Universitat Ramon Llull

DOCTORAL THESIS

Title	Structure-function study on Rpn10 monoubiquitination
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Al Bon Iver per ajudar-me amb les reaccions d'ubicuitinació.
Al Henry David Thoreau per ensenyar-me la importància de les coses.
A la Mareta per estimar-me tant.

AGRAÏMENTS

Lab: espai on jugar, pintar, cridar, saltar (Jump! Jump! Jump!), caminar, córrer, cantar, fer bromes, dir tonteries, *mentre treballes*.

PRESENT

La primera persona en qui penso ets tu, Ali. Pocs mesos després d'haver-nos conegut al 2010, vas entrar totalment a la meva vida. Estàvem tu i jo, i els nostres problemes i les nostres rallades i les nostres xerrades i la relativització de les situacions que ens duien cap a la tranquil·litat. Amb tu, he crescut. Et miro i sé com estàs, em mires i... Tot i ser molt diferents, el que sento per tu és molt gran. T'estimo Alixixi. Gràcies per deixar-me aprendre, dia rere dia, al teu costat.

Marie. I.LO.V.E.Y.O.U. I have been so lucky that one day you decided to come to Barcelona. A mi derecha en la poyata, a mi izquierda en el escritorio. Always ready to help, to listen to, to share. You've been more than a labmate, you are my teacher, my partner in inventing companies, my friend.

Anne, **I** think of you, I **c**an **h**ear your voice, how you **l**augh...und Tränen. Your heart **i**s so big. Secretly, I've tri**e**d to learn from you. I feel your generosity and *good-person style* and I want you to **b**e close to m**e**. You make me HAPPY when I see you smiling or laughing after I make a *high-level* joke. **DICH**.

B de Bernat. El primer dia que ens vam conèixer vam estar mirant junts el mapa de Boston, buscant on havíem viscut. Molts caps fan por, són autoritaris i fan que els seus treballadors ho passin malament i siguin infeliços. Passem masses hores treballant al dia com per permetre'ns el luxe de no estar bé a la feina. Tu, Bernat, m'has deixat gaudir tant d'aquesta feina que la majoria de divendres volia que arribés el dilluns.

Els planetes es van aliniar i a la Clara em van portar. Parlem la mateixa llengua. Admiro la teva maduresa, serenitat i tonteria! Gràcies per escoltar-me sempre que ho he necessitat i per ser còmplice de tots els meus side-projects –Mou, Happybox i HappyWeek (pam! màrketing! ;)). Espero poder compartir molts més moments amb tu ¡ɐuəu oɯɪɬsə,ɬ.

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PASSAT

Tot va començar amb l'Héctor Ruíz al lab de la Marta Pascual de Biologia. Primeres PCRs i la clau per anar al lab del Radu Popa a Portland i el del Roberto Kolter a Boston. Gràcies Héctor per confiar en mi i ficar-me de ple en aquest món. *Cosinet ;)*

AGRAÏMENTS

Acabar aquesta etapa és dir adéu a la Pilar d'aquests anys. Per això necessito escriure agraïments; quan sigui gran voldré recordar les persones més importants que han estat a prop durant la tesi.

Dani, recuerdo nuestros cafés matutinos en el bar de la Harvard Medical School antes de empezar el día, tus bromas y nuestra confianza. Contigo descubrí que me encanta la investigación. Tienes un pedacito de mi corazón. ¡Esto es lo que hay, Francisco!

Aunque no estuvieras, estabas. Galleguinho de la muerte, estoy tan contenta de haber compartido contigo mi tiempo en Boston. Las beer hours, los cafés upstairs, el Mathlab, nuestras charlas científicas… No sé si lo recordarás pero fuiste tú quien me avisó de que en el lab next door, en el Parc, buscaban a alguien. Gracias por estar a mi lado y contagiarme las ganas de ser Leonardo da Vinci.

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Juliol 2010, arribo al lab del Bernat i del Tim. És el primer dia i estic a punt de conèixer a la Paqui, al Toni, la Vero, la Marta Guerra, l'Òscar, la Marta Isasa... Buf. Quasi, quasi, només escrivint el teu nom m'emociono, Marta. Ets pura vida. Ets personalitat i sensibilitat extremes. Ha estat un privilegi tenir-te com a mestra. Estic molt contenta d'haver compartit amb vosaltres la primera etapa del doctorat. Gràcies a tots per haver-me ensenyat i ajudat amb els meus primers experiments de biologia molecular i per fer-me sentir tan a gust entre vosaltres. Marta Guerra, tinc un carinyo per tu molt especial. Penso en tu i m'entra molt bon rollo.

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PERFET I FUTUR

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Tito. Tito. Tito. Tito. Tito. Tito. Tito. Tito. Tito. Tito. Tito. Tito. Tito. Tito. Tito. Tito. Tito. Tito.
Membres del tribunal, li hauríeu de donar al Tito el títol de doctor perquè m'ha escoltat taaaantes vegades explicar què és el proteasoma, Rpn10, l'ubicuitinació, que s'ho sap tot de memòria. Amor, ets una droga. Vull evitar dir que "sense tu això no hagués estat possible", perquè la Mar em mata jejeje Sense tu hagués estat diferent i l'única cosa que sé del cert és que tenint-te al meu costat sóc hiperfeliç.

Com diu el Raimon, qui perd els orígens, per la identitat i mentre us tingui a prop, sabré qui sóc!

Endavant i sense por!

RESUM

TITOL:

Estudi estructura-funció sobre la monoubicuitinació de Rpn10.

RESUM:

L'homeòstasi cel·lular depèn, en part, de la proteòlisi regulada pel sistema Ubicuitina-Proteasoma (UPS). Aquesta proteòlisi inclou la degradació de les proteïnes defectuoses o innecessàries per la cèl·lula i consta de dues etapes. En un primer pas, molècules d'ubicuitina s'uneixen covalentment a un grup amino (ε-NH2) d'alguna lisina del substrat diana.

Aquest procés depèn de l'activitat enzimàtica de tres enzims, E1, E2 i E3. Tot seguit, el proteasoma s'encarrega de la seva degradació. El proteasoma és un complex multiproteic en forma de túnel de 2,5 MDa present en el nucli i el citoplasma de totes les cèl·lules eucariotes i Archaebacteria.
La senyal típica de degradació és una cadena d'ubicuitines que es forma a través d'enllaços isopeptídics entre la glicina G76 a la part C-terminal de la molècula i la lisina K48 de la següent ubicuitina.

Avui dia, s'han descrit tres receptors proteasomals de substrats poliubicuitinats, Rpn10, Rpn13 i Rpn15. La subunitat Rpn10 (Rpn10 en llevat de gemmació i S5a en humans), que també es troba en fraccions citosòliques, s'uneix a les cadenes de poliubicuitina mitjançant el domini UIM (Ubiquitin-interacting motif). Aquest domini és essencial per l'ubicuitinació del propi receptor. Els enzims involucrats en tal modificació són Uba1 (E1), Ubc4 (E2) i Rsp5 (E3). La poliubicuitinació de Rpn10 depèn de la participació d'una quarta ubicuitina lligasa, Hul5, i comporta la seva degradació proteasomal. Alhora, s'ha vist que una fracció de Rpn10 està conjugada a una sola molècula d'ubicuitina. Aquest tipus de modificació post-traduccional s'anomena monoubicuitinació. Quan Rpn10 està monoubicuitinat, deixa d'interaccionar amb substrats poliubicuitinats, afectant l'activitat proteolítica del proteasoma.

Resum

Desxifrar els mecanismes pels quals Rpn10 es monoubicuitina ha estat l'objectiu d'aquesta tesi. La hipòtesi inicial és que la proteïna monoubicuitinada pateix un plegament provocat per l'interacció entre l'ubicuitina unida a Rpn10 i l'UIM. Aquest plegament bloquejaria l'UIM i no permetria l'addició de noves ubicuitines per part de la lligasa Rsp5. Per intentar validar la hipòtesi, hem començat optimitzant la reacció de monoubicuitinació de Rpn10 *in vitro* i hem trobat la seqüència mínima indispensable per obtenir tal monoubicuitinació. Hem observat que la meitat N-terminal de Rsp5, és dispensable per l'ubicuitinació de Rpn10 i hem vist que l'augment de la monoubicuitinació de Rpn10 causa un defecte en el creixement de les cèl·lules. Finalment, hem aconseguit poliubicuitinar Rpn10 *in vitro*, sense la participació de Hul5, mitjançant una mutació *en bloc* de la seqüència precedent l'UIM. Hem vist que aquesta seqüència està intrínsecament desestructurada i que la seva flexibilitat podria ser la raó per la qual l'UIM quedés inaccessible quan Rpn10 està monoubicuitinat.

Un coneixement en profunditat del sistema ubicuitina-proteasoma és fonamental per trobar la cura de les patologies humanes derivades de seu mal funcionament. La feina que fem al nostre laboratori és la base per a que en un futur malalties que semblen estar relacionades amb l'UPS -malalties autoinmunes, neurològiques, càncer, cardiopaties...- tinguin un millor tractament.

PARAULES CLAU:

Monoubicuitinació, Rpn10, proteasoma, Rsp5, ubicuitina, proteïna desordenada.

RESUMEN

TÍTULO:

Estudio estructura-función sobre la monoubicuitinación de Rpn10.

RESUMEN:

La homeostasis celular depende, en parte, de la proteólisis regulada por el sistema Ubicuitina-Proteasoma (UPS). Esta proteólisis incluye la degradación de las proteínas defectuosas o innecesarias para la célula y consta de dos etapas. En un primer paso, moléculas de ubicuitina se unen covalentemente al grupo amino (ε-NH2) de alguna lisina del sustrato diana. Este proceso depende de la actividad enzimática de tres enzimas, E1, E2 y E3. Acto seguido, el proteasoma se encarga de su degradación. El proteasoma es un complejo multiproteico en forma de túnel de 2,5 MDa presente en el núcleo y el citoplasma de todas las células eucariotas y Archaebacteria.
La señal típica de degradación es una cadena de ubicuitinas que se forma a través de enlaces isopeptídicos entre la glicina G76 en la parte C-terminal de la molécula y la lisina K48 de la siguiente ubicuitina.

Hoy día, se han descrito tres receptores proteasomales de sustratos poliubicuitinados, Rpn10, Rpn13 y Rpn15. La subunidad Rpn10 (Rpn10 en levadura de gemación y S5a en humanos), que también se encuentra en las fracciones citosólicas, se une a las cadenas de poliubicuitina mediante el dominio UIM (Ubiquitin-interacting motif). Este dominio es esencial para la ubicuitinación del propio receptor. Las enzimas involucradas en tal modificación son Uba1 (E1), Ubc4 (E2) y Rsp5 (E3). La poliubicuitinación de Rpn10 depende de la participación de una cuarta ubicuitina ligasa, Hul5, y conlleva su degradación proteasomal. Asimismo, se ha visto que una fracción de Rpn10 está conjugada a una única molécula de ubicuitina. Este tipo de modificación post-traduccional se llama monoubicuitinación. Cuando Rpn10 está monoubicuitinada, deja de interaccionar con sustratos poliubicuitinados, afectando la actividad proteolítica del proteasoma.

Resumen

Descifrar los mecanismos por los cuales Rpn10 se monoubicuitina ha sido el objetivo de esta tesis. La hipótesis inicial es que la proteína monoubicuitinada sufre un plegamiento provocado por la interacción entre la ubicuitina unida a Rpn10 y el UIM. Este plegamiento bloquearía el UIM y no permitiría la adición de nuevas ubicuitinas por parte de la ligasa Rsp5. Para intentar validar la hipótesis, hemos empezado optimizando la reacción de monoubicuitinación de Rpn10 *in vitro* y hemos encontrado la secuencia mínima indispensable para obtener tal monoubicuitinación. Hemos observado que la mitad N-terminal de Rsp5 es dispensable para la ubicuitinación de Rpn10 y hemos visto que el aumento de la monoubicuitinación de Rpn10 causa un defecto en el crecimiento de las células. Finalmente, hemos conseguido poliubicuitinar Rpn10 *in vitro*, sin la participación de Hul5, mediante una mutación *en bloc* de la secuencia que precede al UIM. Hemos visto que esta secuencia está intrínsecamente desestructurada y que su flexibilidad podría ser la razón por la que el UIM quedaría inaccesible cuando Rpn10 está monoubicuitinada.

Un conocimiento en profundidad del sistema ubicuitina-proteasoma es fundamental para encontrar la cura de las patologías humanas derivadas de su mal funcionamiento. El trabajo que hacemos en nuestro laboratorio es la base para que en un futuro enfermedades que parecen estar relacionadas con el UPS -enfermedades autoinmunes, neurológicas, cáncer, cardiopatías...- tengan un mejor tratamiento.

PALABRAS CLAVE:

Monoubicuitinación, Rpn10, proteasoma, Rsp5, ubicuitina, proteína desordena

SUMMARY

TITLE:

Structure-function study on Rpn10 monoubiquitination.

SUMMARY:

Cellular homeostasis depends, partially, on the proteolysis regulated by the Ubiquitin-Proteasome System (UPS). This proteolysis includes the degradation of misfolded, damaged or unnecessary proteins for the cell and comprises two phases. In the first stage, molecules of Ubiquitin are attached covalently to a target substrate at the amide group (ε-NH2) of some lysine residues. This process depends on the enzymatic activity of three enzymes, E1, E2 and E3. Afterwards, the proteasome takes care of the degradation of the substrate. The proteasome is a barrel-shaped multiprotein complex of 2,5 MDa present in the nucleus and cytosol of eukaryotic and Archaebacteria cells. The canonical degradation signal is a chain of ubiquitins, which is built via isopeptide bonds between the glycine G76 at the C-terminus of the molecule and lysine K48 of the next Ubiquitin.

So far, three Ubiquitin proteasomal receptors, Rpn10, Rpn13 and Rpn15, have been described. The Rpn10 subunit that can also be found in an extraproteasomal pool, binds Ubiquitin chains by means of the Ubiquitin-binding motif (UIM). This domain is also essential for the ubiquitination of Rpn10 itself. The enzymes involved in this modification are Uba1 (E1), Ubc4 (E2) and Rsp5 (E3). Rpn10 polyubiquitination requires an additional Ubiquitin ligase, Hul5, and promotes Rpn10 degradation. However, a fraction of Rpn10 has been shown to be conjugated to only one Ubiquitin molecule, a type of post-translational modification that is called monoubiquitination. When Rpn10 is monoubiquitinated, its capacity to bind polyubiquitinated substrates is impaired, affecting the catalytic activity of the proteasome.

The goal of this thesis is to decipher the mechanisms by which Rpn10 is monoubiquitinated. The initial hypothesis was that the monoubiquitinated protein

undergoes a change in its conformation caused by the interaction between the Ubiquitin bound to Rpn10 and the UIM. This change would block the UIM and would not allow the conjugation of new ubiquitins by the ligase Rsp5. To validate the hypothesis, we started by optimizing the reaction of monoubiquitination of Rpn10 *in vitro* and we have determined the minimal sequence of Rpn10 required for this ubiquitination. We have shown that the N-terminus of Rsp5 is not essential for Rpn10 ubiquitination and observed that an increase in the levels of monoubiquitinated Rpn10 causes a slow-growth defect. Finally, we have been able to polyubiquitinate Rpn10 *in vitro,* independently of Hul5, via a mutation *en bloc* of the sequence that precedes the UIM. We have seen that this sequence is intrinsically unstructured and that its flexibility could be the reason why the UIM could be blocked when Rpn10 is monoubiquitinated.

A detailed knowledge of the Ubiquitin-Proteasome System is essential to find the cure for the pathologies that derive from its malfunction. The work we do in our lab is the base for that, in a future, diseases that seem to be related to the UPS - autoimmune, heart or neurological diseases, cancer,…- have a better treatment.

KEYWORDS:

Monoubiquitination, Rpn10, proteasome, Rsp5, Ubiquitin, disordered protein

TABLE OF CONTENTS

Table of contents

INTRODUCTION

Ubiquitin: the kiss of death tags for destruction

Post-translational modifications are regulatory processes altering the composition of a protein, usually through the covalent addition of a small molecule to one of the amino acid residues. Phosphorylation, methylation, acetylation, glycosylation, lipidation, ubiquitination, SUMOylation and proteolysis are some of the modifications that proteins undergo (Hochstrasser, 2000; Pickart, 2001).
Ciechanover was the first to describe a post-translational modification finding that a small molecule, Ubiquitin (termed APF-1 initially), formed covalent conjugates with endogenous reticulocyte proteins (Ciechanover *et al*, 1980). Since then, the Ubiquitin (Ub) field seems to be ever expanding. Ub is an essential protein of 76 amino acids (~8KDa) that participates in complex and diverse regulatory roles and its involvement in multiple processes makes it one of the most versatile signaling molecules in the cell.

Ubiquitination consists of a multi-enzymatic cascade that covalently links Ub to a wide pool of proteins by means of the activity of three enzymes, the Ubiquitin-activating enzyme (E1), the Ubiquitin-conjugating enzyme (E2), and the Ubiquitin ligase enzyme (E3). This enzimatic system is involved in the regulation of multiple and diverse cellular processes, such as proteasomal-dependent protein degradation, antigen processing, apoptosis, biogenesis of organelles, cell cycle and division, DNA transcription and repair, differentiation and development, neural and muscular degeneration, morphogenesis of neural networks, modulation of cell surface receptors, the secretory pathway, response to stress and extracellular modulators, ribosome biogenesis or viral infection (Deshaies & Joazeiro, 2009; Raiborg & Stenmark, 2009; Ulrich & Walden, 2010; Zinngrebe et al, 2013).

Ubiquitin belongs to the Ubiquitin-like proteins (UBLs) family, which are proteins modifiers of around 8 to 20 KDa that are related in sequence to Ub, structurally resemble it and are also covalently linked to other proteins to regulate their functions in eukaryotes via an enzymatic cascade that resembles ubiquitination (Schwartz & Hochstrasser, 2003; Pickart & Eddins, 2004). Some of them are Sumo, Nedd8, ISG15 and Fat10 (van der Veen & Ploegh, 2012).

Proteolytic elimination is one of the most common fates of ubiquitinated proteins, making Ub an ideal regulator. Ubiquitin can modify the localization, stability and properties of its target protein. A proteomics approach to identify

Introduction

Ubiquitin conjugates from *Saccharomyces cerevisiae* lysate showed that Ub modifies around a thousand different proteins (Peng *et al*, 2003). There is an extensive ubiquitination machinery in human cells with more than four to five thousand proteins that can be modified by Ub suggesting that the complexity of protein regulation by ubiquitination could be comparable to that of phosphorylation (Kim *et al*, 2011b; Wagner *et al*, 2011).

The secondary structure of Ub consists of a fold dominated by a β-sheet with five anti-parallel β-strands and one α-helix segment. This fold was termed the B –grasp (β-GF), because the β –sheet appears to grasp the α-helix region (Overington, 1992). The first and last strands are adjacent and parallel to each other; the two strands flanking the former ones are in an anti-parallel position. There is an additional strand that packs against the conserved third strand at the margin of the core β-sheet and the α-helix is packed against one face of the sheet (Burroughs *et al*, 2007).

The characteristic topological feature of Ubiquitin is stabilized by hydrophobic interactions conferring a strong stability and resistance to temperature, pH changes and proteolysis (Ibarra-Molero et al. 1999; Lenkinski et al. 1977; Schlesinger et al. 1975).

Ubiquitin is one of the most conserved polypeptides in eukaryotes: only four of its amino acids differ among yeast, plants and animals, which shows that during evolution, Ub has been extremely refractory to amino acid changes (Glickman & Ciechanover, 2002; Catic & Ploegh, 2005; Zuin *et al*, 2014). Strinkingly, only sixteen of the 63 surface residues of Ub are essential for vegetative growth in yeast. These essential amino acids are found in two distinct functional patches near Ile^{44} and Phe^{4} of the Ubiquitin globular domain and in the C-terminus (Sloper-Mould *et al*, 2001). The mutation of the rest of non-essential positions, even though not deeply characterized, are likely to cause a reduced phenotypical fitness.

Three of the essential residues, Leu^{8}, Ile^{44} and Val^{70}, are known as the hydrophobic patch and are required for proteasomal binding and degradation and endocytosis (Shih *et al*, 2000; Sloper-Mould *et al*, 2001). Mutation of pairs of Leu^{8}, Ile^{44} and Val^{70} inhibits degradation of the polyubiquitinated substrates by blocking the binding of Ub chains to ubiquitin receptors in the proteasome.

A second surface region consisting of residues Gln^{2}, Phe^{4}, and Thr^{12} is required for endocytosis. The mutation of Ile^{44} and Phe^{4} may prevent the interaction with different endocytic proteins (Sloper-Mould *et al*, 2001).

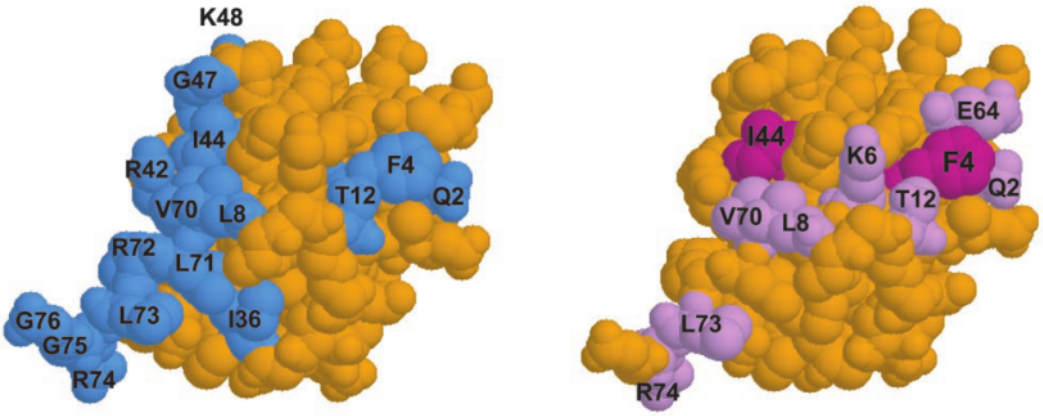


Figure 1. Ubiquitin residues essential for life and internalization.
3D strucuture of Ubiquitin. Left, essential amino acids are highlighted in blue. Right, key residues that promote endocytosis are highlighted in magenta. In pink, residues that play a minor role in internalization.

Ubiquitin can be conjugated to substrate proteins or to itself by means of a covalent isopeptide bond between the C-terminal glycine 76 of Ubiquitin and the amino group of a lysine residue of the protein substrate, a process known as ubiquitination. Additionally, Ubiquitin can bind to specific surfaces, such as Ubiquitin-binding domains, and can also form non-covalent interactions either with Ubiquitin moieties or with Ubiquitin chains (Dikic *et al*, 2009).

Protein conjugation by Ubiquitin-like proteins is not exclusive of eukaryotes: Ubiquitin-systems play also an important regulatory role in prokaryotes and Archaea. The existence of prokaryotic sulphur-carrier proteins involved in molybdenum cofactor (MoaD) and thiamin (ThiS) biosynthesis structurally related to Ubiquitin, sharing a conserved carboxy-terminal Gly-Gly motif, suggest a common origin (Lake *et al*, 2001; Burroughs *et al*, 2009). Regarding the thiamine synthesis, ThiF, a homolog of the E1 enzyme that activates Ubiquitin, adenylates the C-terminus of ThiS. Similarly, MoeB activates the C-terminus of MoaD to form an acyl-adenylate.

Introduction

A homologous conjugation system has recently been reported in Bacteria. TtuB, a bacterial Ubiquitin-like protein found in *Thermus thermophilus* that functions as a sulfur carrier in tRNA thiouridine synthesis, was found to be covalently attached to target proteins by means of its glycine at the C-terminus (Shigi, 2012).
Regarding archaea, SAMPs (small archaeal modifier proteins) are Ubiquitin-like protein modifiers, in the archaea *Haloferax volcanii*, that just require an E1 Ubiquitin activating enzyme competent for sampylation (Humbard *et al*, 2010). However, SAMPs show very low sequence identity (9%) with eukaryotic Ubiquitin (Bienkowska et al., 2003; Darwin, 2009). Interestingly, mutants showing decreased proteasome activity in *Haloferax volcanii* accumulate SAMP conjugates, indicating that SAMPs may play a role in proteasome-dependent degradation.

The genome of *Caldiarchaeum subterraneum* has been sequenced showing that this archaea species harbors an Ubiquitin-like protein modifier system consisting of one single-copy Ubiquitin gene, one Ubiquitin activating enzyme, one Ubiquitin conjugating enzyme, one RING-type Ubiquitin-protein ligase and one deubiquitinating enzyme (DUB) with structural motifs specific to eukaryotic protein system, a system clearly distinct from the prokaryote-type system previously described in Haloferax (Nunoura *et al*, 2011). These five genes in *C. subterraneum* are organized in an operon-like cluster, representing the most simplified genetic arrangement encoding an eukaryote-like Ubiquitin signaling system (Nunoura *et al*, 2011). However, in eukaryotes, the organization of the Ubiquitin-system is substantially different: Ubiquitin is encoded by a multigene family containing three primary members: head-to-tail repeats encoding a polyubiquitin precursor protein (approximately from 4 to 15 Ubiquitin molecules) and two fusions with L40 and S27 ribosomal proteins (Sharp & Li, 1987; Finley *et al*, 1989; Catic & Ploegh, 2005; Ozkaynak *et al*, 1984). Eukaryotic genomes are also provided with a full set of Ubiquitin signaling factors, E1, E2 and E3 enzymes that define a consistent hierarchical structure.

Distinct ubiquitination products generate diversity in Ubiquitin signaling ubiquitination

Ubiquitin contains 7 lysines (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63) that can all be linked to the C-terminus of another Ub or to the N-terminal methionine, resulting in the formation of polymers (Figure 2). When the length of the chain is large enough, containing at least four ubiquitins, the substrate is polyubiquitinated. When there is a single Ubiquitin molecule attached to the protein, the substrate is monoubiquitinated. When the protein is conjugated to one single Ub molecule at more than one lysine, the protein is multi-monoubiquitinated. Both in yeast and in mammalian cells, Ubiquitin linkage types are equally represented (Peng *et al*, 2003; Xu *et al*, 2009; Ziv *et al*, 2011). Depending on the length and type of linkage, the targeted substrate will have one fate or another (Husnjak & Dikic, 2012).

Monoubiquitination (and multi-monoubiquitination) plays a role in the regulation of endocytosis, lysosomal targeting, meiosis and chromatin remodeling while polyubiquitination plays a role in targeting substrates to the proteasome, immune signaling and DNA repair.

Ubiquitin chains can contain one or more than one type of linkage. The first case refers to homotypic chains in which just one lysine participates in the conjugation of Ubiquitin. The second situation refers to the use of distinct lysine residues to connect Ubiquitin moieties- Lys 6/11, Lys 27/29, Lys 29/48 or Lys 29/33 (Kim *et al*, 2007). There is another scenario in which Ubiquitin is connected to other Ubiquitin-like modifiers such as Sumo-2 and Sumo-3 giving rise to heterologous Ub chains (Tatham *et al*, 2001). Finally, Ub-Ub linkages can also be formed on Met1 producing linear chains. In these chains, the C-terminal Gly of Ubiquitin is linked to the Met1 of the next Ubiquitin (Kirisako *et al*, 2006).

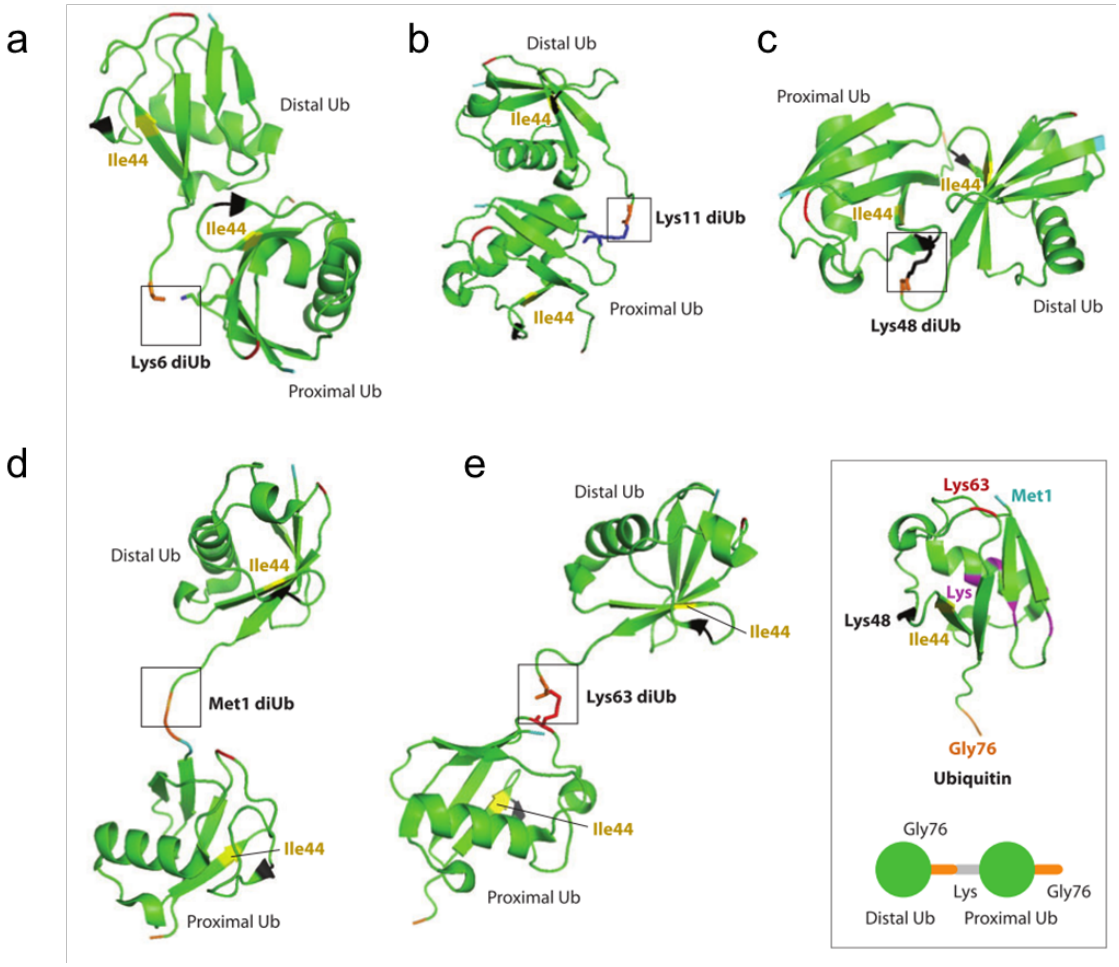


Figure 2. Different Ubiquitin linkages.
Ubiquitin can form eight different homotypic chains. **a, b** and **c** show diubiquitins adopting a compact conformation. **d** and **e** show diubiquitins in an extended conformation. Linkages are shown inside squares and a rectangle at the right. Adapted from (Husnjak & Dikic, 2012).

Ubiquitin moieties interact at different levels with adjacent ubiquitins. When the only interaction takes place in the linkage site, the Ub chain adopts an "open conformation". That is the case for linear and K63 chains (Komander *et al*, 2009b; Tenno *et al*, 2004). In contrast, adjacent ubiquitins in K6, K11 and K48 chains interact with each other and adopt a "compact conformation" (Bremm *et al*, 2010; Tenno *et al*, 2004; Varadan *et al*, 2002).

However, the conformation of these chains is dynamic. Indeed, when the chains are in complex with Ubiquitin-binding domains their conformations change, alternating between open and closed states (Dikic *et al*, 2009). The conformational properties adopted by the different linkages allow the

substrates to be recognized by downstream receptor proteins making the linkages determine the fate of the ubiquitinated substrates. Ubiquitination becomes then a source of signals that has power over the function of a myriad of proteins.

In yeast, Ubiquitin linkages are represented as follows: K6, 10.9%; K11, 28%; K27, 9%; K29, 3.2%; K33, 3.5%; K48, 29% and K63, 16% (Xu *et al*, 2009). In mammalian cells, linear, K6, K27, and K33 linkages represent not more than 0.5%; K11, 2%; K29, 8%; K63, 38% and K48, 52% (Dammer *et al*, 2011).

The Ubiquitinome pattern is not static. For instance, K11 chains are important in both degradative and nonproteolytic pathways. Depending on the stage of the cell cycle, the nature of the linkages can change. During mitosis, K11 chains promote proteasomal degradation. At that moment of the cell-cycle, the E3 ligase Anaphase-Promoting Complex (APC/C) assembles K11-linked chains in substrates such as Securin and Cyclin B1 before degradation (Jin *et al*, 2008). In contrast, other studies associate K11 chains to substrate endocytosis (Boname *et al*, 2010).

Bedford and collaborators found that linkages K6, K11, K29 and K48 contribute to proteasomal degradation in mammalian neurons (Bedford *et al*, 2011). K63 chains act in a range of processes including endosomal trafficking to the lysosome, intracellular signaling, and DNA repair (Ikeda & Dikic, 2008). However, K63 chains-linked substrates cannot be degraded by the proteasomes because soluble factors bind these chains and prevent their association with the proteasome (Nathan *et al*, 2013). In yeast, proteasomal degradation is mediated by K6, K11, K27, K29, K33 and K48 linkages, the latter being described as the principal signal for targeting substrates to the proteasome (Xu *et al*, 2009).

The RBR Ubiquitin ligase Parkin builds atypical K27 chains on the mitochondrial protein Miro1 that may have a role in mitophagy (removing of damaged mitochondria) (Birsa *et al*, 2014). Yuan et al, showed that K33 polyubiquitinated Coronin 7, a protein involved in the protein export from the Golgi, regulates protein trafficking (Yuan *et al*, 2014). K29 chains have been found in substrates targeted for lysosomal degradation (Chastagner *et al*, 2006).

When the linkage does not involve any lysine, the chain formed is called linear. In this type of chain, the N-terminal methionine of an Ubiquitin molecule is linked to the C-terminal glycine of the next Ubiquitin. Linear chains are involved in the development of the immune system and the prevention of chronic inflammation (reviewed in Walczak *et al*, 2012).

Introduction

Enzymatic cascade

Eukaryotes possess a distinctive enzymatic apparatus for Ub-modification, comprised of E1, E2 and E3 enzymes, in which the E1 produces E2 thioesterified with Ubiquitin (E2-Ub) and the E3 binds both to E2-Ub and to the substrate (Dye and Schulman, 2007; Scheffner *et al.* 1995). The polyubiquitinated substrates will be (often) subsequently targeted to, recognized and degraded by the Ubiquitin-Proteasome System (UPS) (Figure 3).

