Zemla, A., Thomas, Y., Kedziora, S., Knebel, A., Wood, N.T., Rabut, G., and Kurz, T. (2013). CSN- and CAND1-dependent remodelling of the budding yeast SCF complex. Nature communications *4*, 1641.

Zheng, N., Schulman, B.A., Song, L., Miller, J.J., Jeffrey, P.D., Wang, P., Chu, C., Koepp, D.M., Elledge, S.J., Pagano, M*., et al.* (2002). Structure of the Cul1-Rbx1-Skp1-F boxSkp2 SCF ubiquitin ligase complex. Nature *416*, 703-709.

Zheng, N., Wang, P., Jeffrey, P.D., and Pavletich, N.P. (2000). Structure of a c-Cbl-UbcH7 complex: RING domain function in ubiquitin-protein ligases. Cell *102*, 533-539.

Zhou, P., and Howley, P.M. (1998). Ubiquitination and degradation of the substrate recognition subunits of SCF ubiquitin-protein ligases. Molecular cell *2*, 571-580.

Zimmerman, E.S., Schulman, B.A., and Zheng, N. (2010). Structural assembly of cullin-RING ubiquitin ligase complexes. Current opinion in structural biology *20*, 714-721.