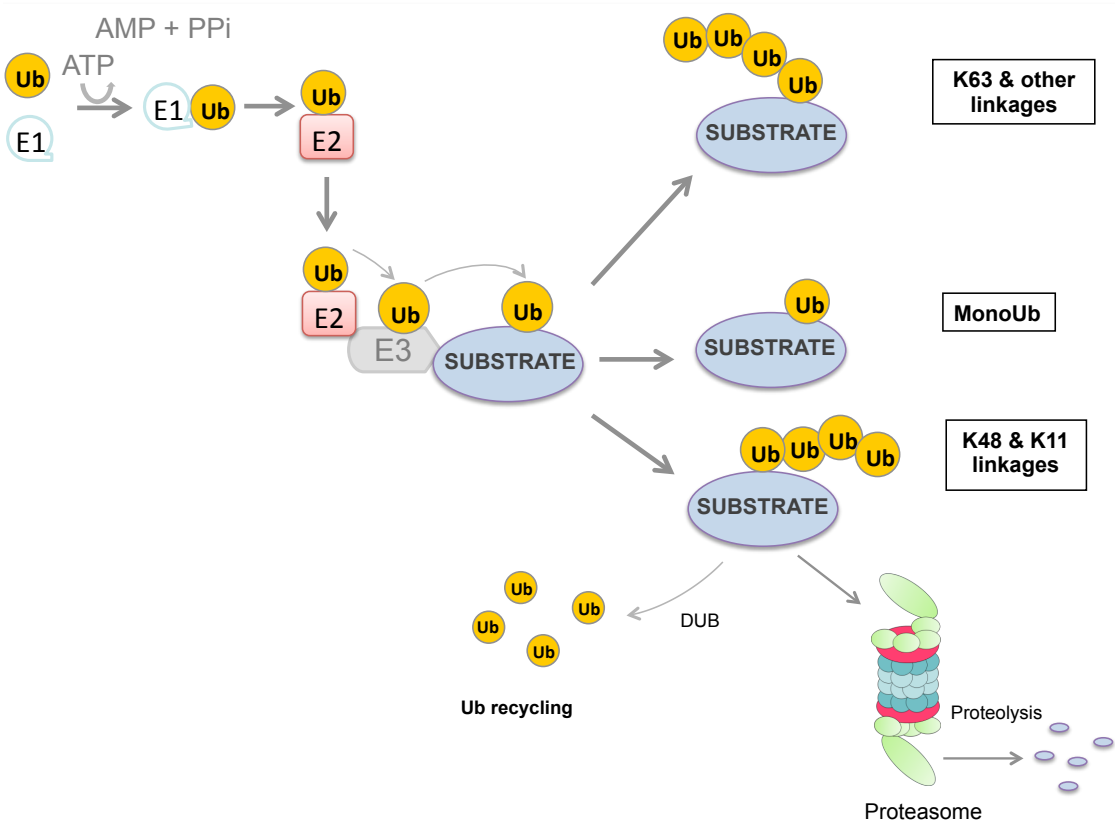


Figure 3. Schematic of the Ubiquitin-Proteasome System and fate of ubiquitinated substrates.
After Ub is activated and transferred from the E1 to an E2, it is attached to an E3 that finally will bind it to a substrate. The linkage topology between ubiquitins determines the fate of the protein. One of the different fates that a polyubiquitinated protein can follow is proteasomal degradation. Several subunits of the proteasome and other factors are involved in this multi-step process. DUBs are deubiquitylases responsible for the recycling of Ubiquitin and chain editing.

These enzymes first catalyze the formation of an isopeptide bond between the C-terminus of Ubiquitin and the amino group of a lysine residue of the protein substrate, leading to monoubiquitination. Multiple lysine residues can be modified with one Ubiquitin leading to multi-monoubiquitination. When the process is repeated several times, elongation occurs, adding Ubiquitin molecules to each other in a growing chain.

The first step is ATP-dependent and is carried out by an Ubiquitin-activating enzyme, E1: a high-energy thioester linkage between Ub and the E1 is generated. Next, the transfer of Ub from the E1 to the active-site cysteine of an Ubiquitin-conjugating enzyme, E2 is catalyzed. Finally, the last step is executed by a member of the Ubiquitin-protein ligase family, E3, resulting in the formation of an isopeptide bond between the C-terminal glycine of Ubiquitin and free lysines either in the target or in the preceding Ubiquitin in the chain (Ciechanover *et al*, 1982; Hershko *et al*, 1983; Glickman & Ciechanover, 2002). In most cases, Ub is conjugated to the epsilon-amino group of a lysine (Glickman and Ciechanover, 2002). In some cases, however, Ubiquitin is conjugated to the NH2-terminal group of the substrate (Ciechanover and Ben-Saadon, 2004), as well as to Cys, Ser, and Thr residues of target proteins (Cadwell and Coscoy, 2005; Ravid and Hochstrasser, 2007).

In yeast, an Ubiquitin chain elongation factor, E4, binds to the Ub moieties of preformed short conjugates and catalyzes Ub chain elongation. Substrates with polyubiquitin chains of more than four Ubiquitins are ready for proteasomal degradation (Crosas *et al*, 2006; Koegl *et al*, 1999).

The structure of the UPS is hierarchical. There is a very reduced number (or, generally, one single representative in unicellular organisms) of Ubiquitin-activating enzymes (E1) that activate Ub and transfer it to a higher number of E2s (Hershko and Ciechanover, 1998; Pickart, 2001). Each of these E2 interacts with several E3s and a much larger number of E3s recognize a set of substrates via their recognition motifs.

This hierarchy is not simply based on a pyramidal structure: there is a whole network of interactions that involves multiple components and raises the complexity of the mechanism to unpredicted levels. A single E3 can interact with two or more distinct E2s, can have several recognition sites targeting different substrates and

the same domain can interact with many substrates. At the same time, different E3s can target one same substrate by recognizing different motifs.

Ubiquitin-activating enzymes, E1

At the start of each ubiquitination cascade is an Ubiquitin-activating enzyme, which activates Ub by binding ATP-Mg^{2+} and Ub. This E1 catalyzes an acyl-adenylation at the C-terminus of Ubiquitin (occupied by a totally conserved Glycine in eukaryotes) (Haas & Rose, 1982). Next, the E1 attacks the adenylated form of Ub by means of its catalytic cysteine creating a complex between the E1 and Ub (Ciechanover *et al*, 1981). At this stage, there is a thioester bond formed between the enzyme and Ubiquitin. Addionally, there is a second Ub bound to the E1 that is adenylated. Finally, the E1 transfers the C-terminus of the activated Ub to a catalytic Cys of an E2 conjugating enzyme catalyzing a thioester bond (Haas *et al*, 1982; Pickart & Rose, 1985).

Ubiquitin activation and conjugation cycle.

The steps involved in Ub activation and conjugation are represented in the following scheme (Figure 4):

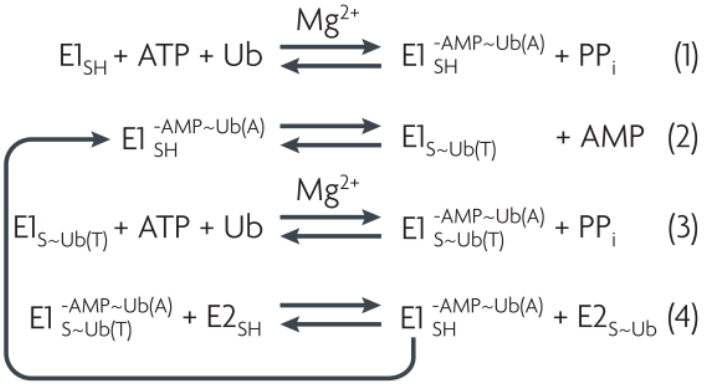


Figure 4. Ub conjugation machinery.
In (1), Ub(A) represents Ubiquitin that is adenylated and forms an Ubiquitin-AMP adduct bound to the enzyme. In (2), the catalytic Cys of E1 forms a thioester linkage with Ubiquitin, Ub(T). In (3), a second Ub is adenylated, being E1 loaded with two ubiquitins. In (4), E1 interacts with an E2 enzyme and Ub is transferred to the E2. The E1-Ub(A)-adenylate complex follows step (2). Adapted from (Dye & Schulman, 2007).

In humans, there are eight E1s that are known to activate UBLs. When the molecule to be activated is Ubiquitin, there are just two E1s or Ubiquitin-activating enzymes: UBE1 (UBA1 in yeast) and UBA6, which are ~40% identical (Pelzer *et al*, 2007; Jin *et al*, 2007). In fact, for many years, Uba1 was thought to be the only Ubiquitin-activating enzyme charging E2s with Ubiquitin. In yeast, only a single E1 has been described to activate Ubiquitin, UBA1.

The origins of E1s start with prokaryotes. The eukaryotic Ubiquitin and the prokaryotic ThiS/MoaD proteins contain the same *β*-grasp fold. The two latter are UBL sulfur-carrier proteins that carry sulphur for incorporation into molybdopterin and thiazole, respectively. In parallel, MoeB and ThiF share sequence homology with the domain of eukaryotic E1s. MoeB catalyzes the adenylation of the C-terminus of MoaD and ThiF adenylates the C-terminus of ThiS (Taylor *et al*, 1998; Burroughs *et al*, 2009).

Ubiquitin-conjugating enzymes, E2

The family of Ubiquitin-conjugating enzymes (E2) is present in all eukaryotes. At least 38 active E2 genes have been identified in humans and 13 genes in *Saccharomyces cerevisiae* (van Wijk & Timmers, 2010; Ye & Rape, 2009).

E2s contain sites through which they interact with E1 and with E3 enzymes. They accept the activated Ub from an E1 and are responsible for Ubiquitin-ligase (E3) selection and substrate modification. E2s can dictate the final product to be mono or polyubiquitinated and the lysine to be modified in the substrate, influencing the fate of the substrate (Ye and Rape, 2009). Active E2s contain a highly conserved Ubiquitin-conjugating (UBC) domain that accommodate the Ub protein through a thioester bond onto its active-site (Burroughs *et al*, 2008; Schulman & Harper, 2009). Unlike E1s, no enzymes containing a UBC domain have been identified in bacteria (Iyer *et al*, 2008).

UBC domains are highly conserved and adopt similar structures. They bear an also highly-conserved cysteine in their active site that binds an Ubiquitin/Ubl protein that

has been previously transferred from the E1. After this step, E2s engage E3s to catalyze substrate ubiquitination.

Not every E2 is active. For instance, UEV proteins are E2 enzymes that, although they contain a UBC domain, lack the active-site Cys residue and cannot bind Ubiquitin (VanDemark *et al*, 2001). However, they regulate active E2s activity and linkage specificity.

Ubiquitin-ligases enzymes, E3

Ubiquitin-ligase enzymes (E3) promote the ubiquitination of either the N-terminus or internal lysine residues of targeted substrates. There are two major types of E3s in eukaryotes: the HECT type and the RING type.

HECT (Homologous to E6AP C-Terminus) enzymes contain a Cys residue that is loaded with an activated Ubiquitin molecule forming an intermediate thioester. Next, Ubiquitin is ligated to the substrate, catalyzing substrate ubiquitination (Figure 5a) (Rotin & Kumar, 2009).

The RING (Really Interesting New Gene) ligase family binds simultaneously the E2-Ubiquitin intermediate and the targeted protein promoting ubiquitination (Figure 5b). RINGs seem to lack a catalytic site.

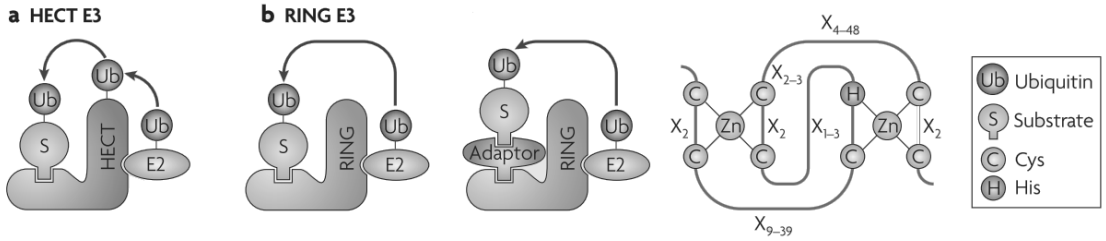


Figure 5. Transfer of Ub by the HECT and RING Ubiquitin ligases.
In **a**, the E2 binds the HECT domain. Next, Ub is transferred from the E2 to the HECT to be finally transferred to the substrate that is interacting with the HECT ligase. In **b**, the E2 interacts with the RING domain and Ub is transferred to the substrate, which binds directly or indirectly, through an adaptor, to the RING ligase. RING E3s are characterized by a Cys-X2-Cys-X(9-39)-Cys-X(1-3)-His-X(2-3)-Cys-X2-Cys-X(4-48)-Cys-X2-Cys sequence. Adapted from Rotin & Kumar, 2009.

The human genome is estimated to encode for over 600 E3s, 300 of which belong to the RING family and 28 to the HECT family. In yeast, around 80 genes were found to encode E3s, 47 of which encode RING ligases and 5 that encode HECT ligases (Li *et al*, 2008). HECT-type E3s contain a HECT domain of ~350 amino acids found at the C-terminus of the protein. The domain can be divided into two parts separated by a flexible linker, the N lobe and the C lobe. The N-terminus contains protein-protein or protein-lipid interaction domains, and this is where the E2 and the E3 interact. Depending on the nature of this N-terminus, HECTs are divided into 3 groups: the Nedd4 family, the HERC family and other HECTs (TRIP12, HUWE1 and UBE3B) (Rotin & Kumar, 2009; Park *et al*, 2009). The C lobe represents one-third of the domain and contains a conserved Cys residue to which the activated Ubiquitin is transferred from the E2 (Scheffner *et al*, 1995).

The structures of the Nedd4 HECT domain and of that domain interacting with Ub suggested a model for the transfer of ubiquitins from the HECT to the substrate, in which a Ub molecule is added from the catalytic Cys of the HECT to a lysine of the substrate. The Ub molecule is then kept close to the catalytic cysteine to promote addition of more ubiquitins (Maspero *et al*, 2011).

Nedd4 family members are characterized by containing two additional domains: C2 domain and WW domains. The C2 domain is a ~120 amino acids calcium-binding domain that binds to phospholipids and mediates intracellular targeting to the plasma membrane, endosomes and multivesicular bodies (MVBs) (Dunn *et al*, 2004). This domain also binds other proteins such as annexin (Plant *et al*, 2000) or the growth factor receptor-bound Grb (Morrione *et al*, 1999). It is also possible that a C2 domain binds the HECT domain of the Nedd4 family, generating inter- and intramolecular interactions (Wiesner *et al*, 2007).

By contrast, the WW domains are ~37 amino acids long protein interaction domains that contain two conserved tryptophan residues, 20-22 amino acids apart. Nedd4 family members can contain between two to four WW domains. WW domains bind to PY (PPXY/PPLP) motifs or phospho-serine/threonine residues in substrate proteins (Dupré *et al*, 2004; Lu *et al*, 1999; Staub *et al*, 1996). WW domains can also bind the HECT domain of the same protein (Gallagher *et al*, 2006). The binding of the HECT either by the C2 domain or the WW domains inhibits its activity and prevents the auto-ubiquitination of these enzymes (Bruce *et al*, 2008).

Misregulation of HECTs-mediated ubiquitination is associated with cancers, neurological disorders, autoimmunity and hypertension (Bernassola *et al*, 2008; Rotin & Kumar, 2009; Metzger *et al*, 2012; Scheffner & Kumar, 2014).

The RING type E3s transfer Ubiquitin to both heterologous substrates and to the RING proteins themselves. The RING domain comprises eight potential metal ligands and binds two atoms of zinc. It is defined by a series of specifically spaced conserved cysteine and histidine residues: Cys-X2-Cys-X(9-39)-Cys-X(1-3)-His-X(2-3)-Cys-X2-Cys-X(4-48)-Cys-X2-Cys, where X is any amino acid. Cys and His residues are buried within the core of the domain, where they help to maintain the overall structure by binding the two atoms of zinc (Borden & Freemont, 1996).

RING fingers serve as a scaffold that brings E2 and substrate together. The RING ligase promotes the transfer of a Ubiquitin molecule from the catalytic cysteine of an E2 to a substrate (Deshaies & Joazeiro, 2009). They can be found as monomers, dimers and multisubunit complexes. The dimerization takes place at the RING finger domain and it can be either homo- or heterogeneous. Some heterodimers contain one RING domain that lack catalytic activity. Activity of RING enzymes can be controlled by covalent modifications (phosphorylation or ubiquitination), by noncovalent binding of proteins, or by competition among substrates.

Deregulation of RING Ubiquitin ligases is often involved in human diseases. Mutations in the E3s, BRCA1 and BRCA2 (breast cancer 1 and 2, respectively) are found in breast and ovarian cancer (Welcsh & King, 2001). The Mdm2 Ubiquitin ligase regulates the tumor suppressor p53. An increased activity of Mdm2 is associated with human cancers (Lipkowitz & Weissman, 2013).

Rsp5 HECT ligase

Rsp5 is the only NEDD4 E3 ligase family member in *Saccharomyces cerevisiae* and is the single E3 encoded by an essential gene (Huibregtse *et al*, 1995). Rsp5 preferentially synthetizes K63 chains *in vitro* and *in vivo* (Kee *et al*, 2005, 2006; Lin *et al*, 2008). However, in the absence of K63, Rsp5 is also able to synthetize shorter K11, K33 and K48 chains (Kim & Huibregtse, 2009). Additionally, Rsp5 has been reported to become auto-ubiquitinated through an intramolecular transfer of Ubiquitin (Lee *et al*, 2009; Huibregtse *et al*, 1995).

Originally, Rsp5 was associated with providing cells with unsaturated fatty acids. Rsp5 was found to polyubiquitinate two transcription factors, Spt23 and Mga2, which activate transcription of Ole1 (Hoppe *et al*, 2000). Ole1 encodes an ER-bound enzyme required for the synthesis of monounsaturated fatty acids, palmitooleic and oleic acids. Later, Rsp5 was found to be involved in several processes such as intracellular trafficking, including endocytosis of plasma membrane proteins and sorting at the multivesicular body (Horák, 2003; Morvan & Froissard, 2004), chromatin remodeling, mitochondrial inheritance (Fisk & Yaffe, 1999) and mediating Golgi to ER trafficking (Jarmoszewicz *et al*, 2012). All this suggests that Rsp5 probably has a multitude of diverse membrane-bound as well as nuclear substrates.

The endosomal sorting complex for transport (ESCRT) consists of four protein complexes (ESCRT-0, -I, -II and –III) that captures ubiquitinated cargoes in the endosome membrane and sorts them to the internal vesicles of multivesicular bodies (MVB) (Katzmann *et al*, 2002). The ubiquitination of these cargoes is generated by specific E3 Ubiquitin ligases (d'Azzo *et al*, 2005). If ubiquitination is inhibited, MVB sorting is abolished resulting in the recovery of cargoes at the vacuolar membrane rather than in the vacuolar lumen.

Ubiquitination and endocytosis of plasma membrane proteins mediated by Rsp5 was first studied through the amino acid permeases, Gap1 and Fur4 (Springael & André, 1998; Galan *et al*, 1996). Lack of ubiquitination of both transporters was observed when reduced levels of Rsp5 were expressed. In some cases, such as for Gap1, evidence exists that indicates that the signal for vacuolar degradation can be polyubiquitination. Rsp5-dependent ubiquitination is also required for the sorting of membrane proteins originating from the Golgi apparatus or the plasma membrane to the MVBs (Katzmann et al., 2002).

Rsp5 is the yeast homolog of the mammalian E3 ligase Nedd4. Like other members of the Nedd4 family, Rsp5 contains an amino-terminal C2 domain, three WW domains and a HECT domain at the C-terminus (Figure 6). The C2 domain was found to be important for subcellular localization to endosomal membranes (Dunn & Hicke, 2001). However, the C2 domain of Rsp5 is not required for the vital function of this enzyme (Wang *et al*, 1999). The WW domains are required for recognition of phosphorylated substrates that undergo endocytosis. Receptor ubiquitination and internalization is impaired when conserved residues in any of the three WW domains are mutated, but cell viability is not affected (Dunn & Hicke,

2001). The third WW domain together with the HECT domain is sufficient to provide its essential function under normal growth conditions (Hoppe *et al*, 2000; Kamadurai *et al*, 2013).

When the N lobe of the HECT domain binds the charged E2-Ub, Ubiquitin is transferred to the catalytic cysteine of the C lobe forming a thioester bond. The C lobe rotates 130 degrees in a way that Ubi can be newly transferred to a substrate lysine (Kamadurai *et al*, 2013; Huibregtse *et al*, 1995).

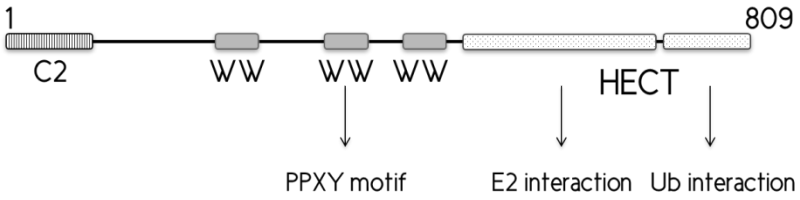


Figure 6. Schematic representation of Rsp5 highlighting its domains.
Rsp5 contains a C2 module, three WW domains and the HECT domain.

Rsp5 interacts with and affects the regulation of numerous substrates in different ways. Here we detail some of these interactions:

- Rsp5 binds and polyubiquitinates the largest subunit of RNA polymerase II, Rpb1, and targets it for degradation (Huibregtse *et al*, 1997).
- Vps9, a protein involved in the yeast endocytic pathway, is monoubiquitinated *in vivo* by Rsp5. Vps9 contains a CUE domain that is required for its monoubiquitination (Shih *et al*, 2003).
- A proteomic *in vitro* ubiquitination screen using a protein microarray platform was developed by Gupta and colleagues to discover substrates of E3 ligases (Gupta *et al*, 2007). They estimated that ~4% of proteins in yeast contain one or more PPXY motifs and ~7% contains LPXY motifs. 72% of the high-confidence Rsp5 substrates was found to bear at least one PY motif, the preferred one being PPXY (the most common residues at the third position, X, were Pro, Ala and Ser).

  Arrestin-related trafficking adaptors (ART) are a family of proteins that target plasma membrane proteins to the endocytic system. Each ART protein contains several PY motif that are used to recruit Rsp5, which ubiquitinates

both the adaptor and the substrate (Lin *et al*, 2008). Bul1 and Bul2 are two arrestin-like proteins that regulate the endocytosis of a subset of plasma membrane proteins. The regulation of these substrates is made after an interaction between Rsp5 and Bul1 or Bul2 that promotes their ubiquitination (Helliwell *et al*, 2001). Gap1, Fur4, Rfa1, Zrt1 and Tat2 are some of these substrates that both do not contain PY motifs and do not bind Rsp5, but that interact with Bul1 and Bul2 (Nikko & Pelham, 2009; Hein *et al*, 1995; Galan *et al*, 1996).

- Smf1 is a manganese transporter that is ubiquitinated by Rsp5. In order that the ubiquitination is achieved, the arrestin-like proteins Ecm21 or Csr2 first need to interact with Rsp5 (Nikko *et al*, 2008).
- Cps1 is a MVB cargo that is ubiquitinated by Rsp5 (Katzmann *et al*, 2004). Although Cps1 does not contain any PY motif, it contains a short sequence (PVEKAPR) that interacts with the C-lobe of the HECT domain of Rsp5. Additionally, residues within the N-lobe contribute to Cps1 recognition and subsequent ubiquitination (Lee *et al*, 2009).
- Cue1 is a substrate of Rsp5 that does not contain any PY motif and does not need any adaptor to be ubiquitinated. However, Cue1 has an Ubiquitin-binding domain (CUE domain) whose interaction with Ub could enable its monoubiquitination (Kang et al, 2003).
- The proteasomal subunit Rpn10 is monoubiquitinated by Rsp5 *in vitro* and *in vivo.* Rpn10 contains an Ubiquitin binding domain, (Ubiquitin-interacting motif or UIM), indispensable for Rpn10 ubiquitination (Isasa *et al*, 2010).

Deubiquitination, a key regulatory reaction in Ubiquitin signaling

Ubiquitination is a process reversed through the action of a large family of deubiquitylases (DUBs), which remove Ubiquitin moieties from polypeptides and polyubiquitin chains. In humans, there are almost 100 different DUBs expressed, 11 of which are not catalytically active (Nijman *et al*, 2005). In *Saccharomyces*

cerevisiae, 24 deubiquitination enzymes are expressed (Reyes-Turcu & Wilkinson, 2009).

DUBs can be divided into five families: Ubiquitin-specific proteases (USP), Ubiquitin C-terminal hydrolases (UCHs), ovarian tumor proteases (OTUs), Josephines and the JAB1/MPN/MOV34 family (JAMMs). In humans, there are ~55 USPs, 4 UCHs, 14 OTUs, 4 Josephines DUBs and 10 JAMM family DUBs (Komander *et al*, 2009a). The first four families contain a cysteine active site. The base of their activity relies on three different amino acids. An aspartic acid polarizes a histidine that will deprotonate the catalytic cysteine that will perform a nucleophilic attack between the carbonyl group of the C-terminus of Ub and the amino group of the substrate lysine.

In contrast, the JAMM/MPN+ family members are zinc metalloproteases. A zinc atom is stabilized by an aspartate and two histidine residues. Zn^{2+} is then bound to a polarized molecule of water that coordinates the fourth link to the metallic atom, facilitating reactivity (Ambroggio *et al*, 2004).

DUBs have both endo- and exo-activity. When they cleave Ub moieties or chains from within an Ub chain, this is called endo-activity. When they hydrolyze the Ubiquitin from the end of a chain, this is exo-activity. The hydrolysis takes place between a lysine of an Ub moiety and the C-terminus of the next Ub. There are many other ways to hydrolase Ub. For instance, DUBs can recognize polyubiquitinated substrates and take off the entire chain at once through the cleavage of the isopeptide bond linking the polyubiquitin chain to the substrate. DUBs can also identify monoubiquitinated substrates and remove a single Ub. Other DUBs remove all the Ub moieties except the one that is bound to the substrate, leading to a monoubiquitinated substrate.

Deubiquitinating enzymes have to deal with chains of distinct linkage. The type of chain can dictate the activity of the DUB. For instance, JAMMs have an exclusive preference for K63-linked chains (Komander *et al*, 2009b). A deubiquitinase enzyme of the OTUs family, Cezanne, shows a K11-linked chains preference, while OTUB1 is K48-linkage-specific and does not hydrolyze linear or K63-linked chains (Bremm *et al*, 2010; Wang *et al*, 2009). In contrast, the USP family hydrolyses all type of linkages and show little specificity for chain types (Faesen *et al*, 2011). Despite this fact, the USP14 hydrolase has a preference for K48-linked, K63-linked

and linear chains, and CYLD for K63-linked and linear chains (Komander *et al*, 2008; Hu *et al*, 2005).

Ubp2 belongs to the USP family and is important for normal trafficking of Rsp5 membrane protein substrates, such as Fur4, to the MVB (Lam *et al*, 2009). This Ub protease interacts with Rsp5 and modulates the auto-ubiquitination status of Rsp5 *in vitro* and *in vivo* (Lam & Emili, 2013; Huibregtse *et al*, 1995; Lee *et al*, 2009) as well as Rsp5-catalyzed substrate ubiquitination (Kee et al, 2005). The interaction between Ubp2 and Rsp5 is stabilized by the co-factor Rup1 (an Ubiquitin-associated (UBA) domain-containing protein that binds Ubiquitin chains). Rsp5 preferentially assembles K63-linked chains, while Ubp2 preferentially disassembles K63 chains, promoting monoubiquitination. Both activities act, for instance, on Spt3, which is monoubiquitinated in an Rsp5-dependent way *in vivo* (Rape *et al*, 2001).

Rpb1, a RNA polymerase II subunit is a substrate of Rsp5 that is poly- and monoubiquitinated *in vivo.* Rsp5 binds the C-terminus of Rpb1 and promotes K63 Ubiquitin chain elongation. Nonetheless, the activity of Ubp2 modifies the substrate resulting in a monoubiquitinated form (Harreman *et al*, 2009). Ubp2 can also efficiently hydrolyze the Ubiquitin-substrate link. This is also the case for another RNAPII-targeting Ubiquitin protease, Ubp3 (Kvint *et al*, 2008).

The Rsp5-Ubp2 association has also been shown to control the levels of monoubiquitination of the proteasome subunit Rpn10 *in vivo* (Isasa *et al*, 2010).

Proteasome 26S

In the 1970s, an intracellular protein degradation pathway in eukaryotes was discovered: the Ubiquitin-Proteasome System (Hershko *et al*, 1983; Varshavsky, 1997). This system includes a large multimeric enzyme that degrades ubiquitinated proteins, the proteasome. The 26S proteasome is a multisubunit protease (2,5 MDa) highly conserved in evolution composed of a barrel-shaped 20S core particle (CP) capped at either end by one or two 19S regulatory particles (RP) (Finley, 2009). The proteasome degrades Ubiquitin–protein conjugates into small peptides in an ATP-dependent manner. For that, the proteasome needs to first recognize the substrates to be degraded, a function held by the subunits in the RP. Next, other

subunits in the RP deubiquitinate, unfold and translocate the substrate into the proteolytic core particle (Tanaka, 2009). Once in the CP channel, the proteolytic active sites degrade the protein (Figure 7).

The general structure of the *Saccharomyces cerevisiae* 26S proteasome has been resolved by Cryo-electron microscopy (Beck *et al*, 2012; Sakata *et al*, 2012; Lander *et al*, 2012; Förster *et al*, 2010).

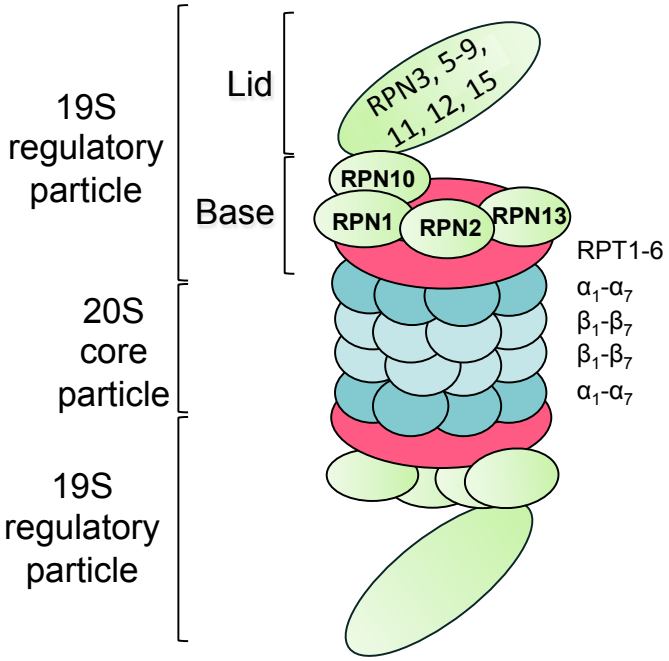


Figure 7 Schematic representation of the 26S proteasome
The subunits of the lid and the base of the RP are shown.

The core particle

The core particle is a complex composed of 28 subunits organized in four stacked rings of subunits (Figure 7 and 8). Each ring contains 7 distinct subunits that are all arranged in a hollow (Groll *et al*, 1997). The two outer rings are known as α-rings, the two inner rings as β-rings, forming a structure α_{1-7}-β_{1-7}-β_{1-7}-α_{1-7}. 3 out of 7 subunits forming the β rings are responsible for the proteolytic activity. Each of these subunits has its unique proteolytic activity: β_1 has caspase-like activity and cleaves peptide bonds after acidic amino acids, β_2 has trypsin-like activity and cleaves after basic residues and β5 is chymotrypsin-like in cleaving after

hydrophobic residues. The two β-rings together create the chamber where the substrates are degraded to become oligopeptides of 3 to 15 amino acids (Tanaka, 2009).

In mammals, there are 4 additional β subunits: β1i, β2i, β5i and β5t (i stands for immuno and t for thymo). These subunits are overexpressed in some tissues of the immune system and they substitute the canonical β subunits, affecting the proteolytic specificity. Proteasomes containing β1i, β2i and β5i subunits are termed immunoproteasomes; proteasomes containing β1i, β2i and β5t are called thymoproteasomes (Tomko & Hochstrasser, 2013).

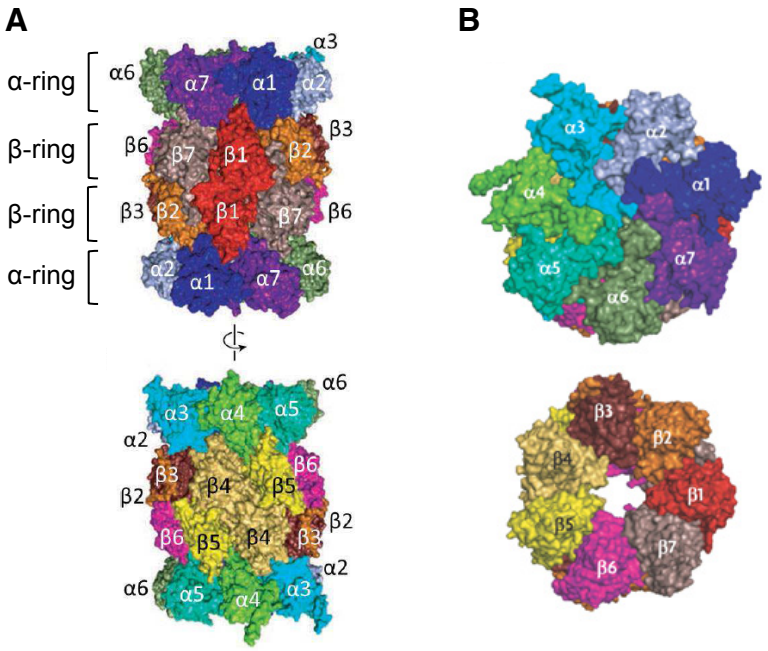


Figure 8. Molecular strucutures of the bovine 20S proteasome.
A) Side view of the 20S proteasome. It consists of two outer rings, the α-rings, and two inner rings, the β-rings. **B)** Top views of the α-ring (closed) and the β-ring (open). Adapted from Murata et al, 2009.

The access of substrates into the inner chamber is regulated by the α-ring. The position of the 7 subunits forming the α-ring blocks or allows the entrance of the substrate. This *gate* exists in a continual alternation between open and closed states (Osmulski *et al*, 2009). When the N-termini of the 7 subunits faces the interior of the ring, the chamber remains closed (Groll *et al*, 2000). To open the gate, additional factors, such as subunits of the RP, need to bind the outer ring of the CP, which allows a shift of the N-termini α subunits (Stadtmueller & Hill, 2011).

The regulatory particle

The 20S proteasomes exhibit a very low proteolytic activity; they need to bind to regulatory particles to be really efficient. The RP is anchored to the α rings at either of the CP ends. Most of the 20S proteasomes in *S. cerevisiae* are capped at either end by the RP. The RP is a 900 KDa complex composed of 19 subunits divided into two subassemblies: 10 subunits form the base, and 9 subunits the lid (Pickart & Cohen, 2004).

The base

The base includes six ATPases from the AAA family, also known as Regulatory Particle Triple A proteins or Rpt1–6, and four non-ATPase subunits, Rpn1, Rpn2, Rpn10 and Rpn13 (regulatory particle non-ATPase). The electron microscopy structure of the proteasome holoenzyme and the lid subcomplex revealed the spatial rearrangement of the diverse subunits (Figure 9 and 10) (Lander *et al*, 2012). The 6 ATPases form an heterohexameric ring organized as follows Rpt1-Rpt2-Rpt6-Rpt3-Rpt4-Rpt5 (Tomko *et al*, 2010). The ATPase ring and the 7 α subunits of the CP form a complex when they contact each other. The ATPases unfold the substrate in an ATP-dependent step and the substrate is then translocated into the proteolytic chamber (Aubin-Tam *et al*, 2011). The C-terminal residues of the ATPases are critical for gate opening and stability of 26S proteasomes (Smith *et al*, 2007).

The 26S proteasome structure from *S. cerevisiae* showed that the ATP ring undergoes conformational changes which could enable the entrance of the substrate through the central pore and its processing. A coordinated ATP-hydrolysis would promote the rotation of the ATPase subunits and give the energy to exert a pulling force to unfold and translocate the substrate partially. The repetition of this cycle would allow the entire translocation into the peptidases (Matyskiela *et al*, 2013).

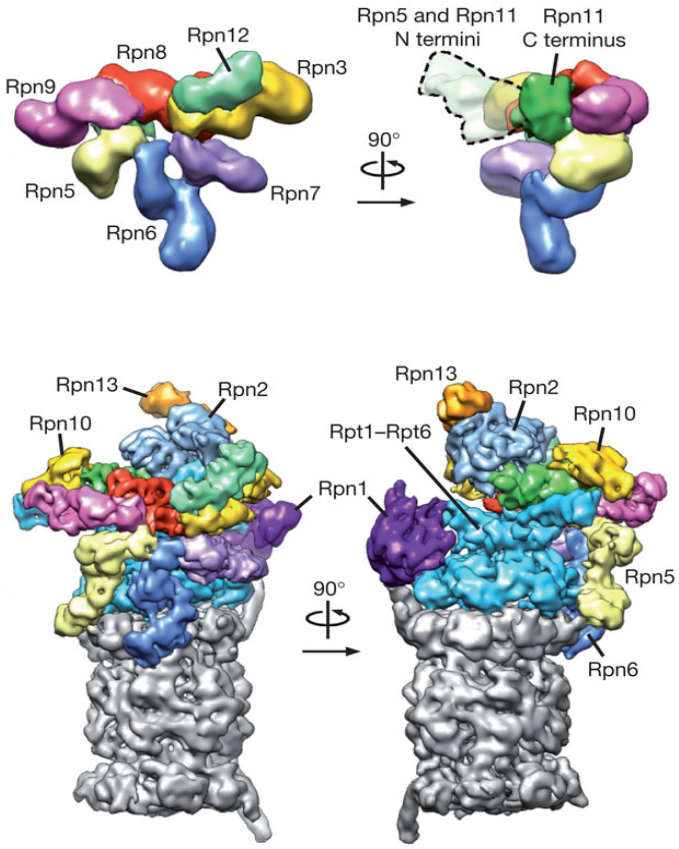


Figure 9. Three-dimensional electron microscopy reconstruction of the regulatory particle.
A negative-stain reconstruction of the isolated lid subcomplex (top) and a reconstruction of the proteasome (bottom). The lid is coloured and the CP is in grey. Adapted from Lander et al, 2012.

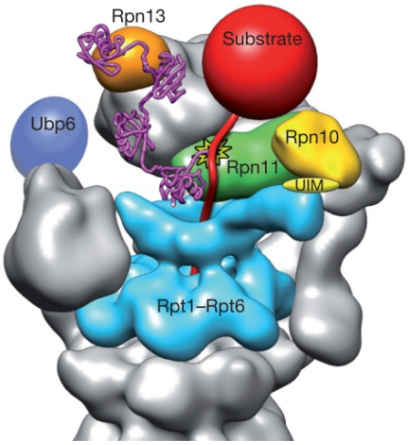


Figure 10. Model for the recognition, deubiquitination and engagement of a polyubiquitinated substrate by the proteasome.
A tetraubiquitinated substrate (red) binds to the Ubiquitin receptor Rpn13 (orange) via the attached ubiquitins (magenta). Rpn11 (green) deubiquitinates the substrate and

Rpt1-Rpt6 subunits translocate it into the core particle. Adapted from Lander *et al*, 2012.

Regarding the Rpn subunits of the base, Rpn1 and Rpn2 are the largest subunits in the proteasome (110 and 104 KDa, respectively). They are composed of 11 α-helical proteasome/cyclosome repeats that are responsible for the interaction between the proteasome and Ubiquitin binding proteins, such as Rpn13 and Rad23 (Elsasser *et al*, 2002; Schreiner *et al*, 2008). Indeed, the repeats in Rpn2 allow the interaction with Rpn13 (He et al., 2012). Rpn1 contacts the outside face of the ATPase ring (subunit α_4) which could help to control substrate docking (Lander *et al*, 2012).

Rpn10 and Rpn13 are both Ubiquitin receptors that bind Ub chains through their UIM and PRU domains, respectively (Verma *et al*, 2004; Mayor *et al*, 2007; Elsasser *et al*, 2004; Husnjak *et al*, 2008).

The UIM of Rpn10 bridges Rpn11 and the lid subunit Rpn9 and also contacts the coiled coil formed by Rpt4 and Rpt5 (Lander *et al*, 2012; Sakata *et al*, 2012). Polyubiquitinated substrates can also bind to extrinsic Ub receptors such as Rad23, Dsk2, and Ddi1, which are Ubiquitin-associated (UBA) and Ubiquitin-like (UBL) domain-containing proteins (Bertolaet et al, 2001; Funakoshi et al, 2002). These proteins have the dual property of binding polyubiquitin, through the UBA domain and of interacting with the proteasome, through the UBL motif (Elsasser *et al*, 2002; Finley, 2009). For instance, Ddi1, Rad23 and Dsk2 interact with Rpn1 through their UBL domain and the leucine-rich-repeat-like (LRR-like) domain in Rpn1 and Rad23 can also bind the proteasome via the UIM of Rpn10/S5a (Elsasser *et al*, 2002; Hiyama *et al*, 1999).

The lid

The lid interacts with both the base and the CP. It is composed of 9 non-ATPase subunits: Rpn3, 5, 6, 7, 9, 11, 12 and 15 (Glickman et al, 1998b). Rpn3, 5, 6, 7, 9, and 12 contain a PCI domain that allows interaction between subunits. Rpn5 and Rpn6 contact with the C-terminus of α1 and α2 subunits, respectively and they both touch Rpn13. Rpn7 interacts with Rpt2 and Rpt6 (Lander *et al*, 2012). Rpn15 has been recently described as a novel intrinsic Ubiquitin receptor

(Paraskevopoulos *et al*, 2014) that bridges the cleft between Rpn7 and Rpn3 (Bohn *et al*, 2013). Rpn8 and 11 subunits contain an MPN domain that allows interaction with PCI subunits Rpn5, 6 and 9 (Tomko & Hochstrasser, 2011; Lander *et al*, 2012). Rpn11 is at the entrance of the pore, surrounded by the base subunits Rpn10 and Rpn13. Rpn11 is a JAMM DUB family member in charge of deubiquitinating proteasome substrates (Verma *et al*, 2002; Glickman *et al*, 1998b). Its activity depends on the interactions with other lid subunits. When the lid is in an isolated form, substrate deubiquitination is avoided, while when the lid is part of the holoenzyme, the DUB activity can take place.

The proteasomal degradation process

First of all, there is an interaction of an ubiquitinated substrate (Ub-S) with a Ub binding receptor (Rpn10, Rpn13 or Rpn15) in the proteasome or with a Ub shuttle receptor (Rad23, Dsk2 and Ddi1) that is recruited to the base (Lander *et al*, 2012) (Figure 11). None of these Ubiquitin receptors is essential for yeast viability (Husnjak *et al*, 2008) and it is likely that additional receptors will be identified in the future. Interestingly, Rpn10 is essential in both mouse (Hamazaki *et al*, 2007) and *D. melanogaster* (Szlanka, 2003) and Rad23 is essential in mouse (Hamazaki *et al*, 2007).

Next, the Ub-S is deubiquitinated by Rpn11, Ubp6 or Uch37. Rpn11 cleaves Ubiquitin chains *en bloc* by a cleavage at proximal Ub (Verma *et al*, 2002). Ubp6 is a base-associated DUB that cleaves within polyubiquitin chains or trims them from their distal end. Ubp6 prevents Rpn11 deubiquitinase activity, which delays proteasomal degradation or allows dissociation of the substrate from the proteasome (Hanna *et al*, 2006). Uch37 is a proteasome-associated DUB that removes ubiquitins one at a time (Lam *et al*, 1997). In contrast with these three proteases, Hul5, a Ubiquitin ligase associated to the proteasome, promotes chain extension on substrates that had been previously ubiquitinated and that are bound to the proteasome (Crosas *et al*, 2006).

Before the substrate is translocated to the CP, it first needs to be unfolded, which is carried out by the Rpt subunits of the base. Having the substrate unfolded, the substrate is translocated to the CP where it is finally degraded into short peptides Sometimes, the proteases in the CP process the protein into a truncated form, creating a new protein that will have different cellular activities. For instance, once

the NF-kß precursor p105 is ubiquitinated, the proteasome degrades the C-terminal region and transforms it into p50, a stable and active subunit of the NF-kß transcription factor (Palombella *et al*, 1994).

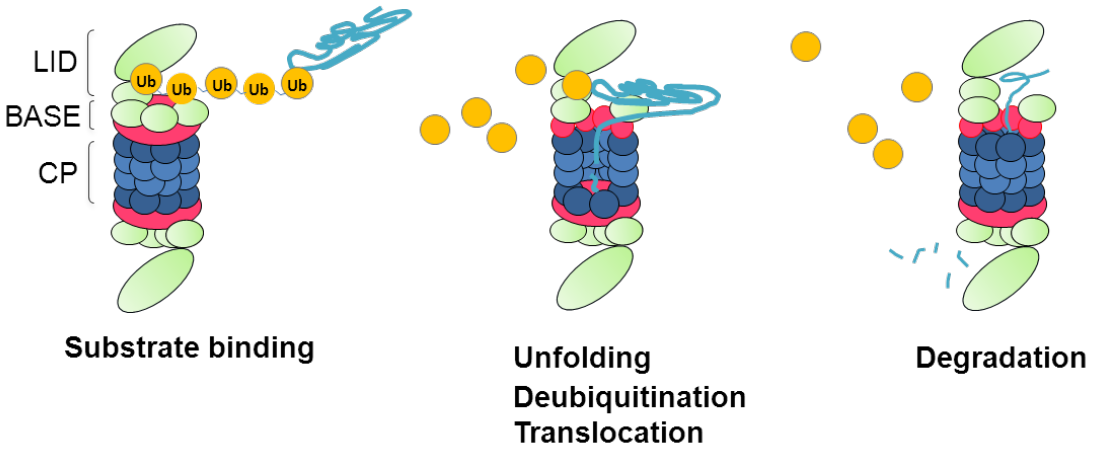


Figure 11. Steps of proteasomal degradation.
Binding, unfolding, deubiquitination, translocation and degradation of a polyubiquitinated substrate.

The regulation and homeostasis controlled by the proteasome is highly precise. It is indispensable that the proteasome degrades solely the proteins that need to be degraded and avoids unwanted removal of other proteins. Depending on the physiological demands, proteasome levels and proteasome composition vary (Lecker *et al*, 2006; Hanna *et al*, 2007). Under proteotoxic stress, there is an upregulation of all proteasome genes. In *S. cerevisiae*, the transcription factor Rpn4 is responsible for the adaptive expression of the subunits of the proteasome (Leggett *et al*, 2002). Under normal conditions, Rpn4 has a very short life of around 2 minutes and is degraded by the proteasome (Ju & Xie, 2004).

The activity of the proteasome is also regulated by its own auto-ubiquitination. Indeed, some Ubiquitin receptors within the human 26S proteasome have been found to be ubiquitinated which impairs the ability of the proteasome to bind, deubiquitinate and degrade ubiquitinated substrates (Jacobson *et al*, 2014). Analogously, in yeast, the ubiquitination in the proteasome of the Ubiquitin receptor Rpn13 causes a strong decrease in Ub-dependent proteolysis (Besche *et al*, 2014).

Dysfunction of the proteolytic activity of the proteasome is associated with many human diseases. Accumulation of toxic aggregated, misfolded proteins leads mainly to neurodegenerative diseases (Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis and spongiform encephalopathies). For instance, Tau is a protein that stabilizes microtubules. It has been suggested that when Tau aggregates, the proteasomal activity decreases resulting in Alzheimer's disease (Keck *et al*, 2003). In parallel, Parkinson disease is characterized by the formation of Lewy Bodies (LB). α-synuclein is the main component of the LB. Depletion of the proteasome subunit Rpt2 results in accumulation of α-synuclein and Lewy Body-like inclusions in mice (Bedford *et al*, 2008).

Although *in vitro* studies showed that tetraubiquitin is the minimum signal for efficient proteasomal targeting (Thrower *et al*, 2000), it has been shown that polyubiquitination is not the only signal to get proteasomal degradation. Indeed, simpler forms of proteasomes have been identified in Archaea and actinobacteria, whilst Ub is just found in eukaryotes suggesting that ancestral proteasomes were able to degrade proteins that were not ubiquitinated (Gille *et al*, 2003; Maupin-furlow *et al*, 2000). For example, Ornithine decarboxylase (ODC) is degraded by the proteasome without being previously ubiquitinated (Murakami *et al*, 2000). Moreover, mono- or multi-monoubiquitinated substrates can also be degraded by the proteasome. For instance, α-globin and ferritin are monoubiquitinated and, subsequently degraded (Shaeffer & Kania, 1995; De Domenico *et al*, 2006). In fact, several studies showed that a protein containing more than 20 residues with a Ub fused to its C-terminus can be degraded (Verhoef *et al*, 2009; Shabek *et al*, 2009). Ub would serve as an anchor to the proteasome and the short substrate would be translocated into the core particle. Furthermore, a recent study proved that monoubiquitinated proteins shorter than 150 residues can be degraded by the proteasome whereas longer ones need to be polyubiquitinated to have the same fate (Shabek *et al*, 2012).

Monoubiquitination

Modification of a substrate by a single Ubiquitin moiety is called monoubiquitination. Monoubiquitination regulates DNA repair, histone function, gene expression, and receptor endocytosis (Hicke, 2001; Di Fiore *et al*, 2003; Hoeller *et al*, 2006; Bergink & Jentsch, 2009).

There are various ways tin which monoubiquitination can occur. E2s, E3s, or the substrate itself determine whether only one lysine on the substrate is modified (monoubiquitination), more than one lysine is modified (multi-monoubiquitination) or if a lysine on the substrate is modified with a chain of Ub molecules (polyubiquitination).

Proteins that contain Ubiquitin-binding domains (UBD) are defined as Ub receptors. These receptors can be monoubiquitinated following a process known as "coupled monoubiquitination" which requires their UBD. Once a UBD protein is monoubiquitinated, it can suffer a change in its conformation due to an intramolecular binding between the UBD and the Ub molecule attached to it. The protein is then intrinsically switched off and is no longer able to bind ubiquitinated substrates (Di Fiore *et al*, 2003; Hoeller *et al*, 2006; Woelk *et al*, 2006).

Eps15 is an endocytic protein that contains two UBDs, specifically two UIMs, and undergoes coupled monoubiquitination by means of two different ligases, Nedd4 and Parkin. When Eps15 is ubiquitinated by a Nedd4, the ligase needs to be first modified by ubiquitination and contain a thiolester conjugated Ubiquitin. Next, the UIM2 of Eps15 binds the Ubiquitin moiety linked to the Nedd4 ligase and then the thiolester-bound Ub is transferred to Eps15 (Woelk *et al*, 2006). When the ligase is Parkin, the UBL domain in Parkin interacts with the UIM of Eps15 and then the E2 transfers the Ub to Eps15 resulting in its monoubiquitination (Fallon *et al*, 2006). Once Eps15 is monoubiquitinated, the UIM interacts intramolecularly with the attached Ub avoiding both further Ubiquitin chain extension in Eps15 and the interaction with ubiquitinated substrates.

Analogously to Eps15, it has been suggested that the UIM of Rpn10 interacts with the ubiquitins linked to the lysines in Rpn10. The intramolecular interaction could suppose a mechanism to both regulate the interaction with polyubiquitinated substrates and prevent Rpn10 from being polyubiquitinated (Isasa *et al*, 2010).

UBD-containing proteins can also be monoubiquitinated in an E3-independent step. The Ubiquitin attached to the active site of an E2 interacts with the UBD and Ub is transferred directly to the substrate (Hoeller *et al*, 2007).

Additionally, monoubiquitination can be generated by restricting polyubiquitination. Rad18 E3 ligase blocks the Ubiquitin-chain synthesis activity of the E2 Rad6, promoting monoubiquitination of the proliferating cell nuclear antigen, PCNA (Hibbert *et al*, 2011).

Moreover, in the process of histone H2A monoubiquitination by the polycomb complex Bmi1-RING1, a model was proposed in which the rigidity of the E2-E3 complex assembled to DNA and nucleosomes promotes K119 specific monoubiquitination (Bentley *et al*, 2011).

Monoubiquitination is also promoted by the catalytic activity of DUBs that trim polyubiquitin chains on substrates leaving just one Ubiquitin molecule (Kee et al., 2005).

The process of monoubiquitination can be induced by an external protein cofactor that modulates enzyme processivity, such as Vps23. When the arrestin family protein Rim8/Art9 is monoubiquitinated by Rsp5, the UBD of Vps23 interacts with the Ub linked to Rim8/Art9, which then prevents its further polyubiquitination (Herrador *et al*, 2010).

A thorough study from our lab on the mechanisms of monoubiquitination has been recently accepted (Puig-Sàrries, & Crosas, 2015).

Rpn10

Rpn10, previously known as Mcb1 for Multiubiquitin chain-binding protein, is a subunit of the proteasome that can also be found in an extraproteasomal pool (van Nocker *et al*, 1996b). Rpn10 is composed of an N-terminal von Willebrand factor A domain (VWA) and a C-terminal Ubiquitin-interacting motif (UIM) that binds to Ubiquitin (Figure 12). The VWA domain is a globular domain thought to be responsible for the lid-base interaction of the proteasome. When Rpn10 is deleted or the VWA domain is mutated, the RP is dissociated into the base and the lid (Verma *et al*, 2004; Glickman *et al*, 1998a; Fu *et al*, 1998). However, Lander and coworkers observed by electron microscopy that the VWA does not contact the

base but bridges Rpn9 and Rpn11 subunits, increasing lid-base affinity (Lander *et al*, 2012). The VWA of S5a, the Rpn10 human orthologue, has been described as a receptor for FAT10, an Ubiquitin-like protein that targets substrates for proteasomal degradation. The proteolysis of targets linked to FAT10 can be accelerated by the UBA-UBL protein, NUBL1 that also binds the VWA. Interestingly, the VWA suffices to enable FAT10 degradation while the lack of this globular domain causes accumulation of FAT10 (Rani *et al*, 2012).

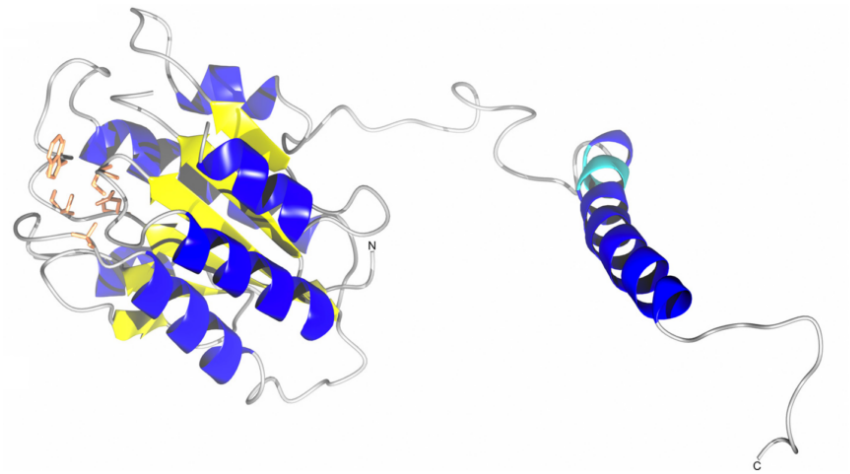


Figure 12. Three-dimensional representation of *Schizosaccharomyces pombe* Rpn10 based on the VWA crystal structure and the homology-modeled C-terminus.
Rpn10 consists of the globular domain, VWA and the UIM domain within an helix. The LALAL motif is represented in cyan. Adapted from (Riedinger *et al*, 2010).

In yeast, Rpn10 contains one UIM while human S5a, and Drosophila Psmd4 orthologues, contain two and three copies, respectively (Young *et al*, 1998). The UIM is a sequence motif of about 20 amino acids that is found in the context of an α-helix and contains 5 hydrophobic residues that form an alternating pattern of large and small side chains, Leu-Ala-Leu-Ala-Leu in S5a, and this pattern is essential for binding Ub chains (Deveraux *et al*, 1994; Young *et al*, 1998). In yeast, the pattern changes slightly to Leu-Ala-Met-Ala-Leu (LAMAL motif). This motif is conserved throughout eukaryotes and it can be found in proteins involved in different processes such as endocytosis, protein trafficking, and signal transduction. All conserved residues are found exposed on one face (Shekhtman &

Cowburn, 2002), nevertheless, UIMs can be partially or totally masked by the folded protein in which they are contained.

Rpn10, together with Rpn13 and Rpn15, functions as the main Ubiquitin receptor of the proteasome (Deveraux *et al*, 1994; Husnjak *et al*, 2008; Schreiner *et al*, 2008; van Nocker *et al*, 1996b; Paraskevopoulos *et al*, 2014). These three subunits are not essential for yeast viability, indicating the presence of other proteasomal Ubiquitin receptors (van Nocker *et al*, 1996b; Husnjak *et al*, 2008; Finley, 2009). Cyclin B1, Sic1, Gic2 and Gcn4 are some of the substrates that Rpn10 targets to the proteasome (Hanna *et al*, 2006; Seong *et al*, 2007; Verma *et al*, 2004). The elimination of a portion of newly synthesized proteins upon oxidative and thermal damage also depends on the Rpn10 subunit (Medicherla & Goldberg, 2008).

An electron cryomicroscopy study localized Rpn10 and Rpn13 in the apical part of the RP (Sakata *et al*, 2012). The surface of the VWA domain of Rpn10 interacts with several proteins in the RP: Rpn1, 2, 9 and 12 (Riedinger *et al*, 2010; Xie & Varshavsky, 2000; Takeuchi *et al*, 1999), while the UIM is close to Rpt4 and Rpt5. Rpn13 interacts only with Rpn2 (Sakata *et al*, 2012). The fact that both Ub receptors are found in an apical position and that they are not necessary to maintain the structural integrity of the proteasome agrees with the survival of their knockouts. In addition to these two receptors, there exist shuttling Ub receptors, including Rad23, Dsk2 and Ddi1 that bind the proteasome through their UBL domain (Bertolaet *et al*, 2001; Funakoshi *et al*, 2002). For instance, Dsk2 binds Rpn1, 10 and 13 (Matiuhin et al, 2008, Funakoshi et al. 2002, Husnjak et al. 2008). In an extraproteasomal context, Rpn10 was seen to have a biological role in restricting the access of Dsk2 to the proteasome by means of an interaction between the UIM and the UBL of Dsk2 (Matiuhin *et al*, 2008).

Rpn10 has high ability to bind K48 Ubiquitin chains and preferentially binds Ub chains rather than monomers (van Nocker *et al*, 1996a, 1996b; Haracska & Udvardy, 1997). Substituting the UIM sequence with five asparagines abolishes chain binding, produces a decrease in the proteolytic capacity of the proteasome and is lethal in mouse (Fu *et al*, 1998; Elsasser *et al*, 2004; Verma *et al*, 2004; Isasa *et al*, 2010; Hamazaki *et al*, 2007).

Quantitative analysis of Ubiquitin conjugates from *Saccharomyces cerevisiae* cultures underscored the importance of Rpn10 on targeting ubiquitinated substrates

to the proteasome. Mayor *et al.* identified around 60 Ubiquitin conjugates that accumulated upon proteasome inhibition in a strain lacking Rpn10, 12 of which also accumulated in a strain carrying an Rpn10 mutant that lacked the UIM (Mayor *et al*, 2007). The substrates that accumulated in *rpn10*Δ, but not in the strain lacking the UIM, probably are dependent upon the VWA domain. This finding agrees with the phenotypes observed when expressing both kind of strains, where *rpn10*Δ strain has a more severe growth defect (Fu *et al*, 1998).

Recently, it has been shown that in the mammalian brain, Rpn10 proteasome association/dissociation is tightly regulated by Id1 protein, having special relevance in dendrite development (Puram *et al*, 2013). Crosas *et al*. reported that the Ubiquitin ligase Hul5 promotes Rpn10 polyubiquitination and subsequent degradation by the proteasome (Crosas *et al*, 2006). Later, a former member of our lab observed the monoubiquitination of Rpn10 (mUb-Rpn10) in both proteasomal and extraproteasomal pools (Isasa *et al*, 2010). Isasa et al found that mUb-Rpn10 levels are regulated by the Ubiquitin ligase Rsp5 and the DUB, Ubp2. On the contrary, it has been suggested that S5a is polyubiquitinated by a large variety of Ub ligases, and even likely by all E3s (Kim & Goldberg, 2012).

Rpn10 is mainly ubiquitinated at lysines K84, in the VWA domain, and K268, at the C-terminus of the protein, although ubiquitins can also be linked at K77 and K99. Importantly, the UIM is indispensable for Rpn10 ubiquitination and mUb-Rpn10 can no longer bind polyubiquitinated substrates *in vitro*, reducing the proteolytic activity of the proteasome (Isasa *et al*, 2010). The inability of mUb-Rpn10 to bind Ub chains could be explained by an intramolecular interaction between the linked Ub and the UIM leading to a self-inactivated state of the protein. Indeed, a 'fold-back' model, based on a structural conformational change in the monoubiquitinated protein promoted by the interaction between a Ubiquitin-binding domain (UIM, CUE…) and Ubiquitin, has been proposed in several works (Polo *et al*, 2002; Hoeller *et al*, 2006; Shih *et al*, 2003). Interestingly, a flexible linker region connecting the VWA domain and the UIM has been described (Wang *et al*, 2005; Riedinger *et al*, 2010). However, a correlation between structural properties and monoubiquitination of the substrate has never been established.

OBJECTIVES

The main objective of this thesis has been to uncover the mechanisms by which Rpn10 is monoubiquitinated by Rsp5. This goal has been divided into the following points:

1. To optimize the Rpn10 ubiquitination reaction.
2. To evaluate the effect of an additional Rsp5 interacting motif on Rpn10.
3. To determine the efficiency of Ubiquitin ligases other than Rsp5 in the reaction of Rpn10 ubiquitination.
4. To assess Ubp2 activity in deubiquitinating Rpn10.
5. To analyze the effect of Rpn10 monoubiquitination *in vivo*.
6. To determine the minimal sequence requirements for Rpn10 ubiquitination.
7. To reproduce Rpn10 monoubiquitination with its human orthologue S5a, using the Rsp5 orthologue, Nedd4.

RESULTS

This project started with my master thesis and continued for four years more. During this time, different initiatives have been approached to better understand the mechanisms underlying Rpn10 ubiquitination. I would like to include, as a first chapter, part of the work I did during the master and beginning of the thesis.

Purification of UbRpn10L85 to analyze K84 ubiquitinated Rpn10

The subunit of the proteasome Rpn10 was found to be ubiquitinated at four different lysines (Isasa et al, 2010). Mass spectrometry analyses of *in vitro* and *in vivo* pools of Rpn10 revealed that Rpn10 is mainly ubiquitinated at lysines 84 and 268 by Rsp5, lysine 84 being the most abundantly modified residue (Isasa et al, 2010). It has been suggested that, once Rpn10 is monoubiquitinated, the linked Ub interacts with the UIM of Rpn10. To investigate whether this hypothesis was true, we decided to analyze Rpn10 monoubiquitination at lysine 84 by X-ray and NMR and see if that interaction existed. To be able to do this characterization, we needed large amounts of very pure protein. For that reason, we decided to prepare a plasmid that would allow us the overexpression and purification of a protein that would mimic the ubiquitination of Rpn10 at lysine 84. This protein consisted of a chimera that included the entire Ubiquitin sequence (residues 1-76) fused by means of a short linker to residues 85-268 of Rpn10 and was N-terminally GST tagged. Normally, the C-terminal glycine residue of Ub forms an isopeptide bond with the side chain of lysine 84 in Rpn10. The stop codon in Ub was mutated to a glycine and the short linker consisted of two other glycines because the result is almost isosteric with the side chain of lysine 84 and the isopeptide bond to the C-terminus of Ubiquitin (Freudenthal *et al*, 2010). We obtained then, by PCR amplification, GST-Ubiquitin-GGG-Rpn10$^{85-268}$, hereafter, GST-UbRpn10L85.
GST-UbRpn10L85 was cloned into a pGEX-4-T vector and transformed into *E. coli* strain BL21. We expressed the chimera as a GST fusion protein that we induced with IPTG and we purified by GST affinity chromatography. The elutions obtained were run in a Coomassie gel and we observed that they contained two bands

(Figure 13). The upper band had a mobility close to the one expected for GST-UbRpn10L85 (54,5KDa), while the lower band appeared to be a contaminant of a molecular weight (MW) of around 34 KDa. It is important to note that Rpn10 properties affect the mobility of the protein when it is run in SDS-PAGE gels, resulting in a considerable different estimated molecular mass from the true one. For this reason, it was reasonable to observe that the protein ran more slowly than expected (60KDa vs. 54,5KDa).

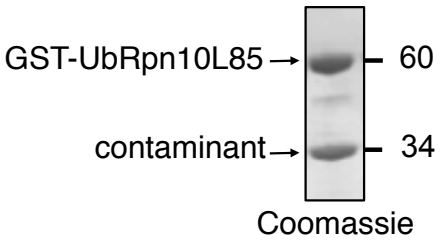


Figure 13. Purification of GST-UbRpn10L85.
Coomassie staining of SDS-PAGE showing GST-UbRpn10L85 and a contaminant.

To analyze the nature of the band with the slower mobility, we did a mass spectrometry analysis, which proved the band to be GST-UbRpn10L85. The conformation of glycine residues has great freedom so that they can provide flexibility for adjacent residues (Yan & Sun Qing, 1997; Serrano *et al*, 1992) This fact led us to hypothesize firstly, that the presence of a contaminant could be due to the truncation of the protein between GST-Ub and Rpn10L85 fragment because of the flexibility given by the three glycines and, secondly, that this contaminant was GST-Ub. In fact, the GST and the Ubiquitin together have a MW of around 34 KDa, which was consistent with the molecular weight of the band observed. However, we could not observe any additional band that could correspond to Rpn10L85 fragment.

To verify our hypothesis, we performed a thrombin cleavage in order to remove the GST tag. If the hypothesis was true and the contaminant was GST-Ub, the product of the thrombin cleavage should include UbRpn10L85, GST and Ubiquitin. The observation of a band (although weak) with a MW close to the one that Ub has (8,5 KDa) made us conclude that the contaminant must have been GST-Ub (Figure 14).

Two additional bands were observed that we presumably associated with UbRpn10L85 (~ 28, 5 KDa) and GST (26 KDa).

The thrombin cleavage resulted in the removal of GST from both, GST-UbRpn10L85 and GST-Ub, which explained the stronger band of the GST alone with respect to the UbRpn10L85 band.

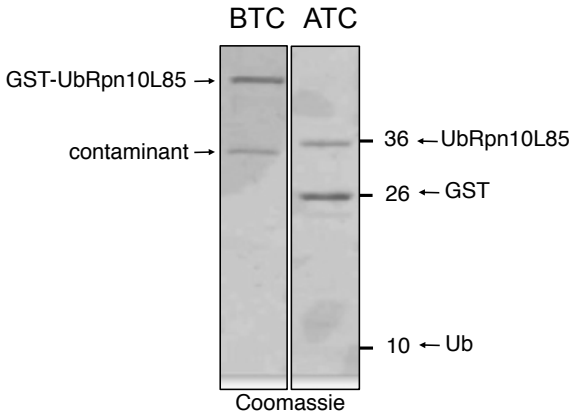


Figure 14. Thrombin cleavage of GST-UbRpn10L85.
Left panel shows GST-UbRpn10L85 and the contaminant before the thrombin cleavage (BTC). Right panel shows the products of the digestion after the thrombin cleavage (ATC).

For NMR and X-ray methods, the protein to be analyzed needed to be as pure as possible. For that, we proceeded to apply a size-exclusion chromatography using a Superdex 200 10/300 column (GE Healthcare), a technique that separates proteins according to their size. The molecular weight of the contaminant appeared to be sufficiently lower than our protein to make separation feasible. We expected that the larger molecule, GST-UbRpn10L85, would elute earlier than GST-Ub and, thus, that we would obtain two different peaks. However, although observing two peaks, a SDS-PAGE gel revealed that the separation was not complete and the contaminant was not fully removed from the full-length protein (Figure 15).

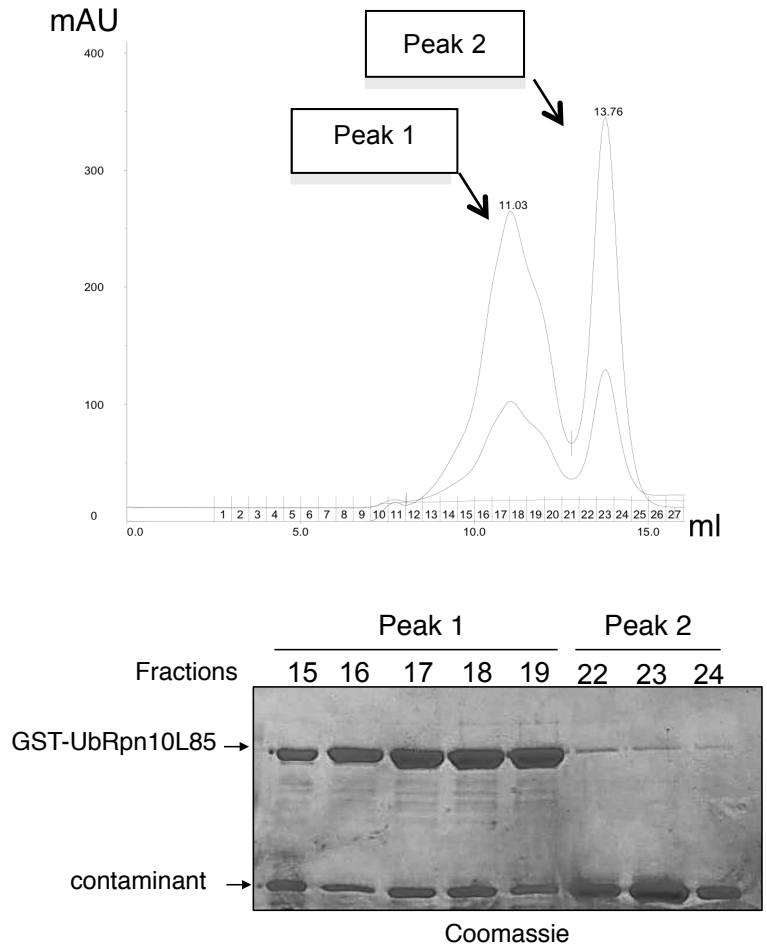


Figure 15. Size-exclusion chromatograpy of GST-UbRpn10L85 using a Superdex 200 column.
Profile of a size-exclusion chromatography. Fractions 15 through 19 (peak 1) and 22, 23 and 24 (peak 2) were loaded on a Coomassie staining of SDS-PAGE.

Next, we concentrated the fractions 15 through 19 that contained mainly the protein of interest and we performed an ion-exchange chromatography (monoQ) with a salt gradient that gradually elutes the proteins. Indeed, this technique separates proteins with different ionic strengths. We analyzed on a Coomassie gel the different elutions that we obtained and we observed that the fractions containing GST-UbRpn10L85 were not completely pure (Figure 16).

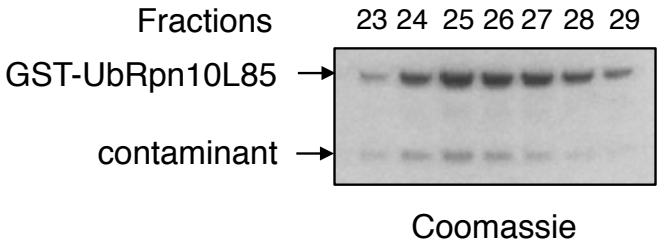


Figure 16. Ion-exchange chromatography.
Coomassie staining of SDS-PAGE of the fractions obtained after performing an ion-exchange chromatography that contained GST-UbRpn10L85.

To further purify the protein, we concentrated the elutions that mainly contained GST-UbRpn10L85 and we performed a thrombin cleavage. In order to remove the GST tag, we bound the elution to new Glutathione Sepharose beads (not shown). Next, we injected our sample into a size-exclusion column (Figure 17). Surprisingly, the Superdex could not elute Ub and UbRpn10L85 separately while all the GST was successfully removed from the fractions.

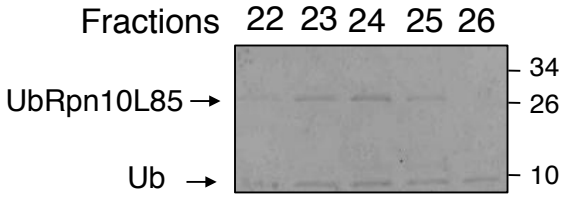


Figure 17. No traces of GST after the size-exclusion chromatography.
Coomassie staining of SDS-PAGE showing the proteins eluted after using a Superdex 200.

Finally, we concentrated the fractions containing mostly UbRpn10L85 and we performed an ion-exchange chromatography (Figure 18) resulting in UbRpn10L85 95% pure. We observed some minor bands that could derive from a degradation of UbRpn10L85.

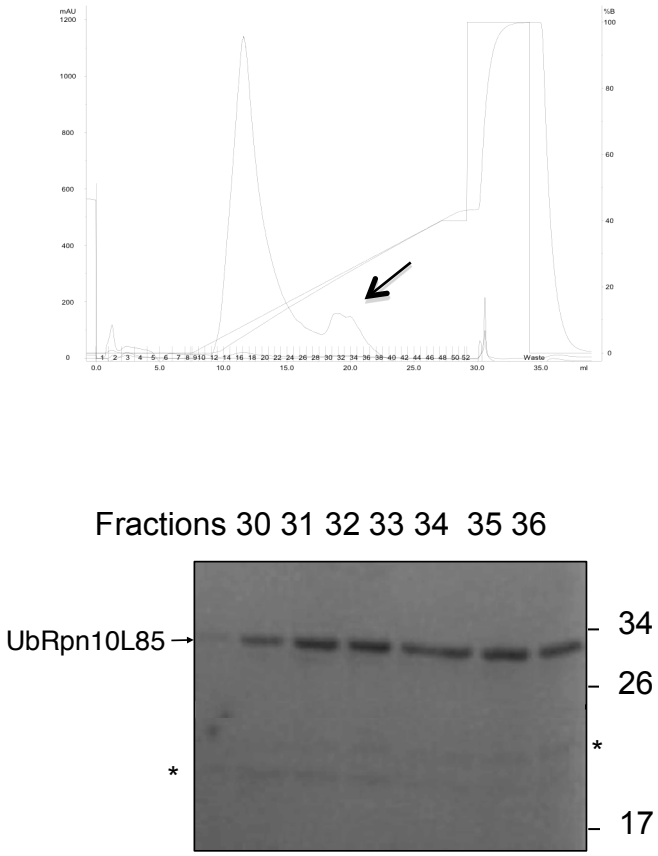


Figure 18. UbRpn10L85 is 95% pure.
Profile of the ion-exchange chromatography. Arrow points the peak including the fractions we ran: fractions 30 through 36. Below, Coomassie staining of SDS-PAGE showing the fractions obtained after running the sample through a monoQ column. Asterisks show some minor bands.

As a summary, after expressing 5 L of culture and finding the right conditions to purify UbRpn10L85, we just could purify 172 µg of 95% pure protein, which did not seem feasible to obtain large enough amounts for X-ray and NMR.

Optimization of the Rpn10 ubiquitination reaction

Rpn10 is ubiquitinated only when a three-step enzymatic cascade catalyzes the reaction. The incubation of Rpn10 with recombinant E1 (Uba1), E2 (Ubc4), E3 (Rsp5) and Ubiquitin results in the mono or multi-monoubiquitination of the substrate. Multi-monoubiquitination consists of the linkage of up to four ubiquitins to

the four *ubiquitinable* lysines in Rpn10, generating di-, tri- or tetraubiquitinated Rpn10 (Isasa et al, 2010).

To study in more detail the mechanisms by which Rpn10 is ubiquitinated, we decided to optimize the *in vitro* enzymatic reaction of Rpn10 ubiquitination that was firstly described by Isasa and collaborators (Isasa et al, 2010).

Before starting the optimization, the conditions we used to perform ubiquitination reaction of Rpn10 were the following:

Rpn10, Uba1, Ubc4 and Rsp5 were purified in the lab while Ub was commercial. We used 50 nM of Rpn10, 285 nM of Uba1, 10.9 μM of Ubc4, 150 nM of Rsp5 and 37,5 μM of Ubiquitin. Reactions were carried out in 100 mM Tris-HCl pH 7.4, 200 mM NaCl, 10 mM ATP, 10 mM $MgCl_2$, 1 mM DTT buffer at 30ºC for 3 hours.

According to Isasa et al, 100 nM of Uba1 from Enzo Life Sciences were enough to carry out the reaction. However, we used to use 285 nM of Uba1 from a purification I did during my visit to the University of Wurzburg, Germany at the lab of Dr. Schindelin (more information in the section Material and methods; (Lee & Schindelin, 2008)). We adjusted that amount in order to reach the same enzyme activity that we had when using the commercial Uba1: 285 nM Uba1 corresponded to 100 nM Uba1 (Enzo) with respect to the activity of the enzyme.

We started the optimization of the reaction by first varying the amount of ligase needed keeping all other components constant (Figure 19A and B). We observed that 50 nM of Rsp5 was the most suitable amount of ligase to produce ubiquitinated Rpn10, since there was little increase of modified Rpn10 when using higher amounts of Rsp5 (Figure 19A and B). Next, we evaluated the concentration of Uba1 needed. During this thesis, two Uba1 enzymes have been used. The first one was purified during my visit to Dr. Schindelin's lab, as mentioned above, and was used in Figure 19A, B and C (Purif. 1). Later, we purified Uba1 in our lab. To assess its activity, we did a titration in an ubiquitination reaction (Figure 19C, Purif. 2). Following the same criteria as for Rsp5, we concluded that working with 87 nM of Uba1 was enough to achieve an adequate ubiquitination reaction (Figure 19C). In a parallel assay, we optimized the amount of Ubc4 required for our reactions. The reaction was not more efficient when using more than 5,7 μM of the conjugating enzyme (Figure 19D).

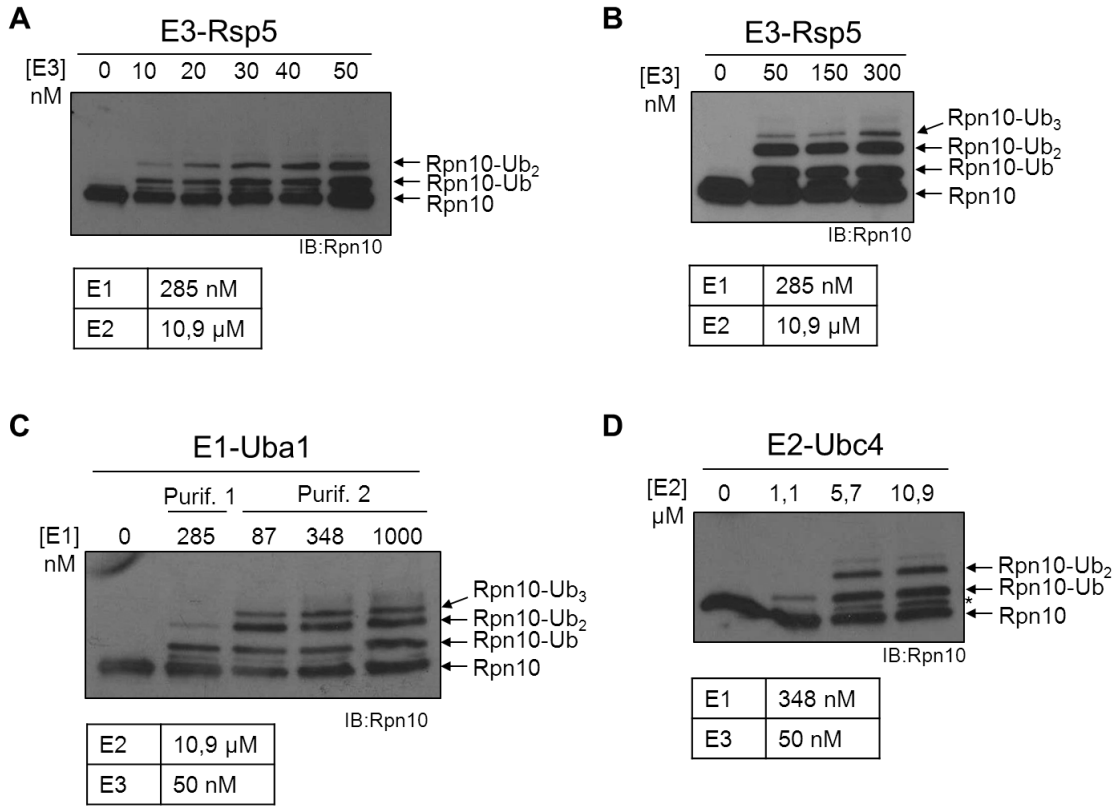


Figure 19. Optimization of the *in vitro* reaction of Rpn10 ubiquitination.
A) and B) Increasing concentrations of Rsp5 were used for Rpn10 ubiquitination reactions. **C)** Rpn10 ubiquitination reactions using different amounts of Uba1 enzyme. Purif. 1 refers to the purification during my visit to Schindelin's lab in Wurzburg. Purif. 2 refers to the purification done in our lab. **D)** Different concentrations of Ubc4 were used for each reaction. Concentrations of the other enzymes used to perform the reactions are included in the panels. Equal amounts of GST-Rpn10 were used for all the reactions, that is 50 nM. Reactions were all incubated at 30ºC for 3 hours.

As a summary, we include below a table detailing the reagent's concentrations of optimized *in vitro* ubiquitination reactions.

Uba1	87 nM
Ubc4	5,7 µM
Rsp5	50 nM
Ub	35,3 µM
ATP	10 mM
DTT	1 mM
Substrate	50 nM
Buffer	-

Interestingly, even under optimal conditions or with an excess of E1, E2 or E3, we never obtained Rpn10 polyubiquitinated.

Effect of the PPAY motif in Rpn10 ubiquitination

As seen in Figure 19 of the previous chapter, Rsp5 is virtually unable to build polyubiquitin chains on Rpn10 (Keren-Kaplan *et al*, 2011; Isasa *et al*, 2010) (Figure 19). This is striking since the ability of Rsp5 to modify substrates by polyubiquitination has been extensively demonstrated (Kim *et al*, 2009; Saeki *et al*, 2009; Kim *et al*, 2011a; Kee *et al*, 2005, 2006; Rotin & Kumar, 2009). We were interested in understanding the basis and to test the hypothesis that features of the substrate, in this case Rpn10, determine the nature of the ubiquitination. First of all, we wanted to investigate whether Rsp5 is able to polyubiquitinate Rpn10 at all. For this reason, we analyzed Rsp5 activity in our Rpn10 ubiquitination assays by evaluating the effect of an additional enzyme interaction motif on Rpn10. To do that, we placed a proline-proline-alanine-tyrosine (PPAY) motif at its N-terminus. A large group of Rsp5 substrates are PPXY-containing proteins, which bind to the WW motifs of Rsp5 and thus promote a productive substrate-enzyme interaction that usually yields polyubiquitination (Gupta *et al*, 2007; Kim *et al*, 2009; Lu *et al*, 2008; Saeki *et al*, 2009). We performed ubiquitination reactions using wild type Rpn10 (Rpn10) and the form carrying a PPAY motif (^{PPAY}Rpn10) and we observed that, whereas the reaction using Rpn10 produced monoubiquitination at 2 or 3 positions (see also Isasa *et al,* 2010), the one using ^{PPAY}Rpn10 resulted in a strong signal of polyubiquitinated forms (Figure 20A). Using different concentrations of Rsp5 we observed that this pattern was reproducible at all concentrations used (Figure 20B). Thus, by adding the PPAY enzyme-binding motif, the pattern of modification of Rpn10 changed substantially, from monoubiquitination to polyubiquitination. The specificity of the reaction catalyzed by Rsp5 was also assessed by a chain topology analysis. Previous work showed that Rsp5 builds K63 Ubiquitin chains on protein substrates (Kee *et al*, 2005; Kim *et al*, 2009; Saeki *et al*, 2009). We carried out reactions with Rpn10 and ^{PPAY}Rpn10 in the presence of WT, K63R, K48R, K63-only and K48-only versions of Ubiquitin. We observed that

the Ub forms containing lysine 63 (WT, K63-only and K48R ubiquitins) were competent in producing polyubiquitination on ^{PPAY}Rpn10, while K48-only and K63R ubiquitins produced short oligoubiquitination or multi-monoubiquitination (Figure 20C). Therefore, in our assays, Rsp5 exhibited a K63-based Ubiquitin-chain synthesis activity, in agreement with the previously characterized topological specificity (Kee *et al*, 2005; Kim *et al*, 2009; Saeki *et al*, 2009). We therefore conclude that the monoubiquitination of Rpn10 does not result from an impaired polyubiquitination activity of Rsp5, but is determined by specific features of Rpn10.

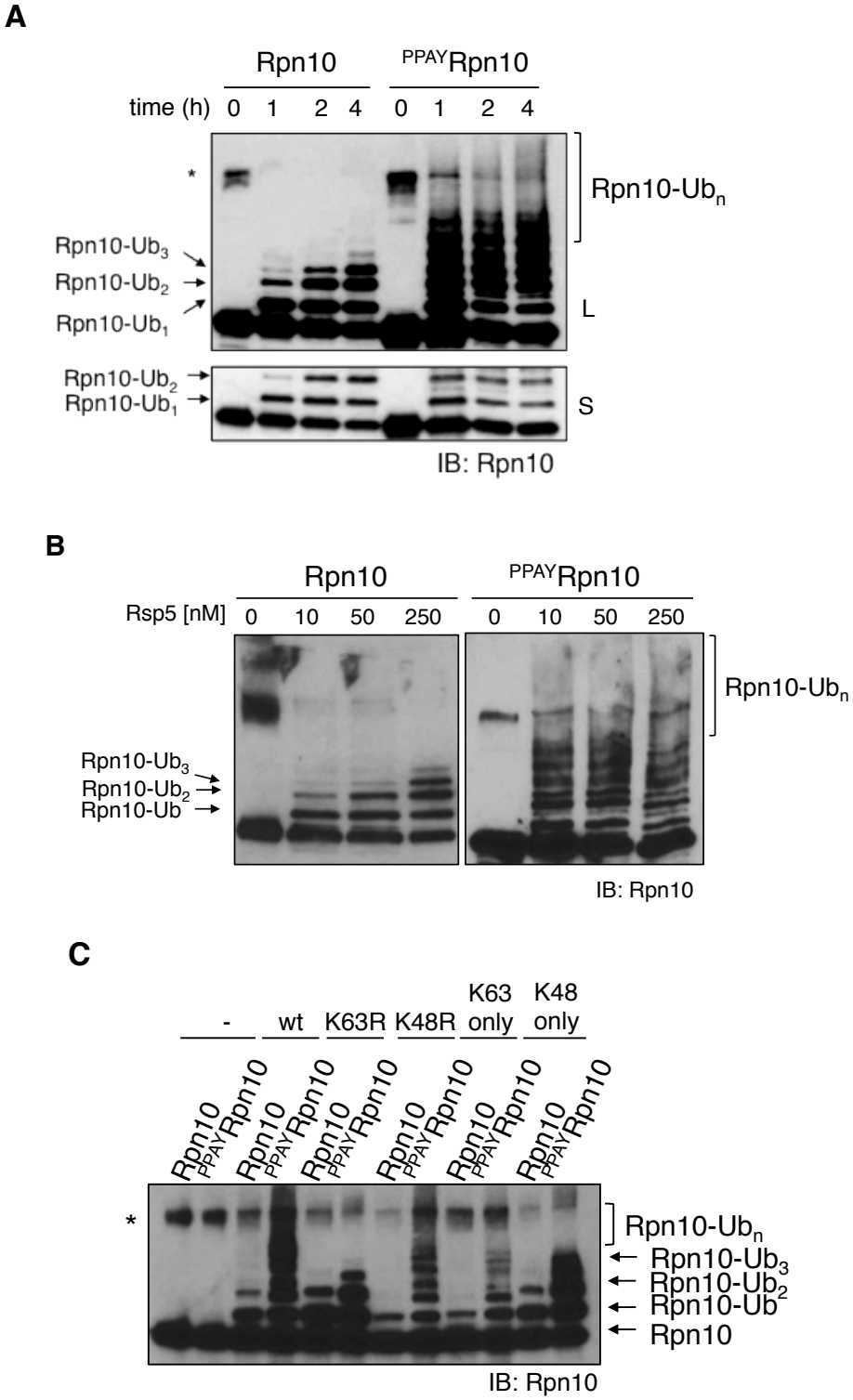


Figure 20. Rsp5 polyubiquitinates ^{PPAY}Rpn10.
A) Ubiquitination reactions of Rpn10 and ^{PPAY}Rpn10 analyzed by western blotting using Rpn10 antibody. Time points were taken as shown. Long (L) and short (S) exposures of the film are shown. **B)** ^{PPAY}Rpn10 is polyubiquitinated using different concentrations of Rsp5. Ubiquitination reactions of Rpn10 and ^{PPAY} Rpn10 using three different concentrations of Rsp5 were analyzed by anti-Rpn10 western blotting. **C)**

Ubiquitination reactions of Rpn10 and ^{PPAY}Rpn10 incubated with WT, K63R, K48R, K63-only and K48-only versions of Ubiquitin.

The Ubiquitin-interacting motif (UIM) of Rpn10 is an indispensable motif that gives Rpn10 the capacity to interact with Ubiquitin conjugates. Moreover, the UIM has been described as a requirement for Rpn10 monoubiquitination (Isasa et al, 2010). For that reason, we wondered whether the UIM motif was still indispensable to guarantee the reaction when the PPAY motif was also present. Considering the ability of Rsp5 to polyubiquitinate substrates that carry a PPAY motif and lack a UIM, it was reasonable to expect a modification in the status of Rpn10 (Figure 21). Interestingly, when the UIM was mutated (we introduced a block substitution from residues 228 to 232 of the UIM (LAMAL → NNNNN) (Elsasser et al, 2004)), the reaction was completely abrogated, for either mutant, Rpn10^{UIM} or ^{PPAY}Rpn10^{UIM}. Thus, the presence of the PPAY motif was not enough to ensure the reaction. This result underscores, once more, the key role that the UIM plays in Rpn10 ubiquitination.

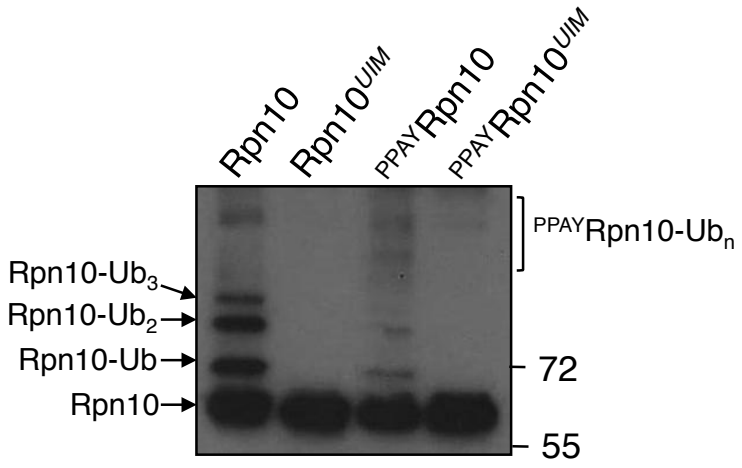
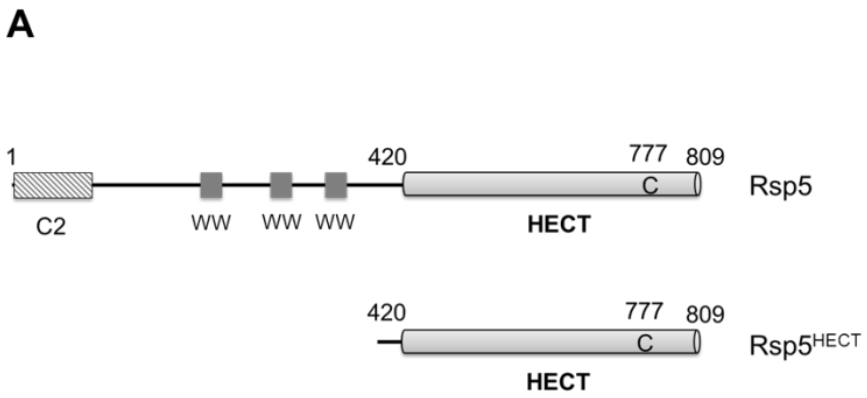


Figure 21. The UIM is still required to ubiquitinate ^{PPAY}Rpn10.
Ubiquitination reaction of Rpn10, Rpn10^{UIM}, ^{PPAY}Rpn10 and ^{PPAY}Rpn10^{UIM} analyzed by anti-Rpn10 western blotting.

The HECT domain of Rsp5 is sufficient for Rpn10 monoubiquitination

The polyubiquitination of ^{PPAY}Rpn10 by Rsp5 most likely resulted from an interaction between the Rsp5 WW domain and the PPAY motif. However, it was also possible that the N-terminal PPAY motif caused an altered conformation of Rpn10, facilitating an aberrant ubiquitination. To approach this point, we analyzed ubiquitination of ^{PPAY}Rpn10 in the presence of an Rsp5 version lacking WW motifs. Therefore, we generated a HECT domain construct (Rsp5^{HECT}), encompassing residues 420 to 809, which includes the catalytic domain but not the WW motifs (Figure 22A). We tested the activity of full-length Rsp5 and Rsp5^{HECT} in reactions using Rpn10 and ^{PPAY}Rpn10 as substrates, and observed that, while full-length Rsp5 showed distinct activities towards Rpn10 and ^{PPAY}Rpn10 (multi-monoubiquitination and polyubiquitination, respectively), Rsp5^{HECT} exhibited monoubiquitination on both these substrates (Figure 22B), suggesting that the N-terminal PPAY motif on Rpn10 does not cause aberrant ubiquitination. Moreover, with these assays we could show that Rsp5^{HECT} was even more efficient in Rpn10 monoubiquitination than the full-length Rsp5 protein.



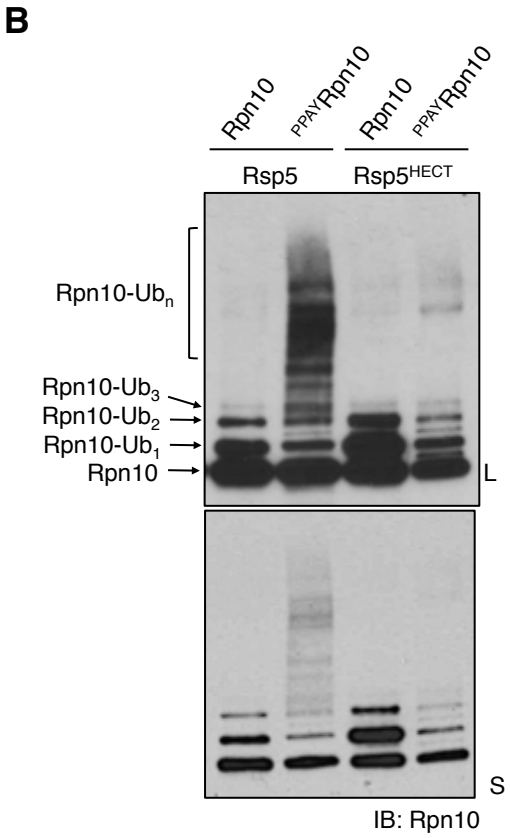


Figure 22. Rpn10 is monoubiquitinated *in vitro* by the HECT domain of Rsp5. A) Schematical representation of Rsp5 and Rsp5^{HECT}. Rsp5 contains an amino-terminal C2 domain, three WW domains and the HECT domain. The catalytic cysteine of the HECT is shown (C777). Rsp5^{HECT} encompasses residue positions 420 and 809. **B)** Reactions of ubiquitination of Rpn10 and ^{PPAY}Rpn10 incubated with Rsp5 full-length or Rps5HECT. Long (L) and short (S) exposures of the film are shown.

Of the five HECT Ubiquitin ligases found in yeast, only Rsp5 has been proved to be the major E3 that ubiquitinates Rpn10, so far (Isasa et al, 2010). Hul5 and Ufd4 ligases have been already ruled out (Isasa *et al*, 2010), while Tom1 and Hul4 have never been studied from the point of view of Rpn10 ubiquitination. In order to shed light on this subject, we studied the efficiency of these two ligases in the ubiquitination of Rpn10. After performing ubiquitination reactions, we observed that Rsp5^{HECT} appeared to be much more efficient than the HECT domain of Tom1, in the reaction of Rpn10 monoubiquitination. Parallel assays using Rsp5^{HECT} and Tom1^{HECT}, showed that, despite the fact that Tom1^{HECT} shows higher catalytic activity than Rsp5^{HECT}, assessed by the level of auto-ubiquitination (Figure 23A), Rsp5^{HECT} was much more competent in ubiquitinating Rpn10 (Figure 23B and C).

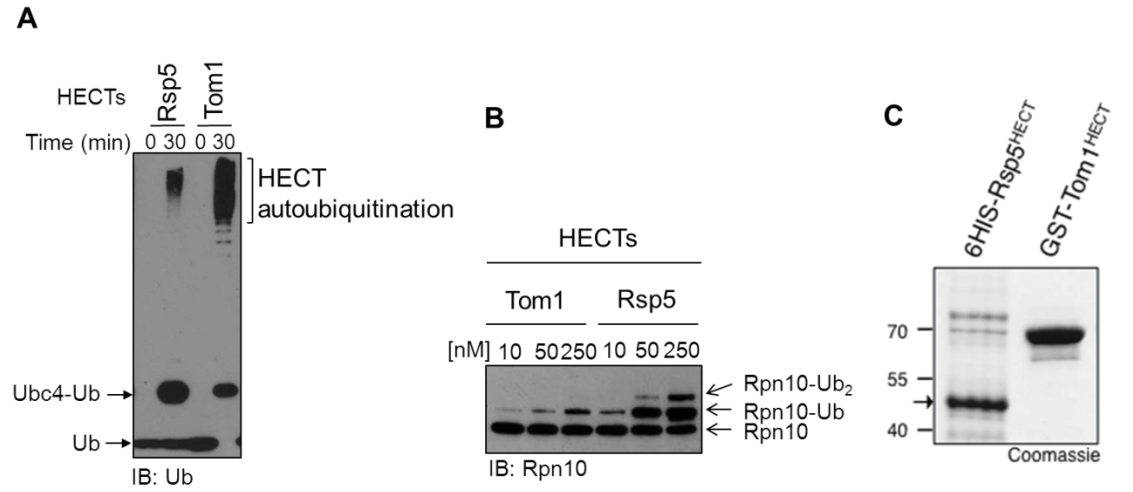


Figure 23. Rsp5 is more efficient than another Ubiquitin ligase in ubiquitinating Rpn10.
A) Autoubiquitination reaction of Rps5HECT and Tom1^{HECT} with Ub WT analyzed by western blotting using an anti-Ub antibody. **B)** Rpn10 ubiquitination assays using Rsp5^{HECT} or Tom1^{HECT} at different concentrations analyzed by western blotting using an Rpn10 antibody. **C)** 2 µg of purified Rsp5^{HECT} (black arrow) and Tom1^{HECT} were resolved by SDS-PAGE and detected using Coomassie blue staining.

We also tested the efficiency of the HECT domain of the Ubiquitin ligase Hul4 in ubiquitinating Rpn10. Interestingly, Rpn10 was not modified after performing an ubiquitination reaction with this ligase at any concentration used (Figure 24A). To know whether this result was due to a lack of specificity or a lack of activity of Hul4^{HECT}, we assessed the levels of auto-ubiquitination of the ligase. We observed that Hul4^{HECT} was not auto-ubiquitinated and therefore not active, which would explain the absence of reaction in Rpn10 (Figure 24B).

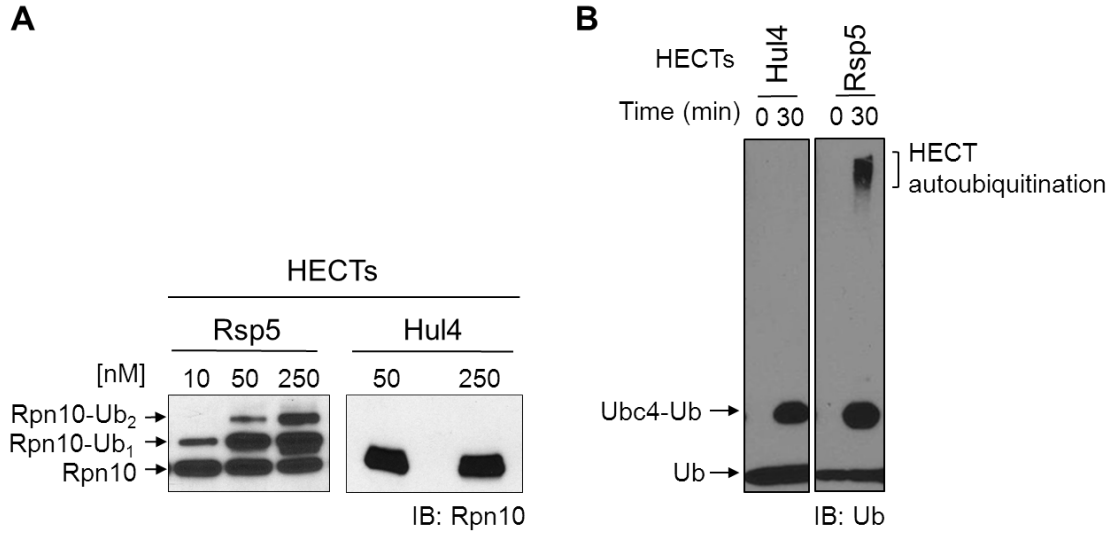


Figure 24. Hul4^{HECT} is not catalytically active.
A) Rpn10 ubiquitination reactions with Rsp5^{HECT} or Hul4^{HECT} at different concentrations. **B)** Autoubiquitination assay of Rps5^{HECT} and Hul4^{HECT} with Ub WT.

From these assays we could conclude that Rsp5^{HECT}, although inactive in the polyubiquitination of Rpn10, is the most efficient HECT ligase in *S. cerevisiae* in ubiquitinating Rpn10, although an active Hul4^{HECT} should be tested before being able to confirm this. Moreover, from the results obtained in Figure 22, we can say that Rsp5^{HECT} showed a stronger activity towards Rpn10 ubiquitination than Rsp5 full-length.

Ubp2 rescues monoubiquitination in ^{PPAY}Rpn10 ubiquitination reactions

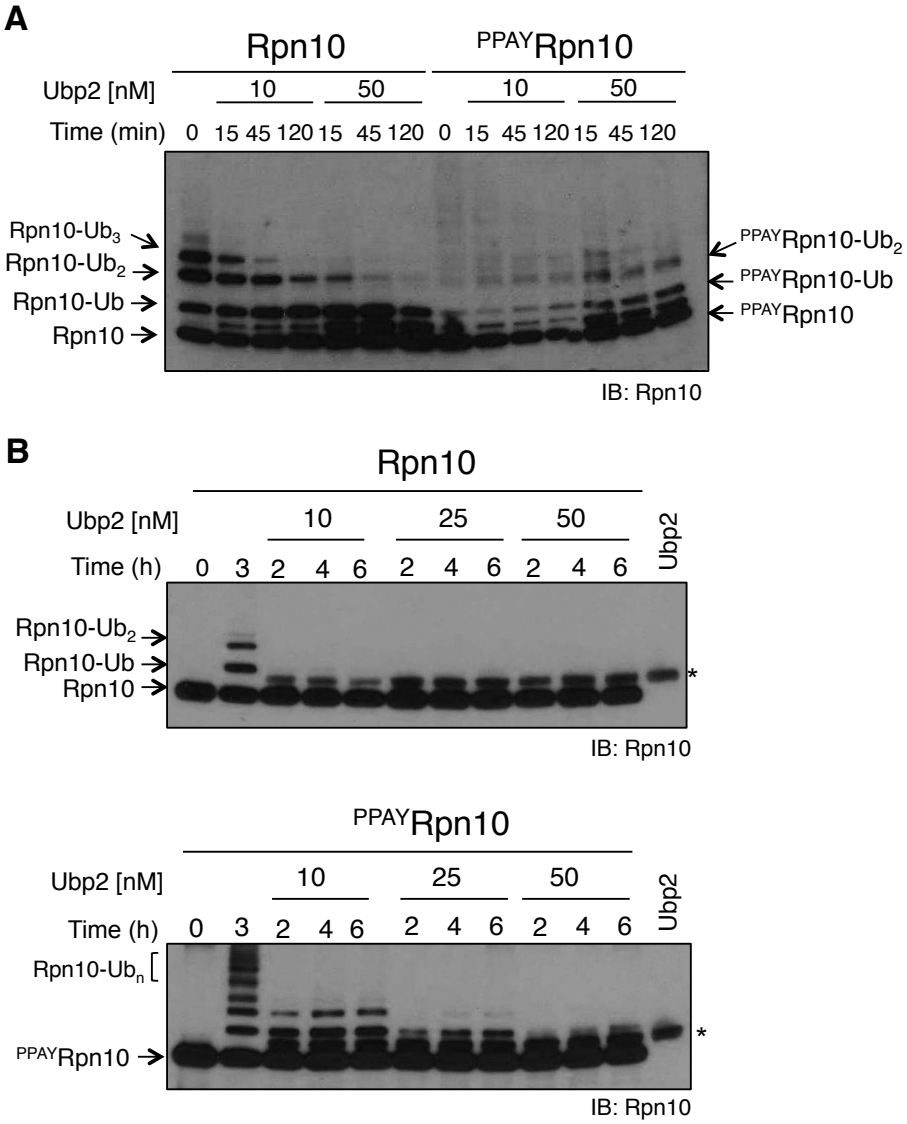
Ubp2 is a deubiquitinating enzyme that exhibits antagonistic activity with respect to Rsp5 and that exists in a complex with Rsp5 (Kee et al., 2005). The hydrolysis takes place between a lysine of an Ubiquitin moiety and the C-terminus of the next Ubiquitin. When the DUB removes all the Ub moieties except the one that is bound to the substrate, the result is a monoubiquitinated substrate. Harreman et al, observed that polyubiquitinated RNAPII was efficiently hydrolyzed by Ubp2, generating monoubiquitinated RNAPII (Harreman *et al*, 2009). It was

previously shown in our lab that the levels of mUb-Rpn10 are regulated by Ubp2 (Isasa *et al*, 2010; Kee *et al*, 2005). Therefore, we next wanted to evaluate whether the polyubiquitination chains formed in ^{PPAY}Rpn10 could be counteracted by Ubp2 activity and consequently, monoubiquitinated Rpn10 could be generated. For that, we did an ubiquitination reaction of both, Rpn10 and ^{PPAY}Rpn10. We incubated the product of these reactions with different amounts of Ubp2 and for different times (Figure 25A and B). Although the ubiquitination reaction of ^{PPAY}Rpn10 was not very efficient, Ubp2 was competent in partially hydrolyzing the Ubiquitin chains. Indeed, after 120 minutes of incubation with Ubp2, most of Rpn10 and ^{PPAY}Rpn10 were mono or diubiquitinated.

To further evaluate the complex formed by Rsp5 and Ubp2, we did an ubiquitination reaction in the presence of Ubp2. We wondered how the reaction would be influenced by the presence of the hydrolase. We observed that Ubp2 was extremely efficient in deubiquitinating Rpn10 considering that no modified state of Rpn10 was visible at all (Figure 25B). Indeed, with only 10 nM Ubp2, whereas 50 nM Rsp5 was present, monoubiquitination of Rpn10 was not observed. In contrast, in a 3 hours' reaction, where Ubp2 is not present, at least mono- and diubiquitination of Rpn10 can be observed (Figure 25A and B, lane 2).

On the other hand, a longer incubation and a higher amount of Ubp2 were needed to obtain the same result when studying the effect of the deubiquitinase on ^{PPAY}Rpn10 (Figure 25B, lower panel). The higher affinity between Rsp5 and the PPAY motif and the subsequent polyubiquitination of the substrate could explain this result. Interestingly, only 10 nM Ubp2 after a 2 hours' incubation were needed to rescue ^{PPAY}Rpn10 mono- and diubiquitination.

Finally, the same experiment was repeated using Rsp5^{HECT} instead of Rsp5 full-length in the presence of Ubp2. Under these conditions, it seemed that Ubp2 was not able to catalyze complete Rpn10 deubiquitination and monoubiquitinated proteins remained. This is consistent with a stronger capacity of Rsp5^{HECT} to ubiquitinate Rpn10 compared to Rsp5 full-length.


A
Rpn10
PPAYRpn10
Ubp2 [nM]
10
50
10
50
Time (min)
0 15 45 120 15 45 120 0 15 45 120 15 45 120
Rpn10-Ub3
Rpn10-Ub2
Rpn10-Ub
Rpn10
PPAYRpn10-Ub2
PPAYRpn10-Ub
PPAYRpn10
IB: Rpn10
B
Rpn10
Ubp2 [nM]
10
25
50
Ubp2
Time (h)
0 3 2 4 6 2 4 6 2 4 6
Rpn10-Ub2
Rpn10-Ub
Rpn10
*
IB: Rpn10
PPAYRpn10
Ubp2 [nM]
10
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Rpn10-Ubn
PPAYRpn10
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IB: Rpn10

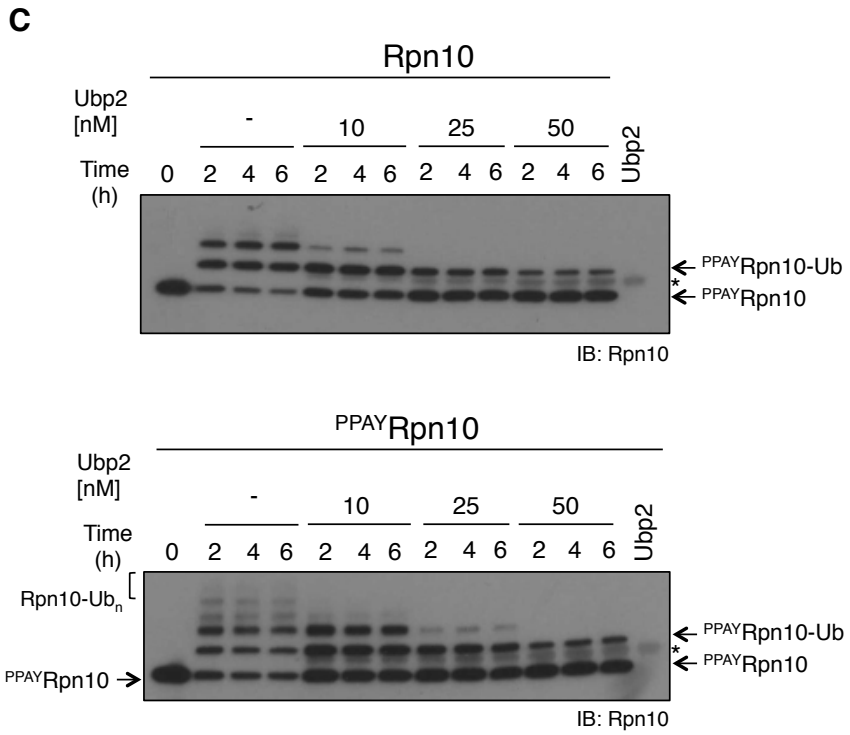


Figure 25. Ubp2 deubiquitinates ^{PPAY}Rpn10.
A) Ubiquitination reaction of Rpn10 and ^{PPAY}Rpn10 (lanes time 0) followed by a time-course deubiquitination reaction with Ubp2. **B)** Time-course ubiquitination reaction of Rpn10 and ^{PPAY}Rpn10 in the presence of Ubp2 and 50 nM Rsp5. The 3 hours' reactions (upper and lower panels) show an ubiquitination reaction of these substrates without Ubp2 to facilitate comparisons. **C)** Time-course ubiquitination reaction of Rpn10 and ^{PPAY}Rpn10 in the presence of Ubp2 and 50 nM Rsp5^{HECT}. The first four lanes (upper and lower panels) show an ubiquitination reaction of these substrates without Ubp2 to facilitate comparisons. Asterisks in Figure 25B and C show a protein present in the Ubp2 purification that crossreacts with the anti-Rpn10 antibody.

We conclude from these experiments that the balance of the activity of these two counteracting proteins, Rsp5 and Ubp2, is important for the final outcome of ubiquitination of Rpn10.

Slow growth defect caused by increased levels of monoubiquitinated Rpn10

The high level of Rpn10 monoubiquitination by $Rsp5^{HECT}$ (Figures 22B and 23B) could be used as a valuable tool for the physiological analysis of Rpn10 monoubiquitination *in vivo*. In a previous study, it was shown that mUb-Rpn10 is inactive as a polyubiquitin receptor, causing a decrease in proteasome activity, thus constituting a direct mechanism of polyubiquitin receptor regulation (Isasa *et al*, 2010). Moreover, the levels of Rpn10 monoubiquitination are tightly controlled *in vivo*, and high levels of Rpn10-Ubiquitin (Rpn10 including C- terminally fused Ubiquitin) cause growth deficiency in yeast (Isasa *et al*, 2010). Our finding here that the HECT domain of Rsp5 alone is sufficient for monoubiquitination of Rpn10 gave us the opportunity to functionally analyze the impact of Rsp5-dependent Rpn10 monoubiquitination without affecting the regulation of numerous PPXY-dependent protein substrates *in vivo* (Gupta *et al*, 2007; Lin *et al*, 2008; Lu *et al*, 2008; Belgareh-Touzé *et al*, 2008; Nikko & Pelham, 2009). Therefore, we placed $Rsp5^{HECT}$ under the control of the GAL4 promoter and tested the capacity of galactose-induced $Rsp5^{HECT}$ to produce monoubiquitinated Rpn10 in a wild-type strain in *Saccharomyces cerevisiae*. With this approach, we observed that Rpn10 monoubiquitination was efficiently induced during several hours of galactose-induced $Rsp5^{HECT}$ expression (Figure 26A).

In order to show more directly that the band with a slower mobility was monoubiquitinated Rpn10, we next tested the effect of increased expression of $Rsp5^{HECT}$ and the catalytically inactive mutant $Rsp5^{HECT\ C777A}$ on Rpn10 and $Rpn10^{K84,268R}$, a mutant involving the two lysine residues mainly targeted for ubiquitination. As expected, when $Rsp5^{HECT}$ was induced in the presence of $Rpn10^{K84,268R}$ monoubiquitination was totally impaired (Figure 26B, lane 8). Interestingly, the induction of $Rsp5^{HECT\ C777A}$ in the presence of Rpn10 resulted in a slight band, revealing the activity of endogenous Rsp5.

With the same purpose to verify the nature of the band above the unmodified Rpn10, we performed an immunoprecipitation. After the induction of $Rsp5^{HECT}$ in a *rpn10*Δ strain carrying Rpn10 and $Rpn10^{K84,268R}$, we immunoprecipitated Rpn10 in order to detect the modified Rpn10 using anti-

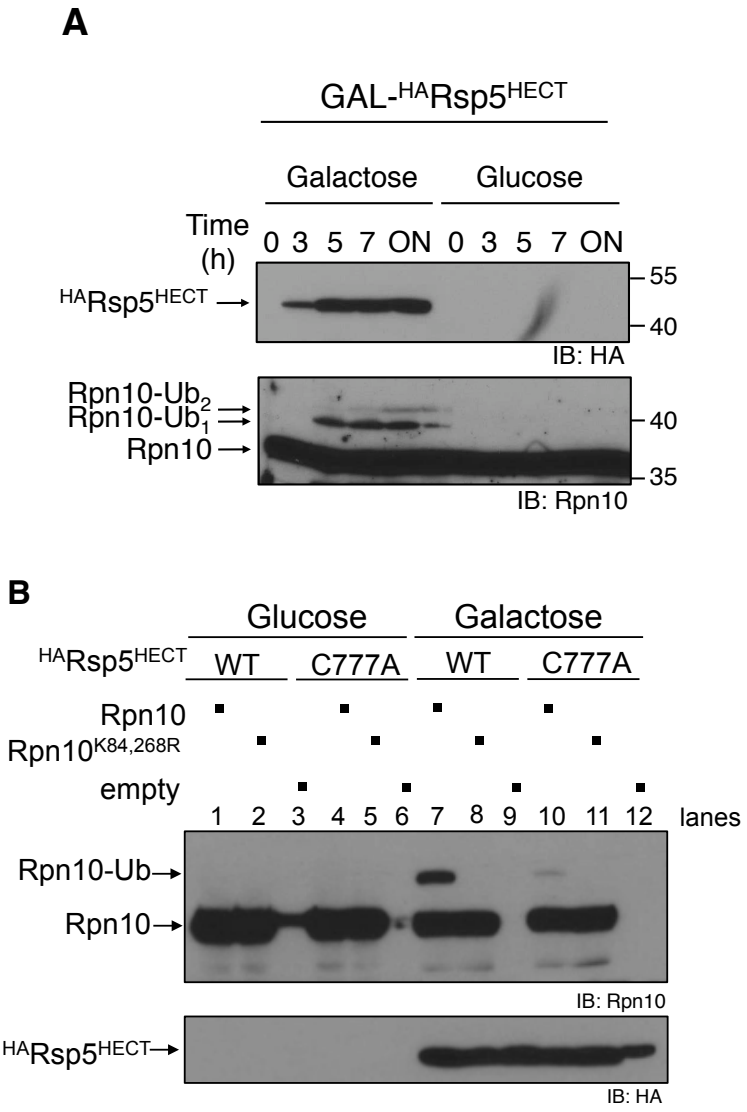
Ubiquitin. Unfortunately, we had problems due to a strong cross-reactivity with Rpn10, which made the approach inconclusive.

Finally, we analyzed the susceptibility of this band to deubiquitination (Figure 26C). As we observed previously (Figure 25; Isasa et al, 2010), Ubp2 deubiquitinates Rpn10. If the band with the slower mobility was mUb-Rpn10, Ubp2 should be able do deubiquitinate it. For this, a whole cell extract obtained after inducing the HECT domain of Rsp5 was incubated in the presence of recombinant Ubp2. We observed that in the control in absence of additional Ubp2, the band with slower mobility decreased with time while the Rpn10 band remained constant (Figure 26C, left lanes). In the presence of recombinant Ubp2, the disappearance of the band was notably accelerated. We reason that the presence of endogenous Ubp2 and other DUBs in the whole cell extract may be responsible for the processing of monoubiquitinated Rpn10 in the control, and that the addition of recombinant Ubp2 results in the increase of the hydrolysis of monoubiquitinated Rpn10 in the extract. Therefore, we conclude that the band above unmodified Rpn10 is monoubiquitinated Rpn10.

Next, we analyzed the effect of Rpn10 monoubiquitination on the steady state levels of polyubiquitinated protein in the cell. It was previously shown in our lab that monoubiquitination of Rpn10 exhibits low affinity to polyubiquitinated substrates *in vitro* and that this results in a decrease in the proteolytic activity of the proteasome (Isasa et al, 2010). Therefore, we expected to see a rich proportion of polyubiquitinated substrates after inducing $Rsp5^{HECT}$ in a strain that carried Rpn10. To analyze the accumulation of polyubiquitinated conjugates, we used the whole cell extracts that we obtained after expressing Rpn10 or $Rpn10^{K84,268R}$ and inducing $Rsp5^{HECT}$ and $Rsp5^{HECT\ C777A}$ (Figure 26B and D). Interestingly, a strong accumulation of polyubiquitin signal was observable after induction (4 hours) of $Rsp5^{HECT}$ in cells expressing wild-type Rpn10 (Figure 26D). This observation suggests that a sudden increase of Rpn10 monoubiquitination correlates with accumulation of polyubiquitinated conjugates, which would imply the incapacity of Rpn10 to bind polyubiquitinated substrates and, therefore, a decrease in proteasome activity.

We next tested the effect of increased Rpn10 monoubiquitination on yeast growth. We expressed Rpn10 and $Rpn10^{K84,268R}$ in a *rpn10*Δ strain that carried $^{HA}Rsp5^{HECT}$ or $^{HA}Rsp5^{HECT\ C777A}$ galactose-inducible plasmids. Cultures were grown

in glucose and galactose for 4 hours and later prepared for a spot assay. Plates with glucose or galactose were grown at room temperature. The induction of Rpn10 monoubiquitination by over-expressing $Rsp5^{HECT}$ resulted in a severe slow-growth phenotype (Figure 26B, lane 7 and E). Notably, both Rpn10 monoubiquitination and the deleterious phenotype were completely dependent on $Rsp5^{HECT}$ activity, since the catalytically inactive mutant $Rsp5^{HECT\ C777A}$ did not show the effect (Figure 26D, lane 10; and 2E). Moreover, a partial rescue of the slow growth phenotype was observed when $Rpn10^{K84,268R}$ was expressed and $Rsp5^{HECT}$ induced (Figure 26E), indicating the high functional impact of Rsp5-induced Rpn10 monoubiquitination on yeast growth.



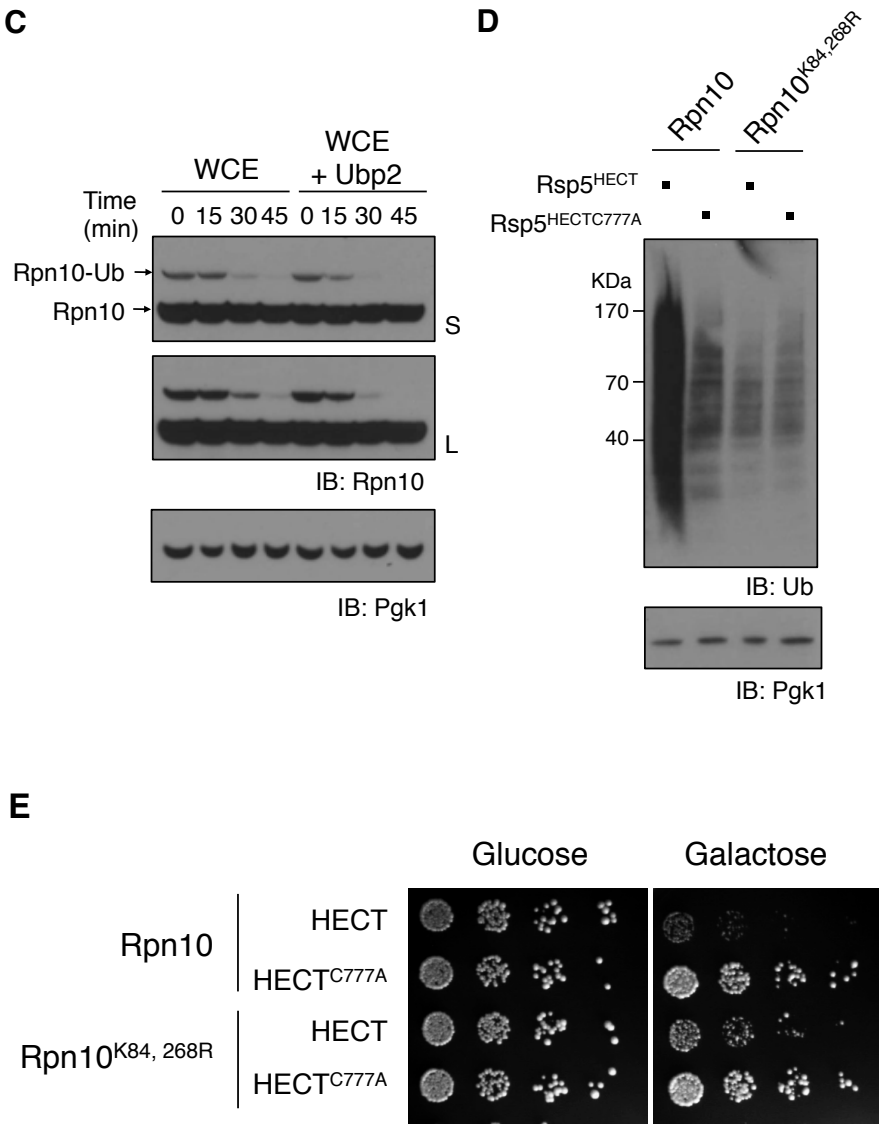


Figure 26. Slow growth phenotype caused by Rpn10 monoubiquitination *in vivo*. **A)** Wild-type strain (FY56) carrying a $^{HA}Rsp5^{HECT}$ galactose-inducible plasmid was grown in galactose or glucose media for the indicated hours. Upper panel shows the induction levels analyzed by western blotting using an anti-HA antibody. Endogenous Rpn10 was analyzed by Rpn10 western blotting, shown in the lower panel. **B)** *rpn10*Δ strain (S72) carrying plasmids Rpn10, $Rpn10^{K84,268R}$, from its own promoter, or the empty plasmid and either the $^{HA}Rsp5^{HECT}$ or $^{HA}Rsp5^{HECT\ C777A}$ galactose-inducible plasmids were grown in glucose and galactose media for 4 hours. Rpn10 was analyzed by Rpn10 western blotting, shown in the upper panel. The lower panel shows the induction levels of Rsp5 proteins analyzed by western blotting using an anti-HA antibody. **C)** The whole cell extract used in panel C carrying plasmids Rpn10 and $^{HA}Rsp5^{HECT}$ was incubated with and without recombinant Ubp2. Reactions were analyzed by Rpn10 western blotting and with anti-pgk1 antibody as a loading control. **D)** Accumulation of polyubiquitin signal is shown for the whole cell extracts used in panel B carrying plasmids Rpn10 and $Rpn10^{K84,268R}$, and $^{HA}Rsp5^{HECT}$ or $^{HA}Rsp5^{HECT}$

^{C777A}. Polyubiquitinated conjugates were analyzed by Ubiquitin western blotting. Blots were also developed with an anti-pgk1 antibody as a loading control. **E)** The same cultures shown in panel B carrying Rpn10 and Rpn10^{K84,268R} and ^{HA}Rsp5^{HECT} or ^{HA}Rsp5$^{HECT\ C777A}$ were diluted to an OD_{600} = 0.04, spotted in 5-fold serial dilutions and grown at room temperature (22ºC), as shown.

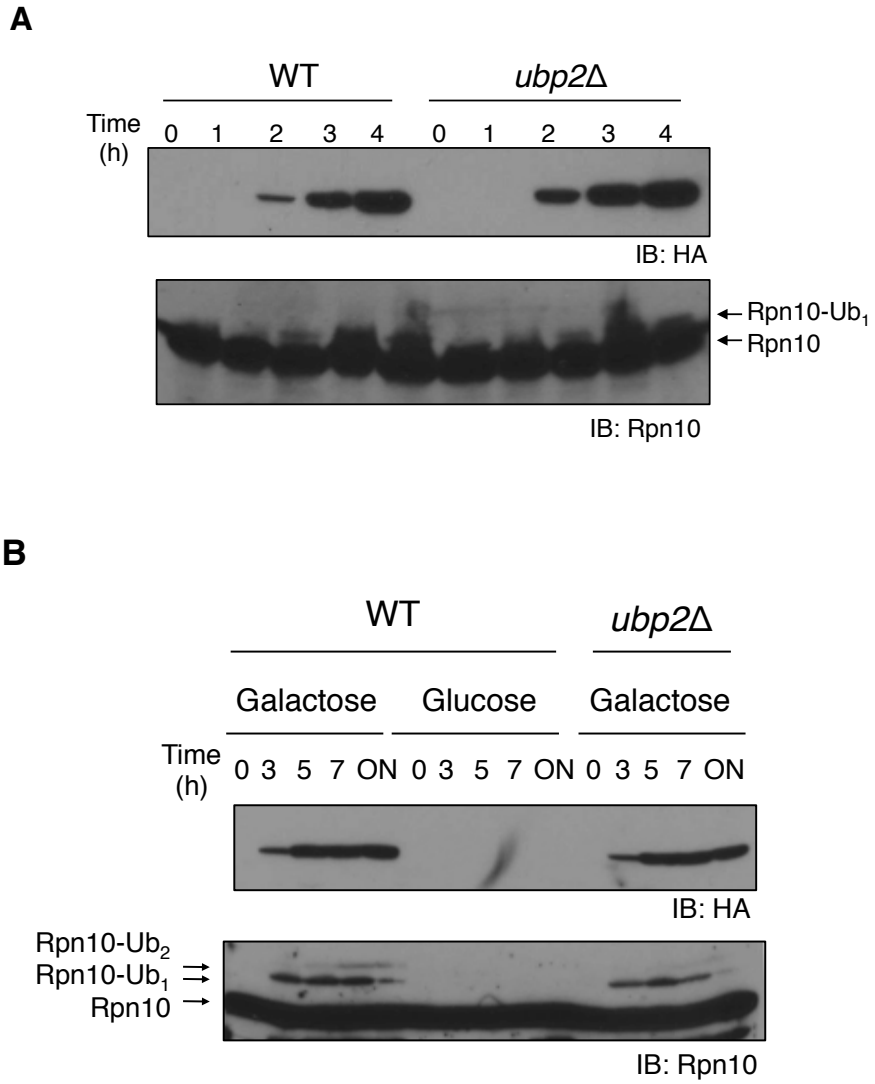
In order to evaluate the effect of the overexpression of two other HECT containing proteins on Rpn10 ubiquitination *in vivo*, we cloned Tom1^{HECT} and Hul4^{HECT} in galactose-inducible plasmids. We transformed each plasmid carrying Rsp5^{HECT}, Tom1^{HECT} and Hul4^{HECT} in a WT strain. Unfortunately, after 4 hours growing the cultures in galactose, no induction was detected by western blot either for Tom1^{HECT} or for Hul4^{HECT}. The experiment was repeated several times with no success. Thus, we cannot draw conclusions regarding the specificity of Rsp5 for Rpn10 ubiquitination *in vivo*.

Induction of Rsp5^{HECT} in other yeast strains

Since Upb2 antagonizes Rsp5 activity, we hypothesized that the absence of Ubp2 might result in a stronger Rpn10 ubiquitination and therefore a stronger growth defect phenotype. To test this hypothesis, we overexpressed the HECT of Rsp5 under control of a galactose-inducible promoter in a wild-type strain and in an *ubp2*Δ strain. We observed that the levels of mUb-Rpn10 were the same in both strains and that both depended on Rsp5 induction (Figure 26A and 27A). In order to make clearer the modification of Rpn10, we repeated the induction reaching the same result (Figure 27B), suggesting that other DUBs may be controlling Rpn10 ubiquitination levels.

Additionally, we tested the effect of Rsp5 induction at 35ºC in a double mutant strain with a thermosensitive mutation for Rsp5 and *ubp2*Δ (*rsp5–1 ubp2*Δ). The *rsp5–1* mutant results in a single mutation change in the HECT domain, which makes the protein deficient in the formation of Ubiquitin-thioester intermediates at 35ºC (Wang et al., 1999). The premise was again that overexpression of Rsp5 would cause an increase in mUb-Rpn10 levels in *rsp5–1 ubp2*Δ strain, only dependent on the induced Rsp5^{HECT}. As previously seen, only when Rsp5 was

induced, the monoubiquitination of Rpn10 was detected, although the intensity of the band was very similar for both the wild-type and the double mutant strain (Figure 27C). We did not expect these results considering that Isasa *et al* observed that levels of mUb-Rpn10 were strongly increased in the absence of Ubp2 (Isasa *et al*, 2010).



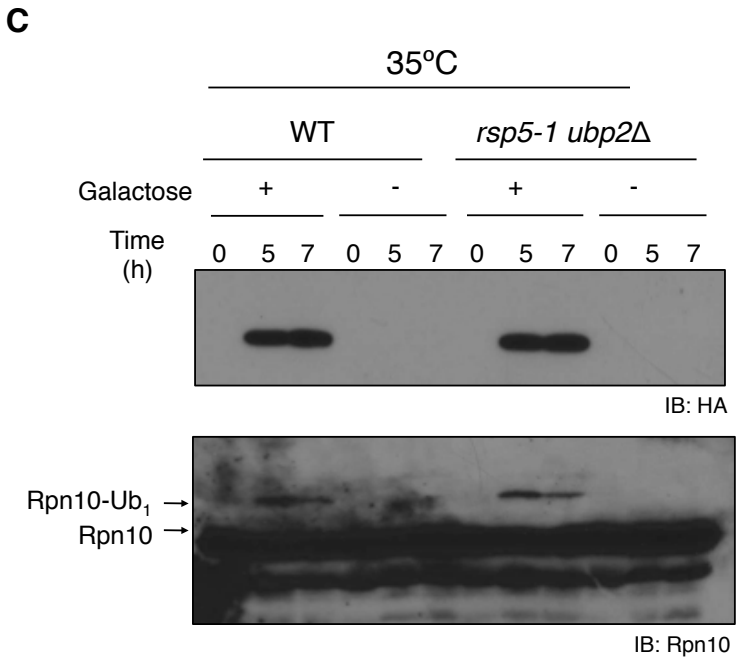


Figure 27. Rpn10 is monoubiquitinated in *ubp2*Δ and *rsp5-1 ubp2*Δ strains.
A) and B) Wild-type strain (FY56) and *ubp2*Δ carrying a ^{HA}Rsp5^{HECT} galactose-inducible plasmid were grown in galactose or glucose media for the indicated hours. Upper panels show the induction levels analyzed by western blotting using an anti-HA antibody. Endogenous Rpn10 was analyzed by Rpn10 western blotting, shown in the lower panels. **C)** Time-course induction of ^{HA}Rsp5^{HECT} in wild-type (FY56) and *rsp5-1 ubp2*Δ strains.

Minimal sequence requirements for Rpn10 monoubiquitination

Having shown that Rpn10 restricts Rsp5 activity to produce Rpn10 monoubiquitination (or multi-monoubiquitination) and that this has important implications for yeast growth, we next aimed to characterize the mechanism underlying Rpn10-driven monoubiquitination. The Rpn10 structure consists of a VWA domain that encompasses an N-terminal region of approximately 190 aa, and a UIM-containing C-terminal region (Lambertson *et al*, 1999; Fu *et al*, 1998). The UIM-containing sequence is found in the context of an α -helix that is flanked by highly flexible linkers (Wang *et al*, 2005; Zhang *et al*, 2009; Riedinger *et al*, 2010). Moreover, the C-terminal end contains lysine residues that are modified by Ubiquitin, as shown in *S. cerevisiae* and *D. melanogaster* (Isasa *et al*, 2010;

Lipinszki *et al*, 2013). With this information, we designed truncated versions of Rpn10 and tested their capacity to undergo monoubiquitination in our standard Rsp5-dependent Ubiquitin ligation reaction. Thus, we tested $Rpn10^{40-268}$, a fragment that contains the VWA domain but lacks N-terminal residues involved in proteasome interaction (Fu *et al*, 1998), and $Rpn10^{195-268}$, which only includes the C-terminal region of Rpn10 including the UIM. Rpn10 can be ubiquitinated at lysines 77, 84, 99 and 268, being K84 and K268 the most abundantly modified. Thus, $Rpn10^{40-268}$ included the four lysines that can be modified whereas $Rpn10^{195-268}$ had only one lysine at the very C-terminus of the protein. We performed ubiquitination reactions with these fragments and we observed that both versions of Rpn10 were efficiently mono- or diubiquitinated by Rsp5 (Figure 28A and B), showing that both the N-terminal region and the VWA domain are dispensable for the reaction of ubiquitination. This is a relevant observation, since the VWA is a large domain, that represents approximately 75% of the whole protein, and plays roles in Rpn10- proteasome interaction and protein degradation (Verma *et al*, 2004; Fu *et al*, 1998). These two fragments were GST tagged and we wondered whether the tag was also a site of Ub attachment. Thus, we cleaved the GST tag in $Rpn10^{195-268}$ and we observed that this fragment, that also lacked the VWA domain ($Rpn10^{195-268}$) behaved as a very good substrate of Rsp5, generating prominent mono- and diubiquitinated forms (Figure 28C and D) in a reaction exquisitely dependent on the presence of the E3 (Figure 28D). The reaction with Ub K0, an Ubiquitin mutant that has no lysines and cannot synthetize Ub chains, produced monoubiquitinated $Rpn10^{195-268}$, while Ub wild-type produced diubiquitinated $Rpn10^{195-268}$ (Figure 28C). This could be explained either by Rsp5 ability to build short Ubiquitin chains in Rpn10 or by the conjugation of Ub to the amino-terminus of $Rpn10^{195-268}$ (Ciechanover and Ben-Saadon, 2004). To confirm the nature of the bands observed in 28C and D, we performed an ubiquitination reaction of $Rpn10^{195-268}$, immediately followed by the addition of Ubp2 (Figure 28E). An evident decrease in the diubiquitinated form of the mutant is observed after 3 hours of incubation with Ubp2 at 10 nM or after 1 hour at 50 nM Ubp2. An incubation of 3 hours with 50 nM Ubp2 seems to almost completely deubiquitinate $Rpn10^{195-268}$. Strikingly, an increase of the monoubiquitinated or the unmodified form of the mutant is not appreciated under these conditions.

Therefore, the reaction of Rpn10 monoubiquitination can be produced in an Rpn10 fragment of 73 amino acid residues. This fragment has a high disposition to disorder, only interrupted by a helicoidal structure that contains the UIM (Figure 28F; (Hofmann & Falquet, 2001; Wang *et al*, 2005; Riedinger *et al*, 2010)). To carry out the prediction of unstructured regions we used the meta-predictor computer program PONDR-FIT. PONDR-FIT uses an amino acid sequence as the input and gives structure (order) or disorder as the output by combining the prediction of different software's predictions.

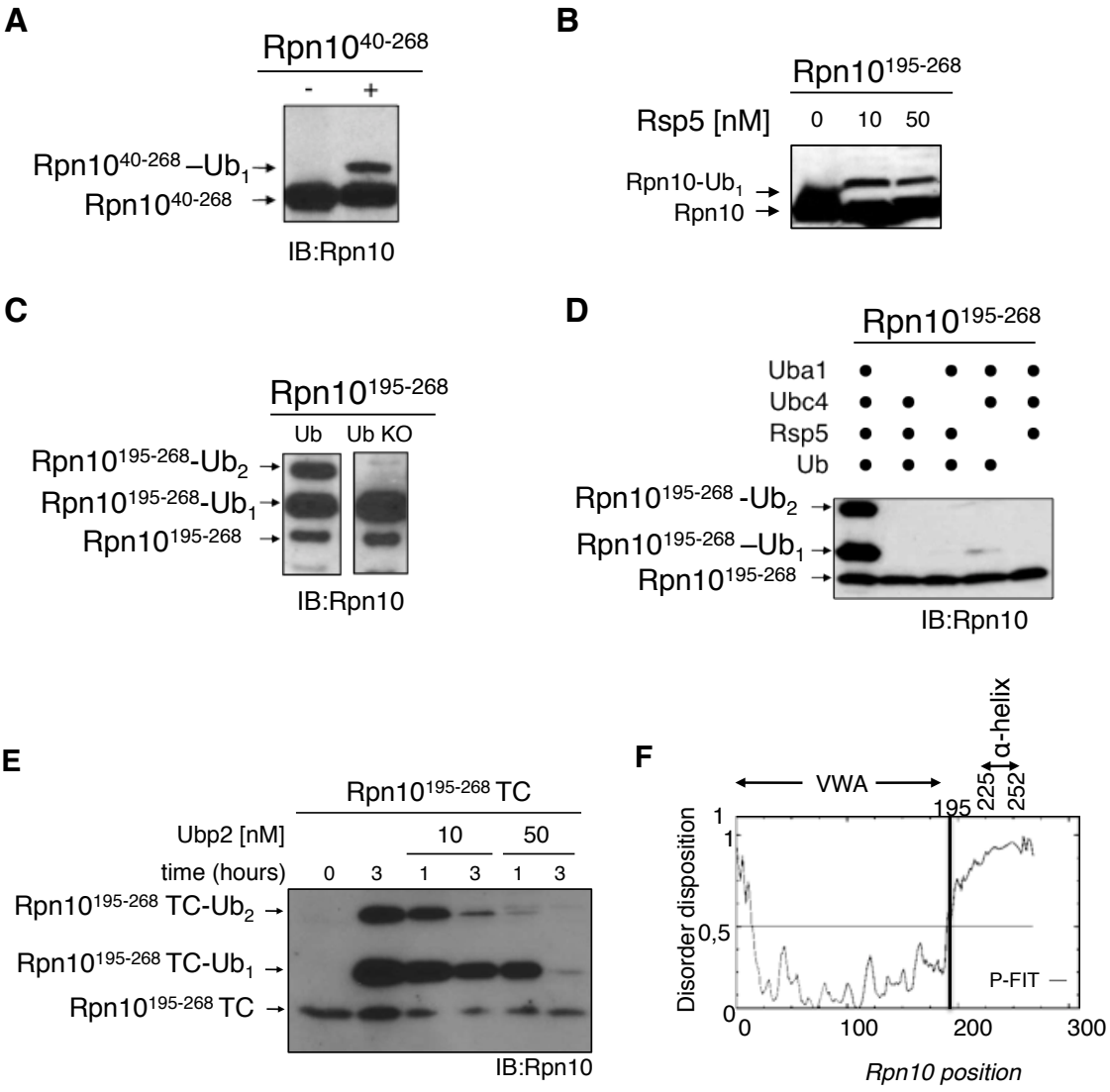


Figure 28. Monoubiquitination is efficiently catalyzed on the C-terminal region of Rpn10.

A) Monoubiquitination reaction of the Rpn10$^{40-268}$ fragment by Rsp5. Left lane, no reaction; right lane, after incubation. **B)** Ubiquitination reactions of Rpn10$^{195-268}$ using 10 nM and 50 nM Rsp5. **C)** Ubiquitination reaction of the Rpn10$^{195-268}$ fragment after cleaving the GST tag using wild-type (left) and Ubiquitin K0 - without lysine residues

(right). **D)** Rpn10$^{195-268}$ ubiquitination reaction in which Uba1, Ubc4, Rsp5 and Ubiquitin were added as indicated. **E)** A 3 hours ubiquitination reaction of Rpn10$^{195-268}$ was performed, followed by an incubation with Ubp2 at different concentrations and during different times. **F)** Prediction of intrinsically disordered residues in Rpn10 of *S. cerevisiae* by PONDR-FIT software. Scores above 0.5 are predicted disordered residues and residues with a score below 0.5 are predicted to be ordered. The VWA domains and the α helix that contains the UIM are also indicated.

In order to map in more detail the minimal sequence requirements for Rpn10 monoubiquitination, we performed additional truncation analysis by generating progressively shorter versions of the fragment Rpn10$^{195-268}$ (Figure 29A), and by testing them in Rsp5-dependent reactions. We observed that the Rpn10$^{208-268}$ fragment was modified by Rsp5, however, a truncation of a further three amino acids, Rpn10$^{211-268}$, resulted in no modification by Rsp5 (Figure 29B), even when doubling the time of incubation (Figure 29C).

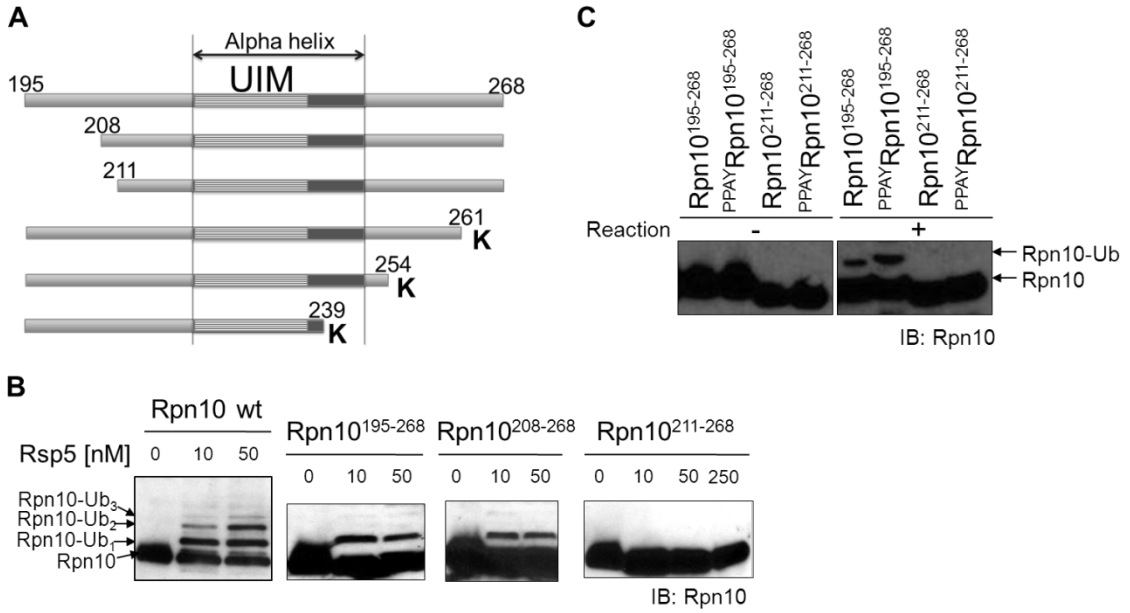


Figure 29. Rpn10 truncation analysis identifies the regions indispensable for Rpn10 monoubiquitination.
A) Schematical representation of the C-terminal fragments of Rpn10 used for ubiquitination reactions. **B)** Ubiquitination reactions of equimolar amounts of Rpn10 wt, Rpn10$^{195-268}$, Rpn10$^{208-268}$ and Rpn10$^{211-268}$ using different concentrations of Rsp5. **C)** Ubiquitination reactions of Rpn10$^{195-268}$, ^{PPAY}Rpn10$^{195-268}$, Rpn10$^{211-268}$ and ^{PPAY}Rpn10$^{211-268}$, run for 6 hours, instead of 3 hours (in **B**) at 30ºC with 10, 50 or 250 nM Rsp5.

Ligases belonging to the NEDD4 ligase family, such as Rsp5, have been shown to be able to ubiquitinate substrates containing an Ubiquitin-binding domains only if this domain is intact (Polo *et al*, 2002; Hoeller *et al*, 2006). For instance, the UIM has been shown to be necessary for the reaction of Rpn10 ubiquitination catalyzed by Rsp5 (Figure 21, 30, and (Isasa et al., 2010)). As it has been analyzed for Rpn10 full-length and ^{PPAY}Rpn10 (Figure 21 and 30), we wished to determine the effect of mutating the UIM in the fragments. For this, we introduced a block substitution from residues 228 to 232 of the UIM to asparagines of Rpn10$^{195\text{-}268}$ and Rpn10$^{208\text{-}268}$, resulting in Rpn10$^{195\text{-}268\ UIM}$ and Rpn10$^{208\text{-}268\ UIM}$. Additionally, we introduced a PPAY motif at the N-terminus of these four fragments, resulting in ^{PPAY}Rpn10$^{195\text{-}268}$, ^{PPAY}Rpn10$^{208\text{-}268}$, ^{PPAY}Rpn10$^{195\text{-}268\ UIM}$ and ^{PPAY}Rpn10$^{208\text{-}268\ UIM}$. The Rpn10 fragments with an intact UIM were monoubiquitinated as expected (Figure 30), while the ones with the UIM mutated were not modified. Although Rpn10$^{208\text{-}268}$ didn't show a very efficient monoubiquitination, no band at all could be seen in the reaction with Rpn10$^{208\text{-}268\ UIM}$ (Figure 30, right panel). Thus, once again, we observed that the UIM is required for Rpn10 ubiquitination and that, also in these short fragments, the PPAY motif is not enough to guarantee the reaction.

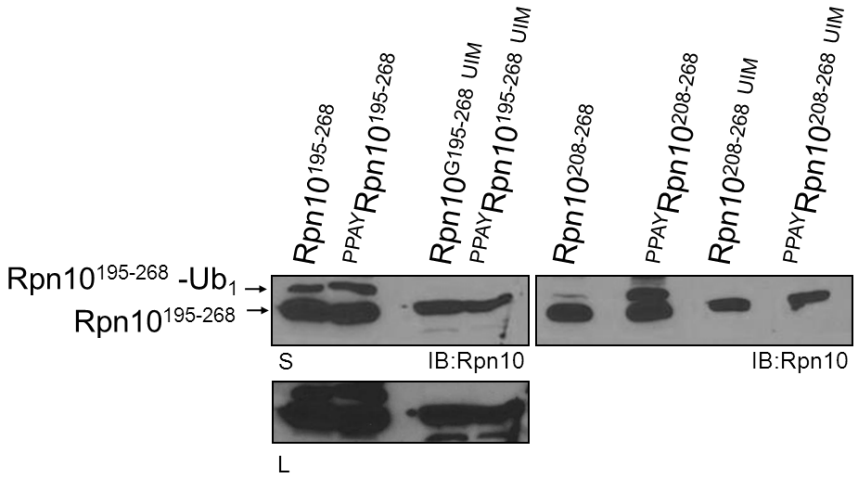


Figure 30. The UIM is required for the ubiquitination of fragments Rpn10$^{195\text{-}268}$, ^{PPAY}Rpn10$^{195\text{-}268}$, Rpn10$^{208\text{-}268}$ and ^{PPAY}Rpn10$^{208\text{-}268}$.
Ubiquitination reaction of Rpn10$^{195\text{-}268}$, ^{PPAY}Rpn10$^{195\text{-}268}$, Rpn10$^{195\text{-}268\ UIM}$, ^{PPAY}Rpn10$^{195\text{-}268\ UIM}$, Rpn10$^{208\text{-}268}$, ^{PPAY}Rpn10$^{208\text{-}268}$, Rpn10$^{208\text{-}268\ UIM}$ and ^{PPAY}Rpn10$^{208\text{-}268\ UIM}$. Lower panel shows a longer exposure (L) of the film.

In light of this, it was possible that the decreased monoubiquitination of Rpn10$^{211-268}$ (Figure 29B and C) could have resulted from an impairment of the Ubiquitin-binding properties of the UIM. To investigate whether this was the case, we analyzed the ability of Rpn10$^{211-268}$, Rpn10$^{195-268}$ and Rpn10$^{195-268\ UIM}$ (carrying a mutated UIM) to interact with polyubiquitin. We observed that Rpn10$^{195-268}$, Rpn10$^{211-268}$ and the UIM from Rnf114 as a positive control were equally capable of binding polyubiquitin, while the UIM mutant Rpn10$^{195-268\ UIM}$ did not (Figure 31).

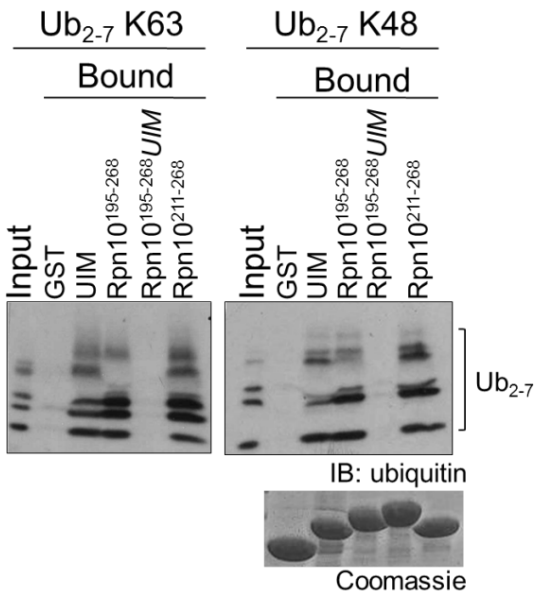


Figure 31. The UIM of Rpn10$^{A211-268}$ is functional.
Binding assay of K63-linked polyubiquitin chains and K48-linked polyubiquitin chains to GST fusion proteins of Rpn10$^{195-268}$, Rpn10$^{195-268\ UIM}$ and Rpn10$^{211-268}$ immobilized on glutathione sepharose beads. Binding to GST was used as a negative control and binding to the UIM of RNF114 (UIM) as a positive control. The input lane contains 1/20th of the poly-Ubiquitin quantity used for the binding experiments. Bound material and input were analyzed by anti-Ubiquitin western blotting. Lower panel shows similar amounts of the proteins bound to the beads in a Coomassie blue staining.

These results indicated that there is no loss of Ub binding of the Rpn10$^{211-268}$ fragment, and suggested a role for the region between amino acids D208 and A211 in the monoubiquitination of Rpn10 that is independent of the Ubiquitin-binding capacity of the UIM.

To further characterize Rpn10 modification, the only lysine residue present in the shortest fragments of Rpn10, K268, was mutated. Fragments $Rpn10^{195\text{-}268\ K268R}$ and $Rpn10^{208\text{-}268\ K268R}$, without lysines, were not ubiquitinated, indicating that K268 is essential for their ubiquitination. Interestingly, $^{PPAY}Rpn10^{195\text{-}268\ K268R}$ without lysines but containing a PPAY motif at the N-terminus was modified (Figure 32). We must point out that all this protein carries a GST tag that contains several lysines and that it is therefore possible that $^{PPAY}Rpn10^{195\text{-}268\ K268R}$ is ubiquitinated at the GST tag. The presence of a PPAY motif, together with the length and the nature of the residues of this fragment, could allow a better interaction with the ligase and, therefore, the ubiquitination of this mutant.

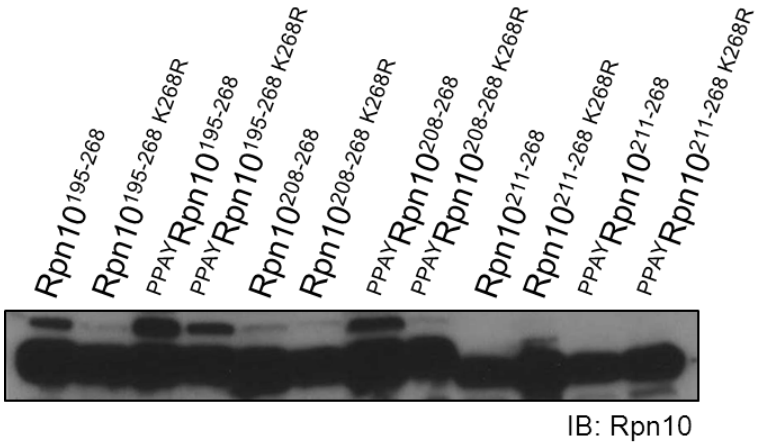


Figure 32. Ubiquitin linkage in Rpn10 fragments.
Ubiquitination reactions of Rpn10 fragments and lysine to arginine mutants.

Next, to determine the C-terminal region required for ubiquitination, we analyzed the behavior of Rpn10 C-terminal truncations at positions Q261, D254 and E239, in which we introduced a lysine residue at the C-terminus (see Figure 29A), as substrates in monoubiquitination reactions. We observed that $Rpn10^{195\text{-}261}$ and $Rpn10^{195\text{-}254}$ were efficiently monoubiquitinated, whereas $Rpn10^{195\text{-}239}$ was not (Figure 33A). We tested the three C-terminal truncations in a polyubiquitin binding assay, and we observed that the $Rpn10^{195\text{-}239}$ truncation showed a notable decrease in polyubiquitin binding (Figure 33B). Therefore, the loss of monoubiquitination of the $Rpn10^{195\text{-}239}$ fragment could be due to a dysfunctional UIM. Additionally, the proximity of the lysine residue to the Ub interacting surface in the $Rpn10^{195\text{-}239}$ fragment (see Figure 29A) could be an additional cause of the

absence of monoubiquitination. Together, these results suggest that the region C-terminal to residue D254 is not needed to get ubiquitinated Rpn10.

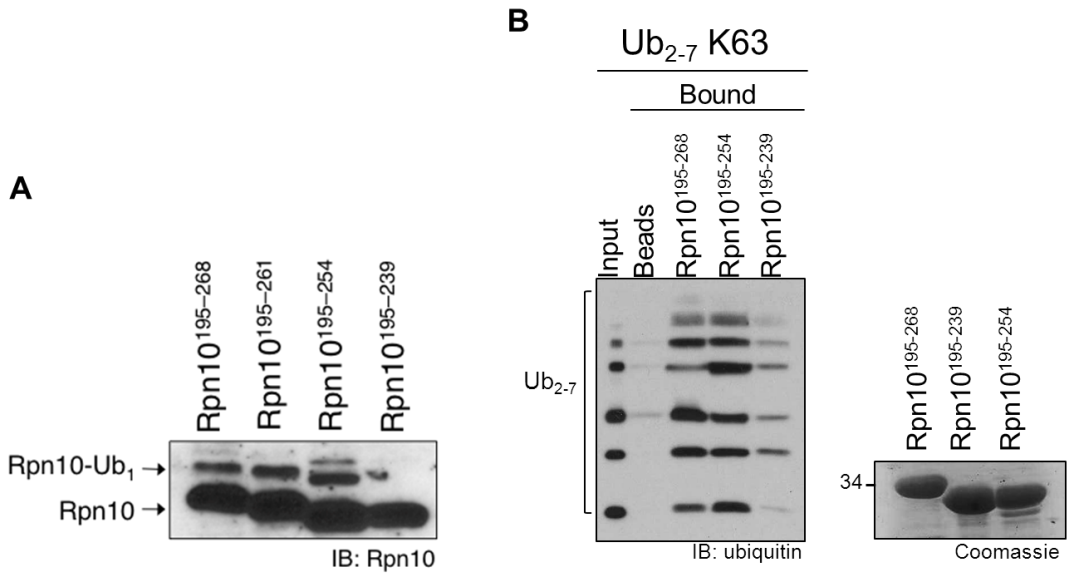


Figure 33. A dysfunctional UIM explains loss of ubiquitination.
A) Ubiquitination reactions of Rpn10$^{195\text{-}268}$, Rpn10$^{195\text{-}261}$, Rpn10$^{195\text{-}254}$ and Rpn10$^{195\text{-}239}$. **B)** Binding assay of GST fusion proteins Rpn10$^{195\text{-}268}$, Rpn10$^{195\text{-}254}$ and Rpn10$^{195\text{-}239}$, immobilized on beads to K63-only polyubiquitin chains (input). 1/20th of the input used per binding was loaded. Bound material was eluted and analyzed by Ubiquitin western blotting. Right panel, coomassie staining of the amounts of fusion proteins used in each assay.

The unstructured linker between the VWA and the UIM prevents Rpn10 polyubiquitination

Since the deletion of amino acids D208, S209 and D210 showed a dramatic effect in our analysis of N-terminally truncated Rpn10 forms, completely abrogating (mono)-ubiquitination, we studied this region in a full-length context (Figure 34).

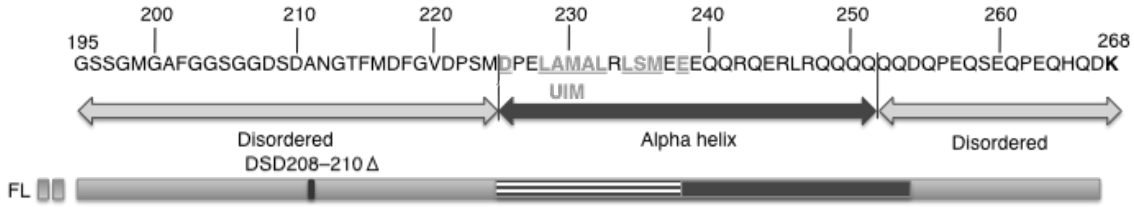


Figure 34. Rpn10$^{195\text{-}268}$ sequence and diagram of full-length Rpn10 with positions D208, S209 and D210 deleted.
Disordered regions are indicated by light grey arrows and the α-helix by a dark grey arrow. The UIM is underlined in the sequence and represented with white and black lines in the diagram.

We deleted residues D208, S209 and D210 (Rpn10$^{DSD208\text{-}210\Delta}$), and investigated the ubiquitination of this mutant protein. We observed that this deletion had an effect on the reaction, producing an oligo/polyubiquitin signal instead of mono-ubiquitination. This was not observed when using Ub K0 that cannot synthetize chains (Figure 35A and B), indicating that the several bands observed when using Ub wild-type are oligo/polyubiquitinated Rpn10$^{DSD208\text{-}210\Delta}$.

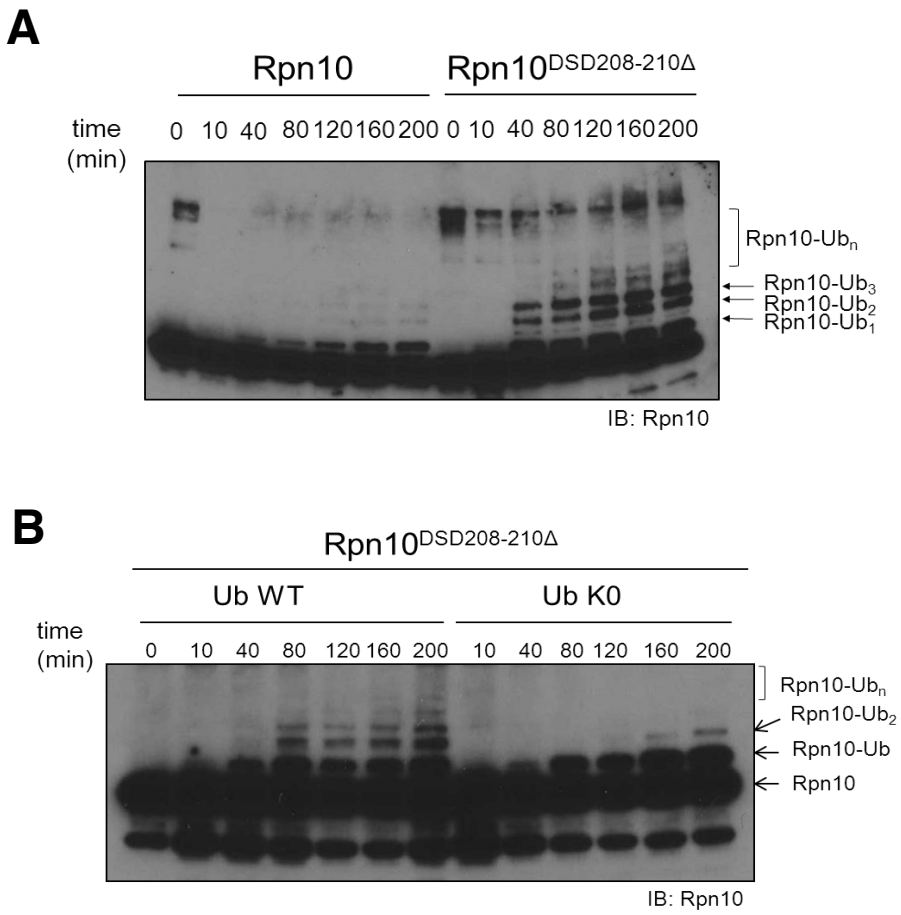


Figure 35. Altering the unstructured linker flanking the UIM promotes polyubiquitination.
A) Time course *in vitro* ubiquitination assay of Rpn10 and Rpn10$^{DSD208-210\Delta}$. Points at indicated times were taken and analyzed by Rpn10 western blotting. Asterisk, unspecific band. **B)** Time course *in vitro* ubiquitination reaction of Rpn10$^{DSD208-210\Delta}$ using wild-type Ubiquitin and Ub K0. Points at indicated times were taken and analyzed by Rpn10 western blotting.

Positions D208, S209 and D210 are part of an unstructured linker between the α-helix 6 of the VWA domain and the UIM- containing α-helix 7 (Figure 36; (Wang *et al*, 2005; Boehringer *et al*, 2012)). Notably, this region contains a Glycine-rich region from positions G195 to G207 (Figure 36).

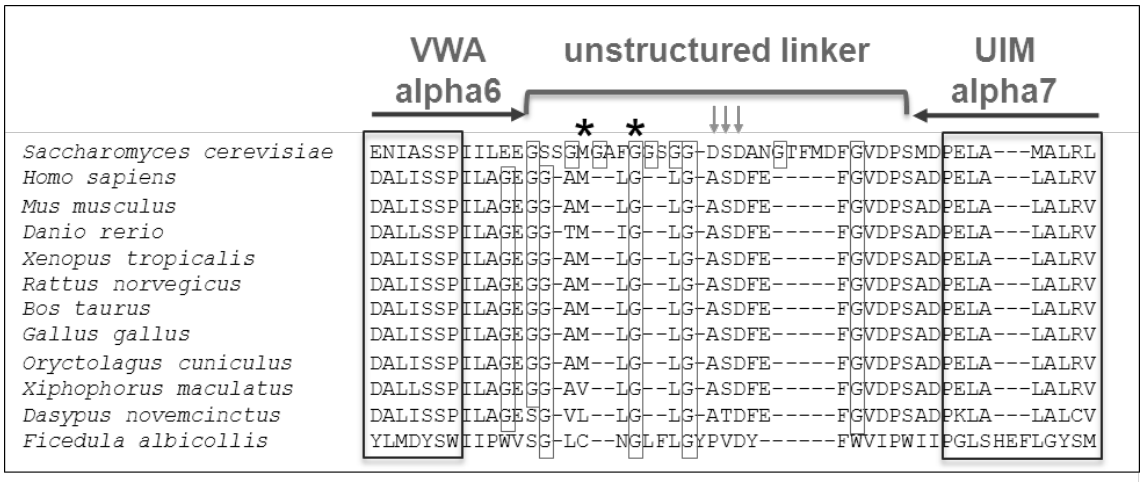


Figure 36. Multiple sequence alignment of Rpn10.
Residues from 183 to 234 are shown in *Saccharomyces cerevisiae*. First and last boxes correspond to the residues within the α-helix 6 of the VWA domain and the UIM-containing α-helix 7, respectively. Conserved glycine residues among species are shown inside light grey boxes. Vertical arrows point D208, S209 and D210 amino acids. The alignment of Rpn10 from 59 species was done with Ensembl software. The most representative species are shown. Asterisks show the conservation of a methionine (residue 199) and a glycine (residue 203) that were mutated in this study.

To examine the importance of this, we generated mutants with a block substitution of the region S196-G204, in the context of full-length Rpn10 (Figure 37). Additionally, we generated point mutations at two different conserved positions, M199 and G203. To create opposing effects with regards to the polarity and the disorder of the segment, we mutated amino acid residues to alanines as well as to serines (Figure 37).

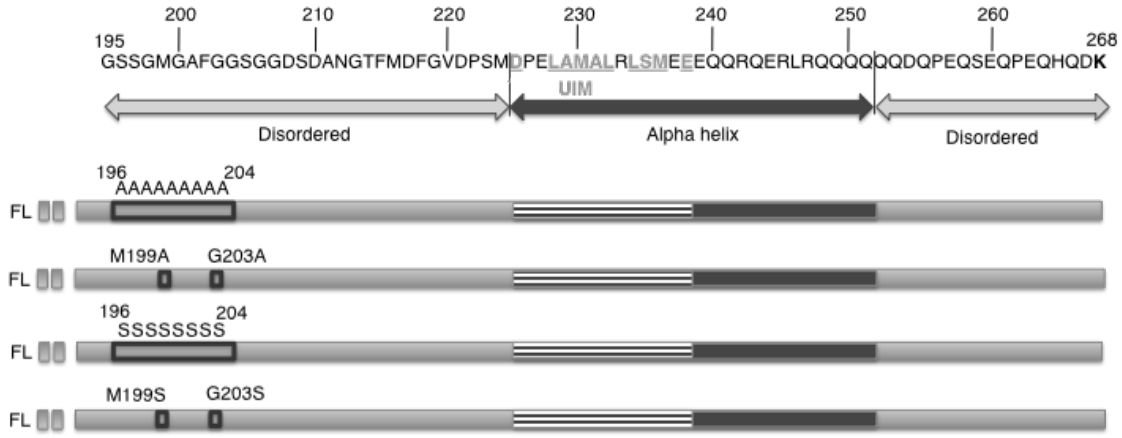


Figure 37. Diagram of the Rpn10$^{195\text{-}268}$ fragment and full-length versions of Rpn10 including the mutations analyzed.
Disordered regions are indicated by light grey arrows and the α-helix by a dark grey arrow. The UIM is underlined in the sequence and represented with white and black lines in the diagram.

Subsequently, Rpn10$^{196\text{-}204A}$, Rpn10^{M196A,G204A}, Rpn10$^{196\text{-}204S}$ and Rpn10^{M196S,G204S} mutants were tested in Rsp5-dependent ubiquitination reactions. We observed that the Rpn10$^{196\text{-}204A}$ mutant underwent a dramatic increase in polyubiquitination, as compared to full-length wild-type Rpn10 (Figures 38 and 39). Rpn10^{M196A,G204A}, Rpn10$^{196\text{-}204S}$ and Rpn10^{M196S,G204S} mutants showed an intermediate effect, which appears to be similar to the one observed with Rpn10$^{DSD208\text{-}210\Delta}$.

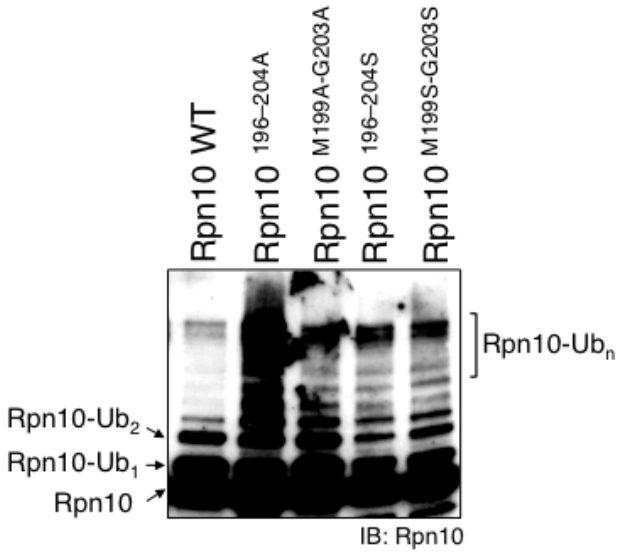


Figure 38. Change in the pattern of ubiquitination of Rpn10 mutants.
Ubiquitination reactions of Rpn10 WT, Rpn10$^{196\text{-}204A}$, Rpn10$^{M199A\text{-}G203A}$, Rpn10$^{196\text{-}204S}$ and Rpn10$^{M199S\text{-}G203S}$.

We further evaluated the ubiquitination of Rpn10$^{196\text{-}204A}$ by performing a reaction with Ub K0 and methylated Ubiquitin that cannot form polyubiquitin chains. This showed that, like in the wild-type protein, two major lysines are ubiquitinated, indicating that the higher molecular weight ubiquitinated forms observed with Rpn10$^{196\text{-}204A}$ are not the result of more extensive monoubiquitination but of a true increase in polyubiquitination (Figure 39B and C). Importantly, this polyubiquitination is observed from the earliest time points onwards and increases with time, whereas polyubiquitination of wild-type Rpn10 is not observed at any time point (Figure 39A).

Finally, we analyzed the competence of Rsp5^{HECT} in ubiquitinating Rpn10$^{196\text{-}204A}$. Although the reaction with Rsp5 full-length was more efficient in polyubiquitinating Rpn10$^{196\text{-}204A}$, the HECT also exhibited polyubiquitination on this mutant. This capacity was valuable to study Rpn10$^{196\text{-}204A}$ polyubiquitination *in vivo,* again without affecting the numerous substrates that depend on the WW within the N-terminus of Rsp5.

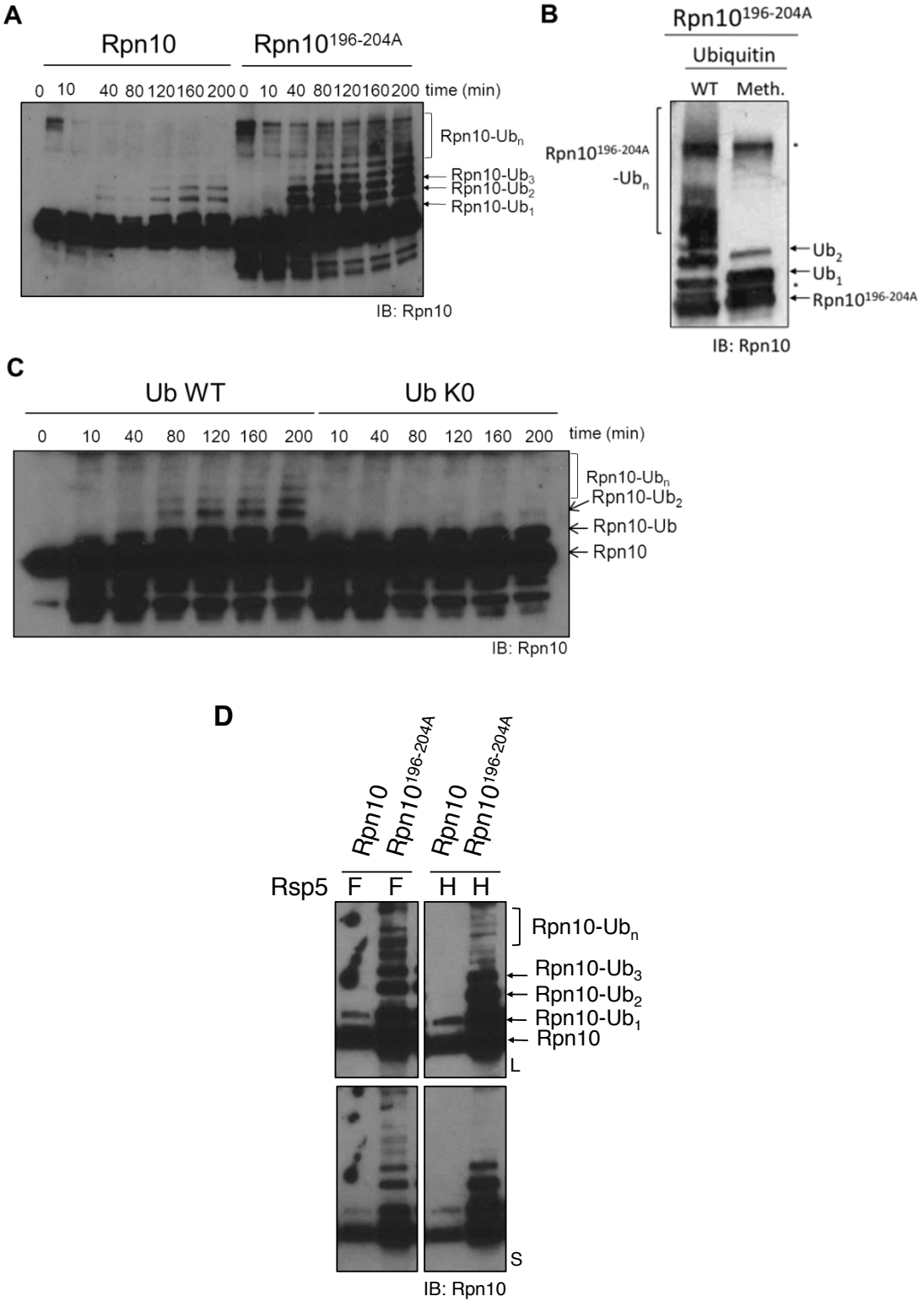


Figure 39. Rpn10$^{196\text{-}204A}$ is polyubiquitinated by Rsp5 full-length and Rsp5^{HECT}.
A) Time course *in vitro* ubiquitination assay of Rpn10 and Rpn10$^{196\text{-}204A}$. Points at indicated times were taken and analyzed by Rpn10 western blotting. **B)** Ubiquitination reaction of Rpn10$^{196\text{-}204A}$ mutant using wild-type and methylated Ubiquitin. Asterisks, unspecific band. **C)** Time course *in vitro* ubiquitination reaction of Rpn10$^{196\text{-}204A}$ using

wild-type Ubiquitin and Ub K0. Points at indicated times were taken and analyzed by Rpn10 western blotting. **D)** Ubiquitination reaction of Rpn10 and Rpn10$^{196\text{-}204A}$ incubated with Rsp5 full-length (F) or Rps5HECT (H). Long (L) and short (S) exposures of the film are shown.

Considering that Rpn10$^{196\text{-}204A}$ was the substrate to undergo the strongest polyubiquitination, we conclude that the disordered region between amino acids S196 to G204 (see Figures 36 and 37) normally prevents Rpn10 polyubiquitination, although residues D208-D210 also contribute. Interestingly, disorder and secondary structure prediction indicated that the Rpn10$^{196\text{-}204A}$ mutation causes a substantial increase in the order of this segment (Figure 40A) and induces a helicoidal structure (Figure 40B).

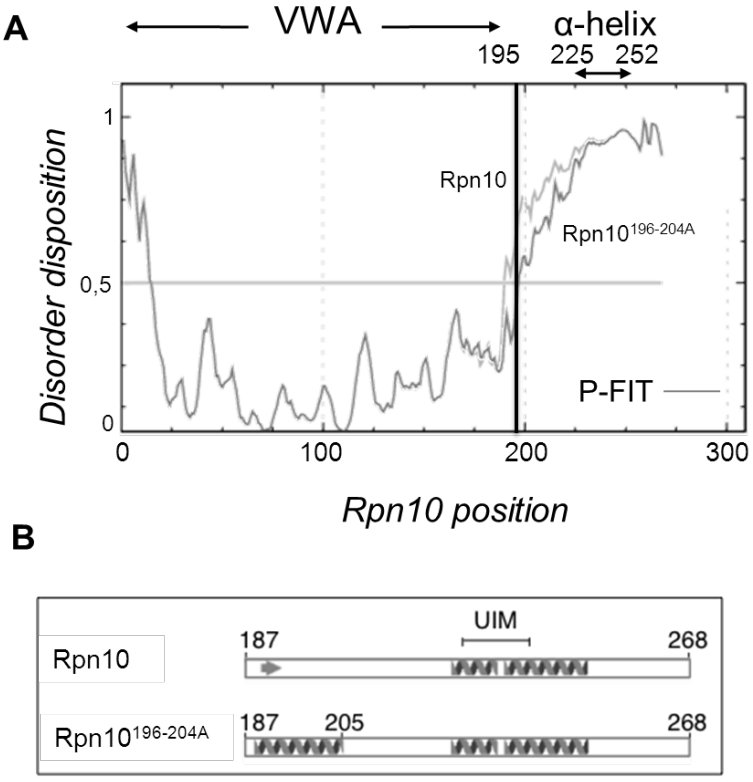


Figure 40. The block-substitution to alanines induces a gain of order.
A) Overlay of the profiles of the predicted intrinsically disordered residues in Rpn10 (light grey) and in Rpn10$^{196\text{-}204A}$ (grey) by PONDR-FIT software. Scores above 0.5 are predicted disordered residues and residues with a score below 0.5 are predicted to be ordered. The VWA domains and the α-helix that contains the UIM are also indicated. **B)** C-terminal prediction of Rpn10 and Rpn10$^{196\text{-}204A}$ secondary structure obtained after running MeDoR, a predictor of secondary structures. The arrow represents a beta-strand; the helices correspond to α-helices.

Finally, we asked what the effect would be, *in vivo*, of the dramatic change in the pattern of ubiquitination exhibited by the Rpn10$^{196\text{-}204A}$ mutant, as compared to Rpn10 and to a mutant with an intermediate effect. Thus, we analyzed the effect of the expression of Rpn10, Rpn10$^{196\text{-}204A}$ and Rpn10$^{DSD208\text{-}210\Delta}$ mutants in strains lacking the RPN10 and RAD23 genes, in which the recruitment of substrates to the proteasome is deficient and shows synthetic slow growth phenotypes which can be rescued by expressing Rpn10 (Chen & Madura, 2002; Isasa *et al*, 2010). We observed a strong decrease of growth in cells expressing the Rpn10$^{196\text{-}204A}$ mutant at 37ºC, whereas the Rpn10$^{DSD208\text{-}210\Delta}$ mutant showed an intermediate effect (Figure 41A).

Moreover, total cell lysates from an *rpn10*Δ strain carrying Rpn10 or Rpn10$^{196\text{-}204A}$ were applied to a Superose 6 column in which separation is based on differences in the size of the analyte. A previous member from our lab determined by immunodetection of Rpn12 and α7 (two subunits of the proteasome) the fractions in which the proteasome elutes. We analyzed by western blotting against Rpn10 fractions of these two cell lysates corresponding to the Rpn10 and the proteasome elution peaks. Rpn10$^{196\text{-}204A}$ and Rpn10 showed the same affinity towards the proteasome *in vivo*, suggesting that the Rpn10$^{196\text{-}204A}$ phenotypes are not caused by a decreased proteasomal association of this mutant (Figure 41B).

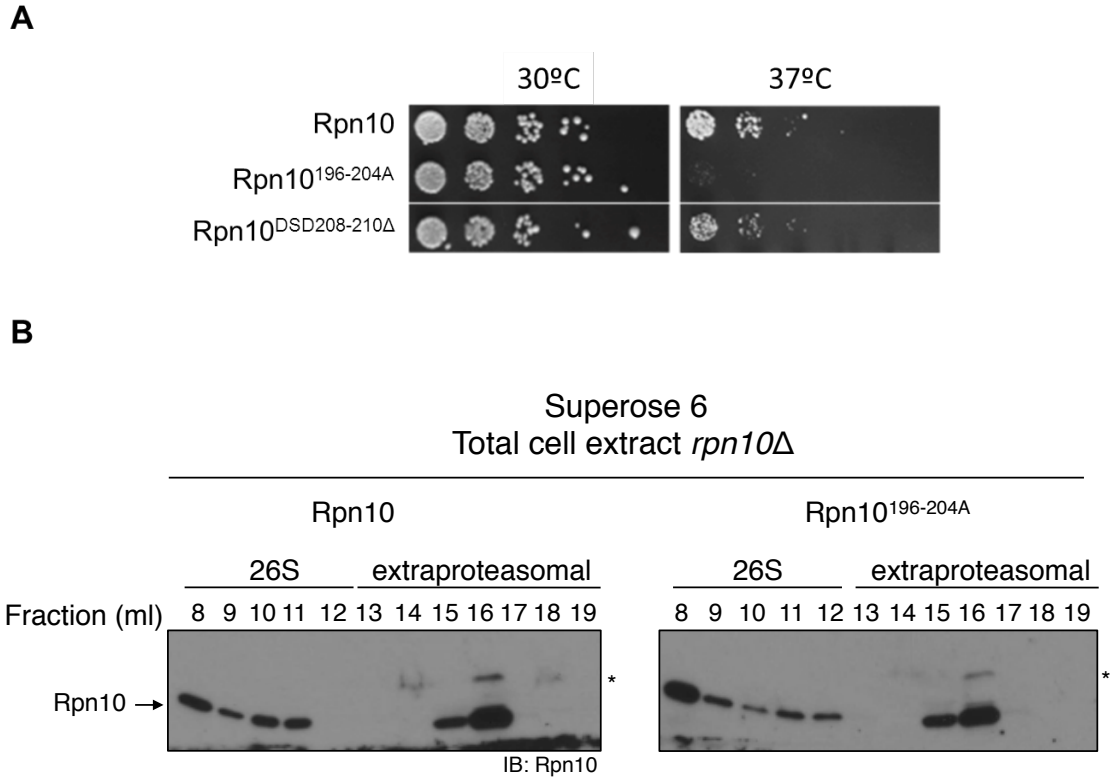


Figure 41. Rpn10$^{196\text{-}204A}$ mutant: effect on cell growth defect.
A) Colony formation assay of *rad23*Δ *rpn10*Δ cells (strain 11d) expressing different Rpn10 forms. Cells were grown at the indicated temperatures. **B)** Total cell lysates from an *rpn10*Δ strain (S72) carrying Rpn10 or Rpn10$^{196\text{-}204A}$, from its own promoter, were applied to a Superose 6 column. Fractions corresponding to the proteasomal and extraproteasomal peaks were analyzed by western blotting against Rpn10. Asterisk, unspecific band.

In order to study whether the polyubiquitination of Rpn10$^{196\text{-}204A}$ and Rpn10$^{DSD208\text{-}210\Delta}$ observed *in vitro* was recapitulated *in vivo*, we induced Rsp5^{HECT} in *rpn10*Δ and *rad23Δ rpn10*Δ strains carrying plasmids expressing, from their own promoter, Rpn10, Rpn10$^{196\text{-}204A}$ or Rpn10$^{DSD208\text{-}210\Delta}$ (Figure 42A-C). Rpn10 monoubiquitination was very difficult to detect *in vivo* unless we induced Rsp5^{HECT}. For this reason, we decided to enhance the ubiquitination of these mutants by inducing the HECT of Rsp5. Although we repeated the inductions 9 times, no ^{HA}Rsp5^{HECT} could be detected by western blot. In addition, unexpectedly, the mutants grown in glucose were not polyubiquitinated but monoubiquitinated as Rpn10. A possible explanation for the observation of the monoubiquitination of Rpn10$^{196\text{-}204A}$ and Rpn10$^{DSD208\text{-}210\Delta}$ was that endogenous Ubp2 was cleaving the

Ubiquitin chains from the mutants. Therefore, in another attempt to induce the HECT and to observe the polyubiquitination of Rpn10$^{196\text{-}204A}$ and Rpn10$^{DSD208\text{-}210\Delta}$, we generated the double mutant *rpn10*Δ *ubp2*Δ. Again, the induction did not work and the mutants were monoubiquitinated (Figure 42D).

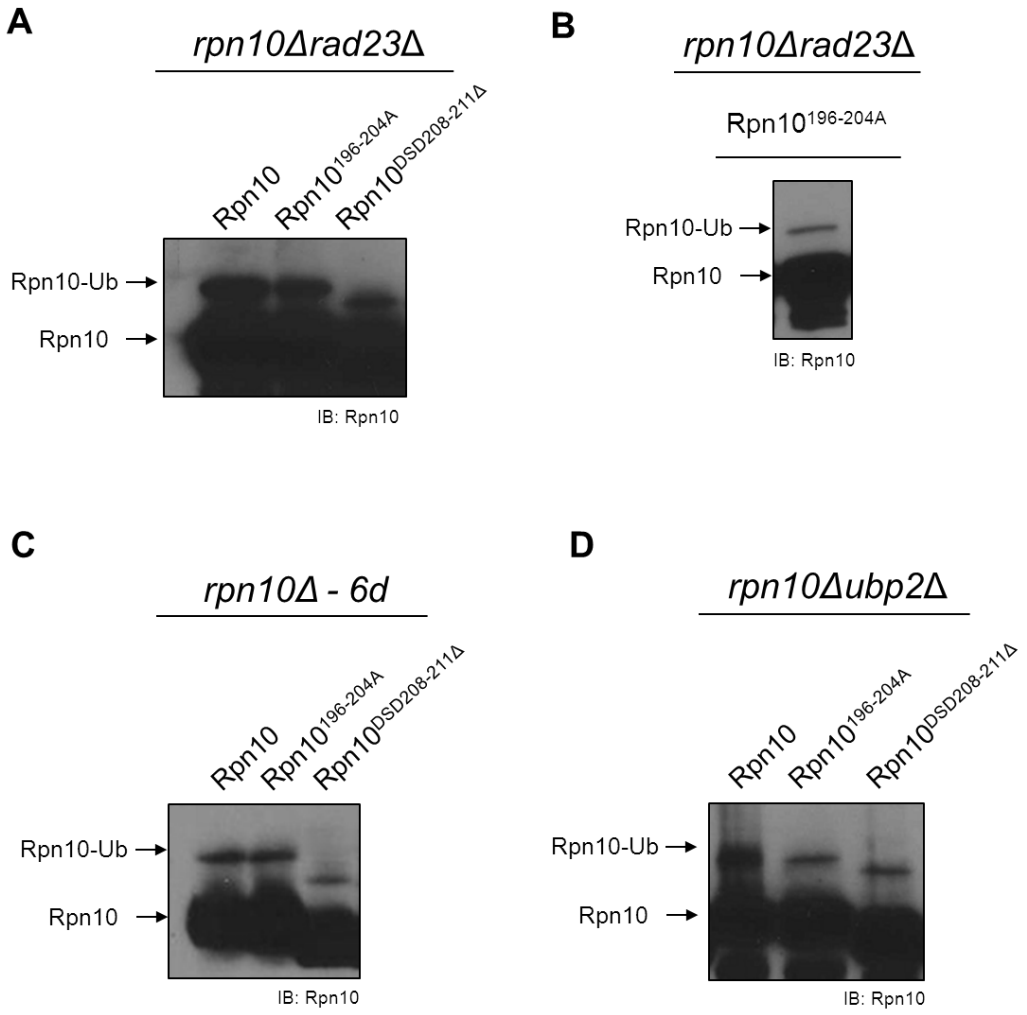
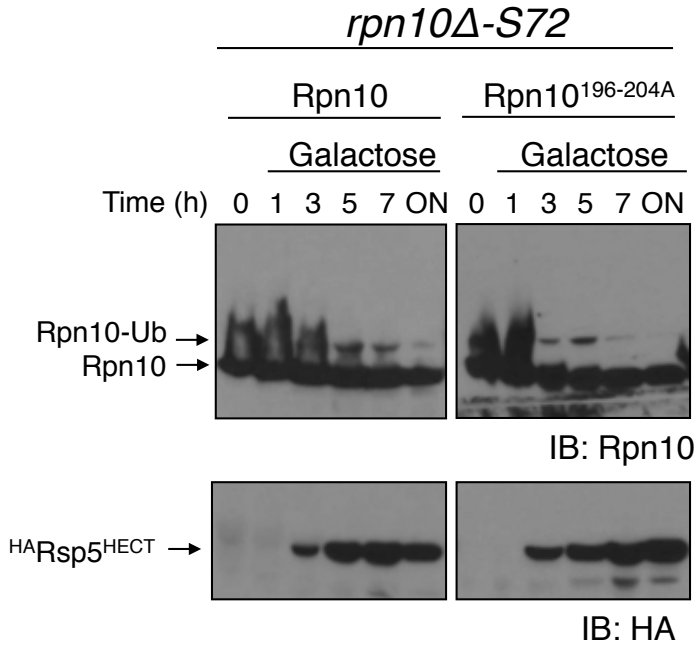


Figure 42. Rpn10$^{196\text{-}204A}$ and Rpn10$^{DSD208\text{-}210\Delta}$ are monoubiquitinated *in vivo*. A), B), C) and D) Different strains, *rpn10*Δrad23Δ, *rpn10*Δ and *rpn10*Δ *ubp2*Δ, were grown in glucose carrying plasmids Rpn10, Rpn10$^{196\text{-}204A}$ and Rpn10$^{DSD208\text{-}210\Delta}$ from their own promoter.

Finally, we tried the induction of Rsp5^{HECT} in an *rpn10*Δ (S72) strain with a different background, carrying plasmids expressing Rpn10 and Rpn10$^{196\text{-}204A}$ and

this proved to be successful. Nevertheless, polyubiquitination of Rpn10$^{196\text{-}204A}$ was not detected, either with galactose or glucose. Instead, the monoubiquitination of this mutant was observed and appeared to be of a similar intensity as the one observed with Rpn10 (Figure 43).

Figure 43. Rpn10$^{196\text{-}204A}$ is monoubiquitinated *in vivo* when the HECT is induced. *rpn10*Δ (S72) strain carrying a ^{HA}Rsp5^{HECT} galactose-inducible plasmid and plasmids



Rpn10 or Rpn10$^{196\text{-}204A}$ was grown in galactose media for the indicated hours. Rpn10 and Rpn10$^{196\text{-}204A}$ were analyzed by Rpn10 western blotting, shown in the upper panels. Lower panels show the induction levels analyzed by western blotting using an anti-HA antibody.

In a preliminary study, we have also investigated the influence of expressing distinct forms of Rpn10 (Rpn10-WT, Rpn10$^{196\text{-}204A}$) on the *in vivo* degradation of a mutant of CPY* a protein with a high turnover rate that is known to be caused by proteasomal degradation (Figure 44). We have previously observed that in a strain carrying the deletion of *RPN10* (*rpn10*Δ), the degradation of CPY* is delayed (Figure 44A and B). When expressing the mutant Rpn10$^{196\text{-}204A}$, the turnover of CPY* is partially rescued (Figure 44B). However, additional assays should be done to obtain reliable statistic data and therefore, draw conclusions.

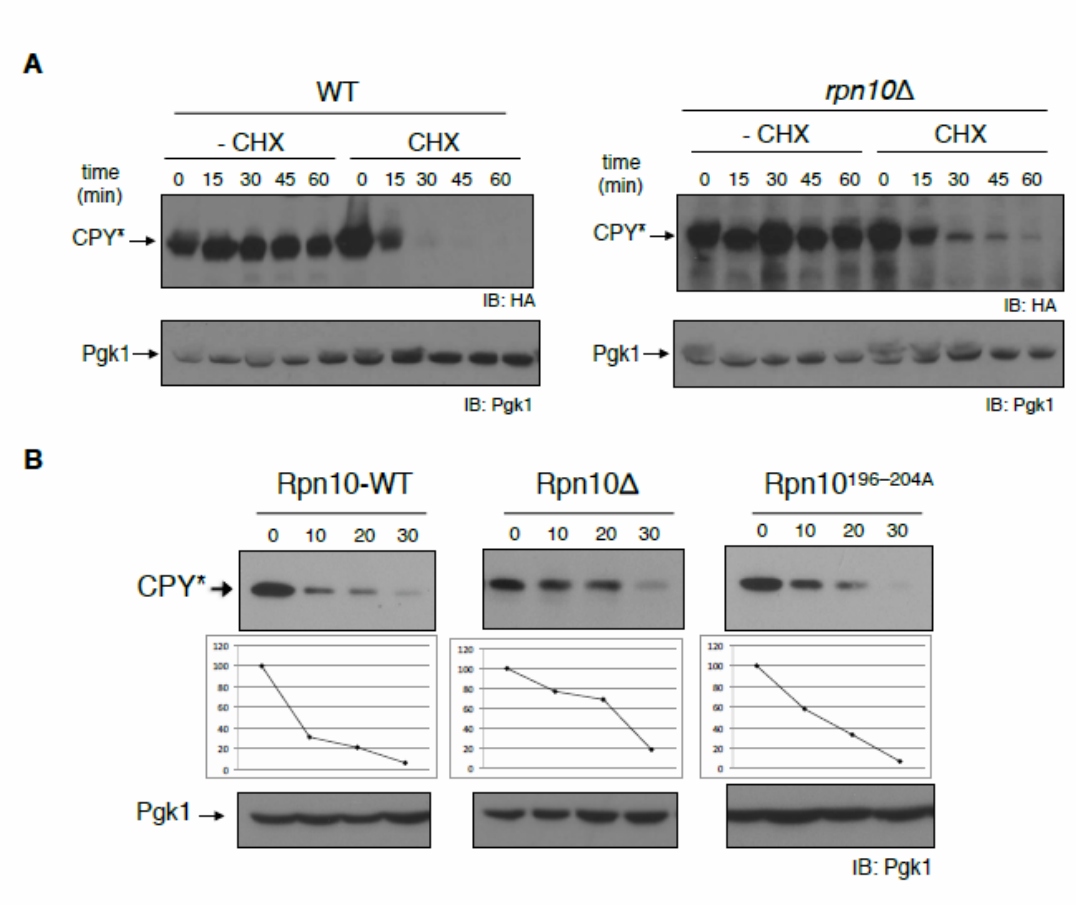
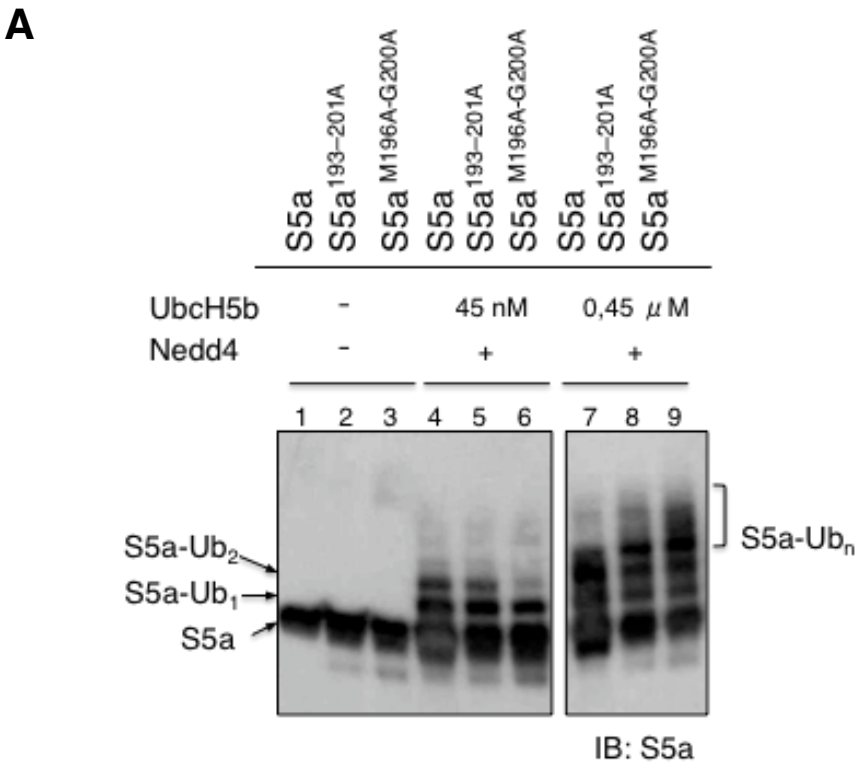


Figure 44. Lack of RPN10 or expression of Rpn10$^{196-204A}$ mutant delays CPY* degradation.

A) Degradation of HA-tagged CPY* was compared in WT (S67) and *rpn10*Δ (S72) strains. Protein synthesis was blocked with 200 µg/ml cycloheximide. Samples were withdrawn at indicated time points. HA-CPY* was analyzed by immunoblotting. Blots were also developed with an anti-pgk1 antibody as a loading control. **B)** Degradation of HA-tagged CPY* was compared in *rpn10*Δ strain carrying Rpn10-WT, empty or Rpn10$^{196-204A}$ vectors. Protein synthesis was blocked with 200 µg/ml cycloheximide. Duration of the treatment with cycloheximide is indicated. HA-CPY* was analyzed by immunoblotting. Blots were also developed with an anti-pgk1 antibody as a loading control. The relative quantification of the CPY* levels in the *rpn10*Δ strain carrying Rpn10, empty vector or Rpn10$^{196-204A}$ was carried out with ImageJ software and intensities were normalized relative to the loading control pgk-1.

Effect of unstructured regions in S5a monoubiquitination

Based on the fact that Rpn10 monoubiquitination is observed in distant species such as *S. cerevisiae* and *D. melanogaster* (Isasa *et al*, 2010; Lipinszki *et al*, 2009), and that the Rpn10 orthologue in humans, S5a, has been shown to be mono- and diubiquitinated in the proteasome (Besche *et al*, 2014), we aimed to reconstitute the monoubiquitination of S5a using human Nedd4 Ubiquitin-protein ligase and the E2 UbcH5b. We observed that at different enzyme concentrations, S5a underwent monoubiquitination, showing abundant S5a-Ub_1 and S5a-Ub_2 forms (Figure 45; lanes 4 and 7). Moreover, we analyzed point mutations at positions M196 and G200, and a block substitution of the G193-G200 segment ($S5a^{M196A,G200A}$ and $S5a^{193\text{-}200A}$ mutants, respectively), corresponding to Rpn10 mutants $Rpn10^{M196A,G204A}$ and $Rpn10^{196\text{-}204A}$, respectively.



B

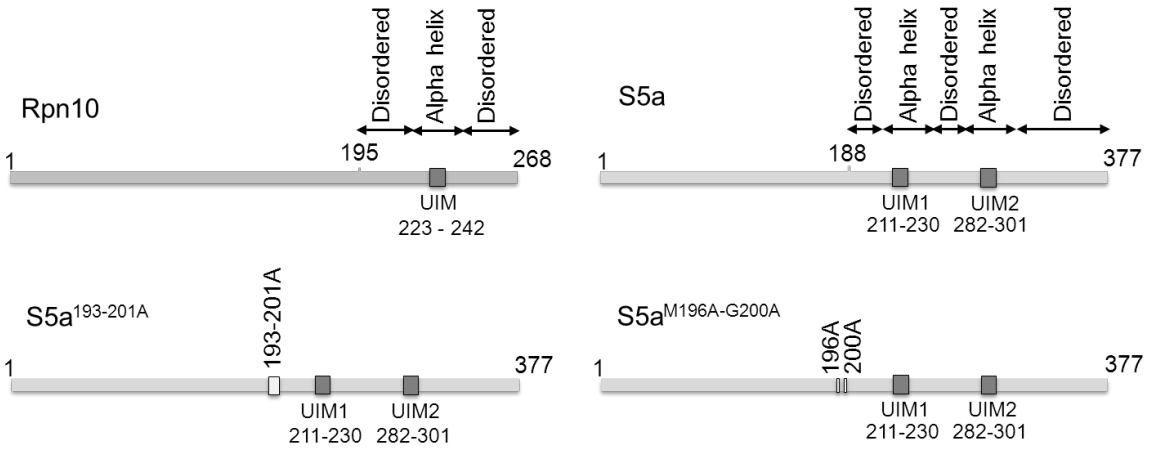


Figure 45. Mutations of the unstructured region before the UIM1 of S5a cause a change in the process of ubiquitination.
A) reactions of S5a, S5a$^{193\text{-}201A}$ and S5a$^{M196A\text{-}G200A}$ carried out for 3 hours at 37ºC. Amounts of UbcH5b are indicated. **B)** Schematical representation of Rpn10, S5a, S5a$^{193\text{-}201A}$ and S5a$^{M196A\text{-}G200A}$. Alpha helix and disordered regions flanking the UIMs are shown.

We observed that mutants and wild-type forms showed distinct behaviors. Whereas S5a showed a pattern of mono- and diubiquitination in all conditions used (Figure 45, lanes 4 and 7), S5a^{M196A,G200A} and S5a$^{193\text{-}200A}$ showed increased polyubiquitination at high concentrations of E2 (Figure 45A, compare lanes 5 and 6 with lanes 8 and 9). Therefore, analogously to the results observed in Rpn10, the region preceding the UIM in S5a has an influence on the ubiquitination pattern.

DISCUSSION

The complexity of the Ub code relies on a highly extended set of enzymatic activities, on the high diversity of Ubiquitin interacting domains, and on the specificity of Ubiquitin surface/Ubiquitin receptor interactions, altogether defining one of the most sophisticated signaling systems in biology (Dikic *et al*, 2009; Komander & Rape, 2012). In the present thesis we have uncovered a link between the capacity of the proteasomal receptor Rpn10 to undergo monoubiquitination and the presence of an unstructured region in Rpn10 protein. To do so, we have dissected the ubiquitination of Rpn10 from different point of views.

The HECT of Rsp5 is sufficient to ubiquitinate Rpn10

In our characterization of the process of Rpn10 monoubiquitination, we have observed that the HECT domain of Rsp5 is sufficient and very efficient in the catalysis of Rpn10 monoubiquitination. Therefore, the HECT domain contains all the required domains to ubiquitinate Rpn10, which are an E2 binding site found at the amino-terminal lobe (N-lobe) and the active-site cysteine that accepts the Ub transferred by the E2 at the carboxyl-terminal lobe (C-lobe) (see Figure 46).

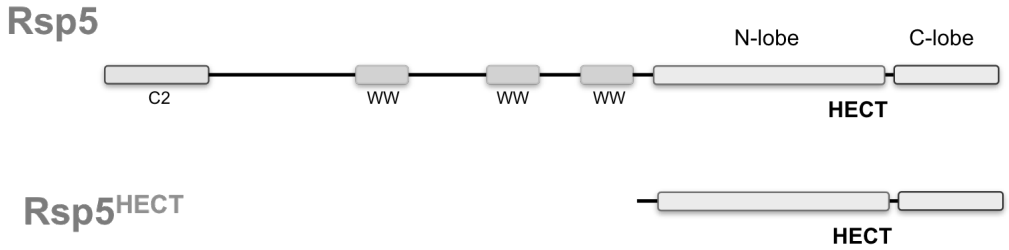


Figure 46. Schematical representation of Rsp5 full-length and Rsp5^{HECT}.

Interestingly, the HECT domain of Rsp5 alone seemed to be considerably more efficient in monoubiquitinating Rpn10 than full length Rsp5 (Figure 22B). Features in Rsp5 could help explain this observation. Firstly, Rsp5 contains a PY motif (LPQY) in the HECT domain that could be binding to the WWs that are found at the N-terminus of Rsp5, leaving the ligase partially in an inactive state (Lam 2009, Lam 2013, Kee 2005, Kee 2006). Secondly, the N-lobe and C-lobe of the

HECT of Rsp5 are connected by a short unstructured linker that might give the flexibility required by the C-lobe to access the lysine on the target substrate (Kim & Huibregtse, 2009). It has been suggested that the linker also facilitates the orientation of the E3 towards the E2 (Huang *et al*, 1999). Taken together, the lack of the N-terminus of Rsp5 in $Rsp5^{HECT}$ could, on the one hand, avoid the inactive state due to the PY-WW interaction, and, on the other hand, provide more freedom to the HECT domain to orient itself towards the target lysine and the E2, facilitating the transfer of an Ub from the active cysteine of the HECT to the lysines within Rpn10 and the binding with the E2, respectively.

On the contrary, $Rsp5^{HECT}$ showed a lower capacity to polyubiquitinate $Rpn10^{196\text{-}204A}$ than Rsp5 full-length (Figure 26D), suggesting that the ability of the HECT to link ubiquitins on a polyubiquitin chain could be lower than the one achieved by Rsp5 full length. This again could be explained by a change in the orientation adopted by the HECT domain with respect to the full-length ligase that would encounter difficulties to transfer a Ubiquitin from the active cysteine of the HECT to the last Ubiquitin of the chain. Another explanation could be related to differences in the self-ubiquitination of the full length and HECT ligases that would affect the ubiquitination of the substrate. There are too many questions to answer before being able to even hypothesize. One very first step to do is to determine the Km for the ubiquitination reaction of Rsp5 and $Rsp5^{HECT}$ towards Rpn10 and $Rpn10^{196\text{-}204A}$.

It would be interesting to assess whether the induction of Rsp5 full-length rather than $Rsp5^{HECT}$ could help observe the polyubiquitination of $Rpn10^{196\text{-}204A}$ *in vivo*.

S. cerevisiae has four additional genes encoding HECT E3 proteins: Tom1, Hul4, Ufd4 and Hul5. It has been already reported that neither Ufd4 nor Hul5 are Ubiquitin E3 ligases for Rpn10 (Isasa *et al*, 2010). Our interest was then focused on the activity of the HECT of the two other ligases, $Tom1^{HECT}$ and $Hul4^{HECT}$. Unfortunately, we observed that *in vitro*, $Hul4^{HECT}$ was catalytically inactive. There is little literature regarding this enzyme and we do not know the reason for its inactivity, although it could be that the protein looses its activity during the purification from E.coli. *In vivo*, none of these two ligases could be induced in a galactose-inducible plasmid, leaving us with no controls showing the effect of overexpression of other HECT domains on Rpn10 ubiquitination. More time should

be invested to achieve the induction of Tom1^{HECT} and Hul4^{HECT} in order to find out whether other HECT ligases are able to ubiquitinate Rpn10 or whether Rpn10 ubiquitination is exclusively dependent on the Rsp5 ligase.

Increased Rpn10 monoubiquitination decreases yeast growth

The inability of the Rsp5^{HECT} to interact with PPXY-containing substrates has provided us a tool to evaluate the functional relevance of increased Rpn10 monoubiquitination in vivo. Notably, by expressing Rsp5^{HECT} in cells we have been able to produce a slow-growth phenotype that is dependent on the monoubiquitination of Rpn10 (Figure 26B and E). This result is in agreement with that observed by expressing a Rpn10-Ubiquitin chimera, in a previous work (Isasa *et al,* 2010). To evaluate the specificity of this phenotype, we have analyzed the behavior of the Rpn10^{K84,268R} mutant, which carries mutations on the two main targeted lysines in the reaction catalyzed by Rsp5 (Isasa *et al*, 2010), and we have observed that the Rpn10^{K84,268R} mutant has a rescuing effect on yeast growth. Thus, these results unequivocally show that increased Rpn10 monoubiquitination decreases yeast growth. This observation is very relevant, considering the importance of the anti-proliferative effect of proteasome inhibition by chemical inhibitors (Goldberg, 2012). We envision inactivation of proteasome receptors as a potential biomedical approach for the inhibition of proteasome activity.

Structural properties in Rpn10 promote Rpn10 monoubiquitination

Commonly, the mechanism of monoubiquitination is viewed as a process in which the enzymatic factors or cofactors involved contain information that impairs or counteracts polyubiquitin synthesis. For example, it has been shown that Rad18 E3 ligase blocks the Ubiquitin-chain synthesis activity of the E2 Rad6 to promote PCNA monoubiquitination (Hibbert *et al*, 2011). Moreover, in the process of histone H2A monoubiquitination by the polycomb complex Bmi1-RING1, a model was

proposed in which the rigidity of the E2-E3 complex assembled to DNA and nucleosomes promotes K119 specific monoubiquitination (Bentley *et al*, 2011). Alternatively, it has been found that properties of the E2 dictate monoubiquitination, as observed in Ube2W in conjunction with FANCL, Brca1-Bard1 and CHIP E3 ligases (Christensen *et al*, 2007; Alpi *et al*, 2008; Scaglione *et al*, 2011). Another interesting model suggests that the activity of deubiquitinating enzymes (DUBs) processes polyubiquitinated proteins to produce monoubiquitination (Kee *et al*, 2005), as we observed in the assays we performed with Ubp2, where monoubiquitination was rescued from polyubiquitinated ^{PPAY}Rpn10 (Figure 25A). Moreover, the process of monoubiquitination can be induced by an external protein cofactor that modulates enzyme processivity (Becuwe *et al*, 2012). Figure 47 (sections A-D) contains different possible models of monoubiquitination, oriented on the factor that regulates the process. We have characterized here another type of monoubiquitination reaction in which the enzymes involved are proficient in polyubiquitin synthesis, and protein polyubiquitination is the default activity that they exhibit. Nonetheless, structural properties found in the substrate could be dictating a mechanism that produces monoubiquitination. We define this type of reaction as substrate-driven monoubiquitination (Figure 47, section E).

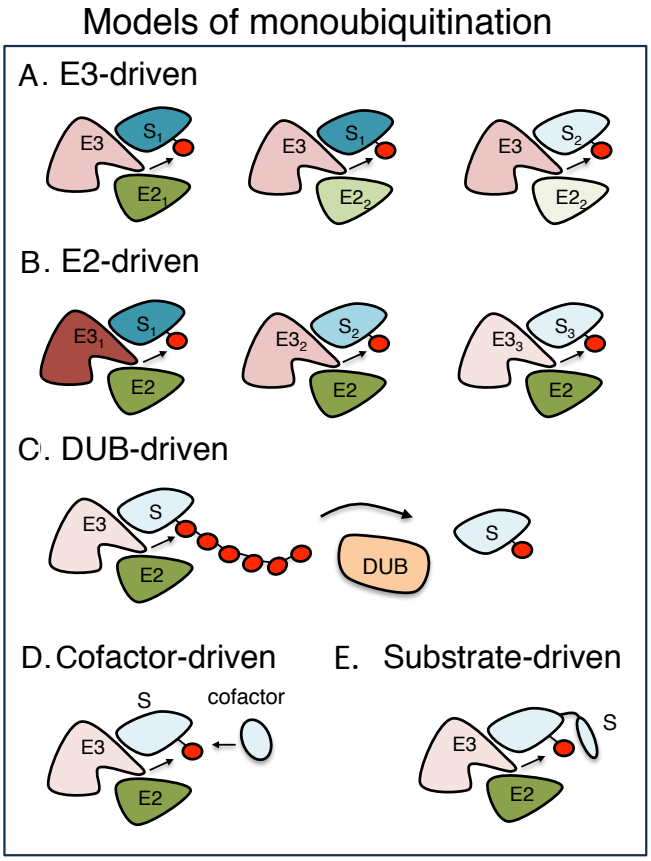


Figure 47. Models of monoubiquitination.
A) E3-driven model: The Ubiquitin ligase determines the specificity. Different E2 enzymes may be involved, with no influence on the product. B) E2-driven model: The Ubiquitin-conjugating enzyme determines the specificity. The E2 binds distinct cognate E3s to promote monoubiquitination to different substrates. C) DUB-driven model: a deubiquitinating activity trims the polyubiquitin chain of a polyubiquitinated protein producing a monoubiquitinated one. D) Cofactor-driven model: a cofactor promotes monoubiquitination of the substrate by preventing the polyubiquitination. E) Substrate-driven model: structural properties of the substrate prevent it to be polyubiquitinated.

Sometimes proteins or regions of proteins lack a well-structured three-dimensional fold. This feature is very common in nature and is involved in several biological processes such as signaling, recognition and regulation (Dunker *et al*, 2000). Disordered proteins can be predicted through bioinformatic tools that analyze the composition of the amino acid sequence. However, the quantitative and qualitative measures of this type of proteins are still not available (Xue *et al*, 2012; Xie *et al*, 2007). To predict the disorder within Rpn10 and Rpn10$^{196\text{-}204A}$, we have used several metapredictors that combine the outputs of several individual disorder predictors. Our data suggests that there is a conserved intrinsically

disordered region linking the last α-helix of the VWA domain (helix 6) and the α-helix that contains the UIM (helix 7; Figures 28F and 36) and that this linker is required for the specificity of the monoubiquitination process. Mutations that change the properties of this region seemed to cause a change on the processivitiy of Rsp5, the Ubiquitin-protein ligase involved in the reaction of ubiquitination, promoting polyubiquitination (Figures 35, 37, 38 and 39). This is the case for $Rpn10^{196\text{-}204A}$, whose polyubiquitination is probably due to the gain of structural order (a predicted helicoidal structure). Indeed, some amino acids are found more frequently in α-helices than others; this tendency is known as helix propensity. Excluding proline, glycine has the lowest helix propensity and alanine has the highest (Pace & Scholtz, 1998). The fact that the linker is glycine-rich and that glycines disrupt helices and adopt flexible conformations agrees with the prediction of disorder. The flexibility given by the glycines could be playing a role in the ubiquitination of Rpn10.

Additionally, the $Rpn10^{M196A,G204A}$, $Rpn10^{196\text{-}204S}$, $Rpn10^{M196S,G204S}$, and $Rpn10^{DSD208\text{-}210\Delta}$ mutations, which don't show an increase in structural order, also show a slight increase of poly (Figure 38). These observations are compatible with a change of flexibility and with a change in E3 interaction in the mutants tested. Therefore, the behaviour of these Rpn10 mutations within the disordered sequence between α-helices 6 and 7, suggests a functional adaptation to promote monoubiquitination. As a matter of fact, eukaryotic genomes are predicted to have a higher fraction of disordered proteins than bacteria and archaea, indicating that the unstructured regions may be participating in more complex processes (Dunker *et al*, 2005).

The polyubiquitination of $Rpn10^{DSD208\text{-}210\Delta}$ appears to be in contradiction with the effect of the deletion of the N-terminus of Rpn10 up to residue 211 ($Rpn10^{211\text{-}268}$), which abolishes monoubiquitination. We speculate that differences in the conformation of the full-length protein $Rpn10^{DSD208\text{-}210\Delta}$ and the short fragment $Rpn10^{211\text{-}268}$ and a different capacity of Rsp5 to interact with both proteins could explain the differences in their ubiquitination. The latter possibility could be easily tested by pull down studies.

Interestingly, the combination of a post-translational modification and a disordered region within the same protein has been described as a mechanism to control

protein autoinhibition. Autoinhibition is the inhibition of the function of a protein domain via interactions with an inhibitory module in the same protein: the catalytic or binding site of the functional domain of the protein is blocked (Trudeau *et al*, 2013). In our context, the functional domain is the UIM that is required to monoubiquitinate Rpn10 and to bind polyubiquitinated substrates. After monoubiquitination, the activity of the UIM could be inhibited due to allosteric mechanisms or direct blocking of the LAMAL motif in the UIM by the ubiquitins linked to Rpn10. The presence of an unstructured linker is, therefore, key to allow the "inactivation" of the UIM.

Although Rpn10 is mostly monoubiquitinated at one position (Isasa *et al*, 2010) three more lysines can be modified with a Ub molecule (multi-monoubiquitination), suggesting that the interaction between the UIM and the substrate-bound Ub does not avoid the ubiquitination of the other three lysines. However, once Rpn10 is multi-monoubiquitinated, the interaction between the UIM and ubiquitins covalently linked to Rpn10 is favoured and, consequently, the UIM is no longer accessible and Rsp5 can build no chain extensions on Rpn10 (Figure 48).

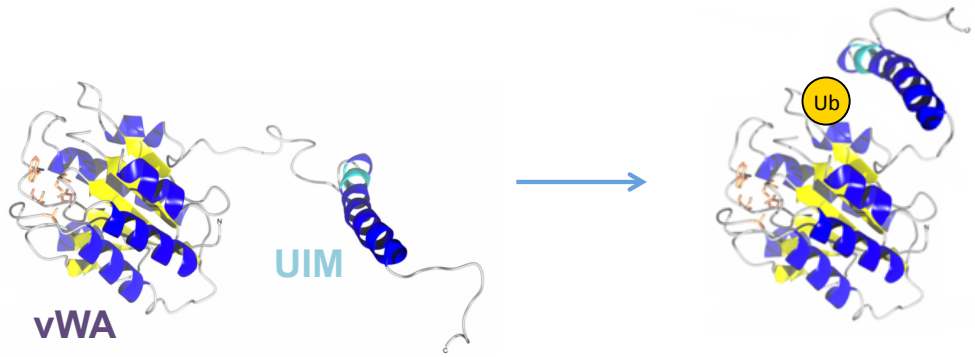


Figure 48. Fold-back model applied to Rpn10.
Left, full-length model of Rpn10 from Riedinger et al. (2011), showing the VWA domain and the C-terminus with the Ub binding domain motif highlighted in cyan. Right, model of monoubiquitinated Rpn10, showing the closed position of the C-terminal arm of Rpn10, derived from Riedinger et al, 2011.

On the contrary, when the flexible region in Rpn10 is changed into an α-helix, as in the Rpn10$^{196\text{-}204A}$ mutant, the C-terminal arm of Rpn10 containing the UIM is no longer free to move or rotate towards the linked ubiquitins in Rpn10 (Figure 49). The fold-back of the protein and therefore, the intramolecular interactions are avoided. As a consequence, the UIM is still accessible and Rpn10 can be repeatedly loaded with Ubiquitin molecules resulting in a polyubiquitinated protein.

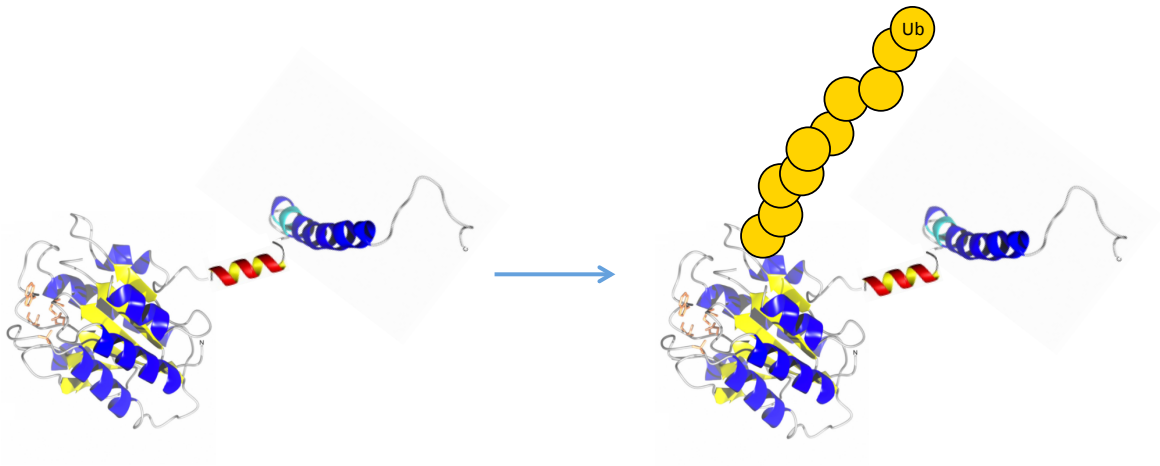


Figure 49. Model showing Rpn10 polyubiquitination of Rpn10.
Left, full-length model of Rpn10$^{196\text{-}204A}$, showing the VWA domain, the C-terminus with the Ub binding domain motif highlighted in cyan with an additional α-helix highlighted in yellow and red derived from Riedinger et al. (2011). Right, model of polyubiquitinated Rpn10$^{196\text{-}204A}$, showing the open position of the C-terminal arm of Rpn10 and the polyUb chain.

A complementary hypothesis that could explain the polyubiquitination of Rpn10$^{196\text{-}204A}$ is that when the unstructured region within Rpn10 looses flexibility, the spatial conformation of the protein is compromised and interactions with other proteins can be altered. Therefore, it could be that Rsp5 and Rpn10$^{196\text{-}204A}$ were able to interact in a way that Rsp5 and Rpn10 cannot and that this interaction facilitates the polyubiquitination of Rpn10$^{196\text{-}204A}$. In fact, the study of the interaction between Rsp5 and both wild-type Rpn10 and mutant could shed light on this hypothesis. Additionally, the rigidity adopted by the mutant could also alter the orientation of its lysines towards the ligase allowing a better transfer of the Ub moieties.

Unfortunately, protein secondary structure predictors have limitations: some metapredictors predicted that the N-terminus of Rpn10 is disordered, while it has been well shown in *Schizosaccharomyces pombe* that the first 190 amino acids are found in a globular domain, the VWA domain. Similarly, the UIM is predicted to be whitin a disordered region, whereas it is part of an α-helix A thorough study, including NMR or circular dichroism approaches, should be addressed to quantitatively determine the extent of order in the sequences of Rpn10 and $Rpn10^{196\text{-}204A}$.

Notably, the mutant exhibiting the strongest polyubiquitination effect, $Rpn10^{196\text{-}204A}$, caused a slow growth phenotype at 37ºC (Figure 41A). Despite the fact that monoubiquitination of Rpn10 is reduced in the polyubiquitinated $Rpn10^{196\text{-}204A}$, we observe the same phenotypical effect as with monoubiquitinated Rpn10 (Figure 26E). A possible interpretation is that the oligo/polyubiquitin chains bound to $Rpn10^{196\text{-}204A}$ mask the UIM, promoting inactivation of this Ubiquitin receptor. As a consequence, old or damaged proteins are less well degraded by the proteasome and are accumulated in the cell. This accumulation would lead to proteotoxic stress and a subsequent growth defect. To address this, we could analyze the steady state levels of polyubiquitinated proteins in the cell when $Rpn10^{196\text{-}204A}$ is polyubiquitinated.

Ubiquitin binding domains as scaffolds for substrate-driven ubiquitination

The control of the activity of substrate receptors Rpn10 and Rpn13 has emerged as a pivotal checkpoint in the regulation of substrate recruitment to the proteasome and of the proteasome pathway. First, Rpn10 monoubiquitination has the capacity to regulate interaction with polyubiquitinated substrates (Isasa *et al*, 2010) and with the ubiquilin type protein Dsk2 (Lipinszki *et al*, 2012). Moreover, Rpn10 can be regulated by its proteasome association status, existing both proteasome-bound and proteasome-unbound forms *in vivo* (Isasa *et al*, 2010; Matiuhin *et al*, 2008; Lipinszki *et al*, 2009; Puram *et al*, 2013). Interestingly, the distinct pools of Rpn10 might have different roles, as suggested by the capacity of

'free' Rpn10 to filter Dsk2 on its way to the proteasome, in yeast and drosophila (Matiuhin *et al*, 2008; Lipinszki *et al*, 2012). Furthermore, controlling the equilibrium of Rpn10 proteasome- bound/proteasome-unbound forms *in vivo* might have profound physiological consequences in mammalian cells. Recently, it has been shown that in mammalian brain, Rpn10 proteasome association/dissociation is tightly regulated by Id1 protein, having special relevance in dendrite development (Puram *et al*, 2013). Interestingly, the ubiquitination of the other main proteasome substrate receptor, Rpn13, has been recently shown to decrease substrate recruitment and 26S activity, and to correlate with situations of proteotoxic stress (Besche *et al*, 2014). Therefore, the inactivation of Rpn10 and Rpn13 by ubiquitination could be a very interesting physiological and biomedical scenario. Further research will be required to determine the link between Rpn10 and Rpn13 ubiquitination and to uncover the functional implications of this putative link.

A common aspect in the process of monoubiquitination of proteins containing UBDs is the involvement of Nedd4 enzymes, such as Rsp5 in yeast and Nedd4.2 in mammals. The Nedd4 Ubiquitin ligase family is highly conserved and involved in multiple and diverse tasks in cell physiology. These Ubiquitin ligases have been involved in the monoubiquitination of UIM containing proteins. It has been proposed that the UIM within these proteins promotes a molecular interaction with the Ub moiety linked to the substrate, generating a fold-back of the protein (Figure 49) (Polo *et al*, 2002; Shih *et al*, 2003; Hoeller *et al*, 2006; Isasa *et al*, 2010). Analogously, the RING Ubiquitin ligase SCF^{MET30} monoubiquitinates the transcription factor Met4. The UIM of Met4 both restricts chain elongation on Met4 and prevents the recognition and proteolysis of ubiquitinated Met4 by the proteasome (Flick *et al*, 2006). The fold-back model could explain the mechanism by which UIM-proteins inhibit their own polyubiquitination. This model implies certain capacity of substrates to undergo a conformational change. However, the requirement of specific structural properties facilitating a conformational change and, thus, preventing Ub chain extension has never been established.

Role of Ubp2 in regulating Rsp5-dependent Rpn10 ubiquitination

The deubiquitinase enzyme, Ubp2, efficiently hydrolyses Ubiquitin-Ubiquitin links from conjugates that have been previously K63-polyubiquitinated by Rsp5 and reverses completely the *in vitro* ubiquitination of certain Rsp5 substrates (Kee *et al*, 2005, 2006). A former member of our lab observed that the incubation of Ubp2, after an *in vitro* ubiquitination reaction, deubiquitinated Rpn10 but not reversed the ubiquitination completely (Isasa *et al*, 2010). We reproduced this experiment and we observed the same pattern (Figure 25A). Regarding ^{PPAY}Rpn10, a mutant that becomes extensively polyubiquitinated, the catalysis of Ubp2 generated a mono- and diubiquitinated version of this mutant (Figure 25A), which is one of the mechanisms by which monoubiquitination can be generated. The incomplete hydrolysis of ubiquitinated ^{PPAY}Rpn10 was similar to the inability of Ubp2 to trim back all the ubiquitins bound to Rpn10.

Interestingly, it seems that the capacity of Ubp2 in deubiquitinating Rpn10 to a greater or lesser extent depends on the conditions under which the reactions are carried out. After we incubated Ubp2 and Rsp5 simultaneously in a reaction with Rpn10, we observed that the deubiquitinating activity of Ubp2 against Rpn10 was at least 5 times higher than the ubiquitinating activity of Rsp5 (Figure 25B). Indeed, although using 50 nM of Rsp5 and 10 nM of Ubp2, Rpn10 was still entirely unmodified. Remarkably, the extensively polyubiquitinated ^{PPAY}Rpn10 needed five times more Ubp2 than Rpn10 to be totally deubiquitinated when Rsp5 was also present. The interaction between the PPAY and the WWs in Rsp5 might be strengthening ^{PPAY}Rpn10 ubiquitination.

However, when Rsp5^{HECT} was used as the ligase and the reaction contained Ubp2 from time 0, recovery of the unmodified Rpn10 was not observed, even when the Ubp2:Rsp5 stoichiometry was 1:1. In this context, ^{PPAY}Rpn10 de was the same as for Rpn10 since the lack of WW in Rsp5^{HECT} does not change the pattern of ubiquitination of ^{PPAY}Rpn10 (Figure 25A and B). These results agree with our observation of the higher capacity of Rsp5^{HECT} in catalyzing the monoubiquitination of Rpn10.

In summary, we have observed that the ubiquitination/deubiquitination equilibrium of Rpn10 depends on the presence or absence of Ubp2 at the beginning of the reaction, which would affect the levels of self-ubiquitinated Rsp5. In a reaction

without Ubp2, Rsp5 can be largely self-ubiquitinated and ubiquitination of the substrate takes place. Once we add Ubp2, cleavage of the Ub molecules from the ligase and the substrate would occur (Rsp5 preferentially conjugates K63 chains to itself, and Ubp2 deubiquitinates Rsp5 (Kim & Huibregtse, 2009; Lam & Emili, 2013)) (Figure 50A). The prior self-ubiquitination of Rsp5 could be an advantage to keep ubiquitinating Rpn10 over Ubp2 deubiquitinating it. Conversely, when Ubp2 is present in a reaction from the beginning, as Rsp5 is self-ubiquitinated and the substrate is ubiquitinated, Ubp2 would be deubiquitinating them. Rsp5 would no longer be able to build long polyUb chains on itself and, therefore, ubiquitinate Rpn10 as efficiently. Here, Ubp2 would hydrolyze all the Rpn10-Ubiquitin links (Figure 25B and 50B).

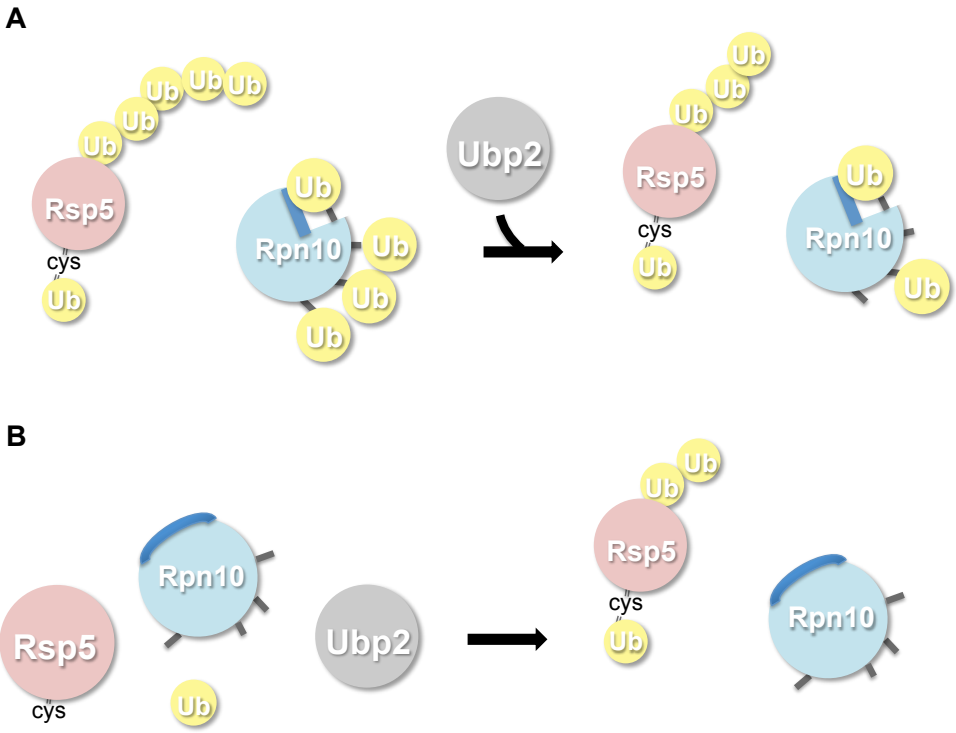


Figure 50. Ubp2 deubiquitinates Rpn10 completely.
A) The addition of Ubp2, after a Rpn10 ubiquitination reaction, catalyzes partial deubiquitination of Rsp5 and Rpn10. **B)** Ubp2 deubiquitinates completely Rpn10 when added simultaneously with the other factors of the ubiquitination reaction.

Kee et al. showed that an UBA-containing protein called Rup1 stabilizes the interaction between Rsp5 and Ubp2 (Kee *et al*, 2005) and in a recent paper, it has

been shown that Rup1 and Rsp5 interact by means of a putative Rsp5-binding motif (PPPSY) and that their interaction increases Ubp2 deubiquitinating activity (Kee 2005, Lam 2009, Lam 2013). Additionally, Lam and collaborators suggested that Rup1 might change Rsp5 conformation from a non-active to an active state, which would stimulate Rsp5 self-ubiquitination and substrate ubiquitination (Lam & Emili, 2013). The presence of Rup1 in *in vitro* ubiquitination reactions was tested in our lab in the past. The catalytic rates of deubiquitination of Rpn10 were not increased by Rup1 presence and, thus, de-monoubiquitination of Rpn10 couldn't be observed (Isasa, 2012). We wonder what would be the role of Rup1 on the Ubp2-Rsp5-Rup1-^{PPAY}Rpn10 complex. Ubiquitination reactions with Ubp2 and Rup1, both included after the reaction, would determine whether Rup1 helps Ubp2 cleave the residual short Ubiquitin chains and the most proximal Ubiquitin moieties within ^{PPAY}Rpn10 or cannot stimulate de-monoubiquitination, as with Rpn10.

Strikingly, in the *in vivo* context, the levels of conjugated Rpn10 after overexpressing Rsp5^{HECT} were not increased in a strain lacking Ubp2- *ubp2*Δ strain (Figure 27A and B). The same result was reproduced after inducing Rsp5^{HECT} in the double mutant *rsp5–1 ubp2*Δ strain at a restrictive temperature (Figure 27C). Isasa and collaborators already reported that Ubp2 is highly active towards Rpn10-monoubiquitin isopeptide bonds but that under some conditions the deubiquitination is not apparent (Isasa *et al*, 2010). Certainly, the equilibrium between mUb-Rpn10/Rpn10 is controlled by other factors such as other DUBs that might act on Rpn10 or Rsp5. High levels of mUb-Rpn10 limit the interaction with substrates, which decreases proteasome activity and produces a severe slow-growth phenotype (Figure 26E). A more complex regulation of Rpn10 ubiquitination is likely to exist.

S5a ubiquitination, a model for Rpn10 ubiquitination?

It is interesting to mention the universality of S5a (Rpn10 human ortholog) as a substrate described by Uchiki and coworkers (Uchiki *et al*, 2009). Uchiki proposed that the ubiquitination of S5a results from its binding to the polyubiquitin chain on the E3 ligase after auto-ubiquitination and suggested that, through this mechanism, S5a can be polyubiquitinated by all E3s when combined with UbcH5 E2 enzyme (Figure 51). Strikingly, when we performed ubiquitination reactions with S5a, Nedd4 and UbcH5b, S5a showed a pattern of mono- and diubiquitination, never reaching the polyubiquitinated state (Figure 45A). This observation collides with the one observed by Uchiki and colleagues as they observed polyubiquitinated S5a when using different E3s such as the RING types, Siah2 and MuRF1, the U-Box type, CHIP and the HECT types, E6AP, and Nedd4 (Uchiki *et al*, 2009). This same lab also showed that the polyubiquitination of the ligase was a requirement to obtain ubiquitinated S5a. S5a contains two UIMs that would interact with the polyubiquitin chain on the self-ubiquitinated ligase. This interaction would allow the proximity of S5a to the highly reactive Ub thioester that is formed between Ub and the active site of the ligase, which would trigger S5a ubiquitination.

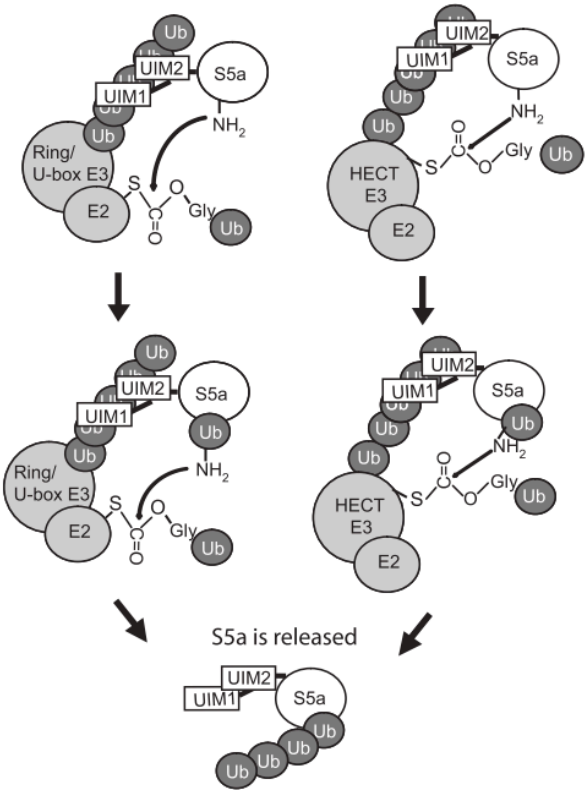
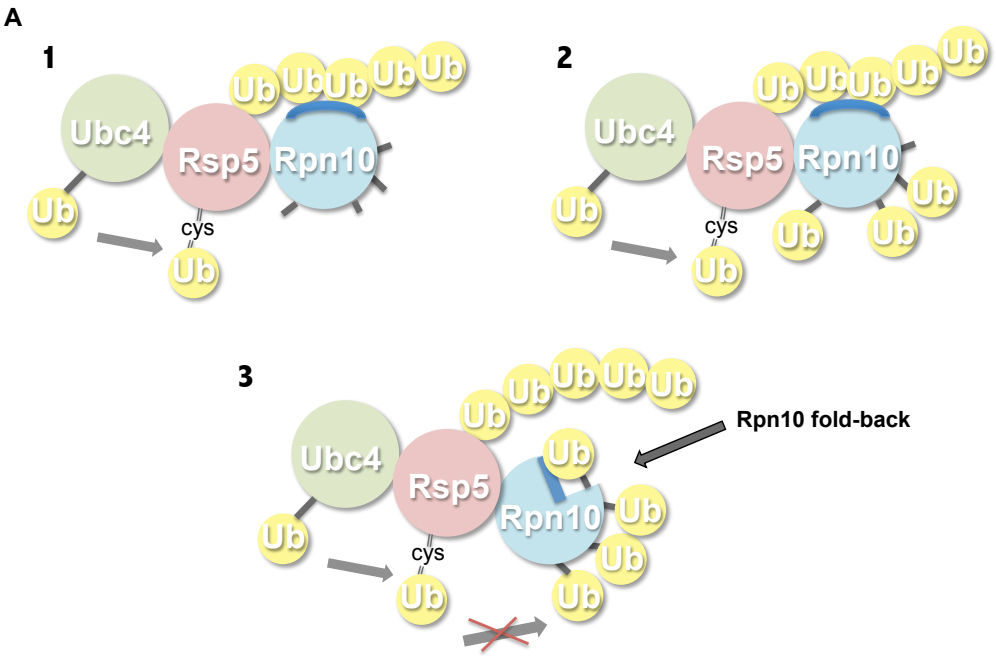


Figure 51. S5a ubiquitination model.
Uchiki's model has been an inspiration to propose the following model for Rpn10 and Rpn10$^{196\text{-}204A}$ Rsp5-mediated ubiquitination

To test whether the polyubiquitination of the ligase was necessary for the ubiquitination of S5a, Uchiki and coworkers performed ubiquitination reactions using methylated Ub, an Ubiquitin mutant that cannot form polyubiquitin chains (Hershko & Heller, 1985). They obtained a monoubiquitinated ligase, which led, under their conditions, to a non-modified S5a, indicating that S5a needs to bind to polyUb chains before being ubiquitinated. Interestingly, a similar reaction using Rpn10 as substrate and methylated Ub produced the same pattern of ubiquitination observed using wild-type Ubiquitin, which is mono- or multi-monoubiquitinated Rpn10 (Isasa *et al*, 2010). In summary, although Rpn10 also contains an UIM that is indispensable to allow its ubiquitination and to interact with Ubiquitin conjugates, it is still ubiquitinated when using ubiquitins that do not form chains (i.e. Ub K0, Ub methylated). Therefore, on the one hand, we would suppose that the mechanism by which Rpn10 is modified is at least partially different from the one followed by its orthologue, S5a. However, on the other hand, S5a and Rpn10 as well as S5a mutants (S5a^{M196A,G200A} and S5a$^{193\text{-}200A}$) and Rpn10 mutants (Rpn10$^{196\text{-}204A}$ and

Rpn10$^{M199A-G203A}$) showed similar behaviours in our experiments, indicating that the mechanism could be similar.

Taking Uchiki's model into account, we propose that the UIM of Rpn10 is able to bind both poly- and monoubquitinated Rsp5. Once Rpn10 is multi-monoubiquitinated, the interaction between the Ubiquitins attached to Rpn10 and the UIM becomes stronger than the one between the Ubiquitins attached to the ligase and the UIM, facilitated by the fold-back of Rpn10 (Figure 52A and B). On the contrary, the Rpn10$^{196-204A}$ mutant cannot fold-back, which enhances the binding between the UIM and the ligase and promotes polyubiquitination of the substrate.



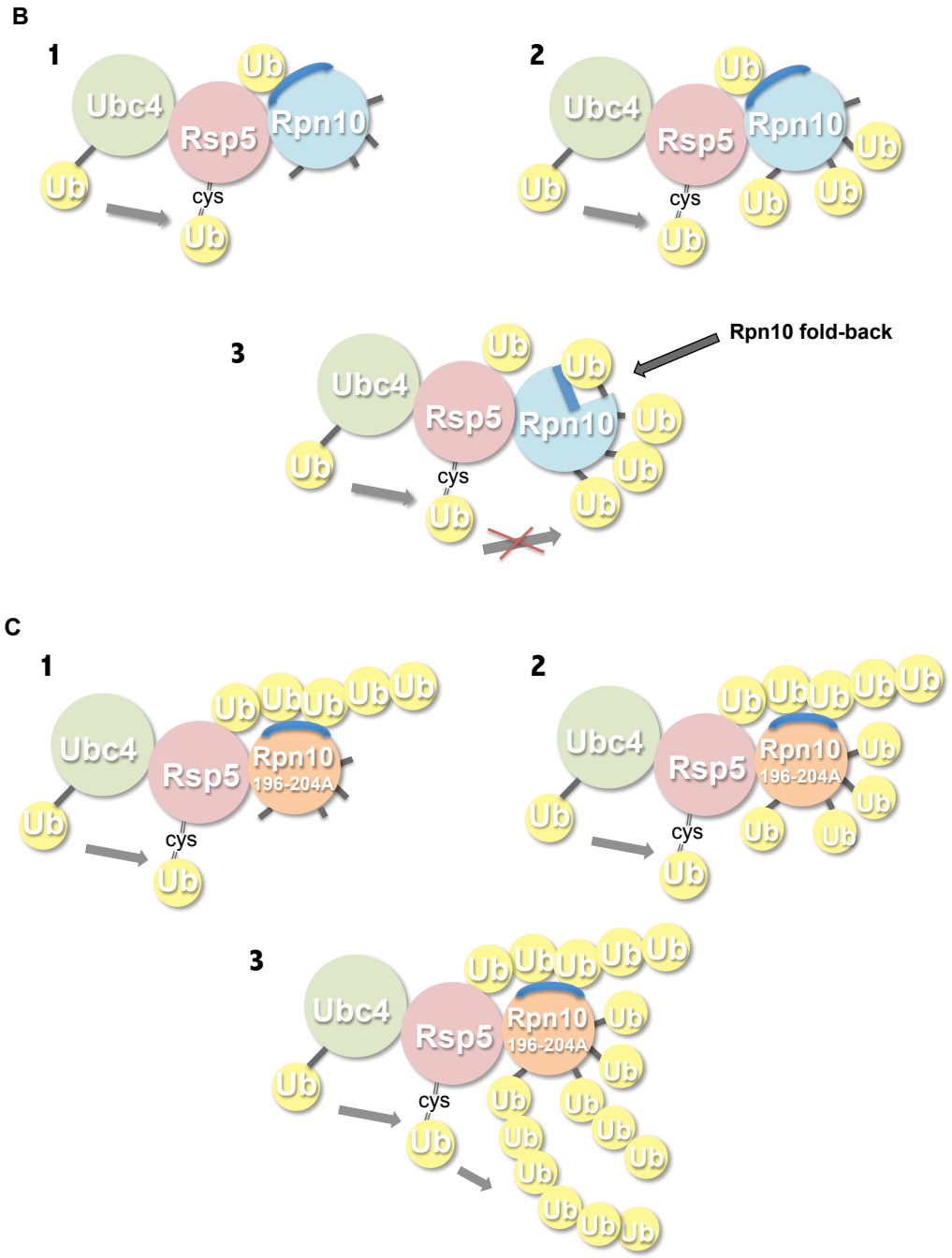


Figure 52. The proposed mechanism of Rpn10 and Rpn10$^{196\text{-}204A}$ ubiquitination. **A) 1-** Ubc4 transfers a Ubiquitin to the active site of Rsp5. Rpn10 binds to Rsp5 and to the growing Ub chain on Rsp5 through the UIM domain, highlighted in cyan. **2-** Rpn10 is multi-monoubiquitinated because of the proximity between the active-site cysteine (cys) and the lysines on Rpn10. **3-** Formation of an intramolecular binding between the UIM and a Ubiquitin bound to Rpn10, favored by a fold-back within Rpn10. The orientation of the lysines has changed and the highly reactive Ub thioester is too far to keep adding ubiquitins to Rpn10. **B)** In contrast with S5a, Rpn10 is multi-monoubiquitinated when using methylated Ubiquitin. Same steps as in **A)1**, with the exception that now, the UIM domain binds only to one Ubiquitin in Rsp5. **C) 1-** As in **A)1**, the mutant Rpn10$^{196\text{-}204A}$ binds to the self-ubiquitinated Rsp5 through the UIM

domain. **2-** Rpn10$^{196\text{-}204A}$ is multi-monoubiquitinated. **3-** Rpn10$^{196\text{-}204A}$ cannot fold-back because of its gain in structural order and, therefore, it is polyubiquitinated.

Disordered regions flank the UIM of proteins that undergo monoubiquitination

Our work suggests a correlation in Rpn10 between the UIM, a flanking conserved disordered region and monoubiquitination. The prediction of conserved UIMs and unstructured regions in Rpn10 orthologues (Figure 53) suggests a conserved mechanism facilitating monoubiquitination. Additionally, disorder can be found anywhere within a protein although it is typically found at the termini, which is the case for Rpn10 orthologues (Uversky, 2013; Dunker *et al*, 2000).

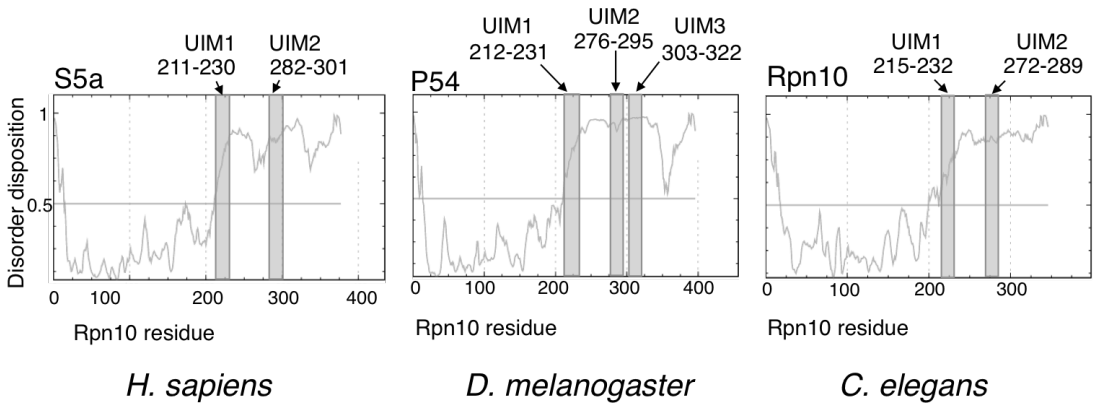


Figure 53. The regions flanking the UIMs show a disposition to disorder.
Plots showing the disposition to disorder of Rpn10 from different species. The rectangle areas correspond to the positions of the UIM1 and UIM2 domains.

We asked whether this correlation could be a trait of Ubiquitin-binding proteins regulated by monoubiquitination. We analyzed the disposition to disorder of Eps15, Vps27, Hrs, Vps9 and Cue1, which undergo monoubiquitination *in vivo* (Polo *et al*, 2002; Shih *et al*, 2003; Hoeller *et al*, 2006). We observed that these proteins show high disposition to disorder in regions flanking the UIM (Figure 54).

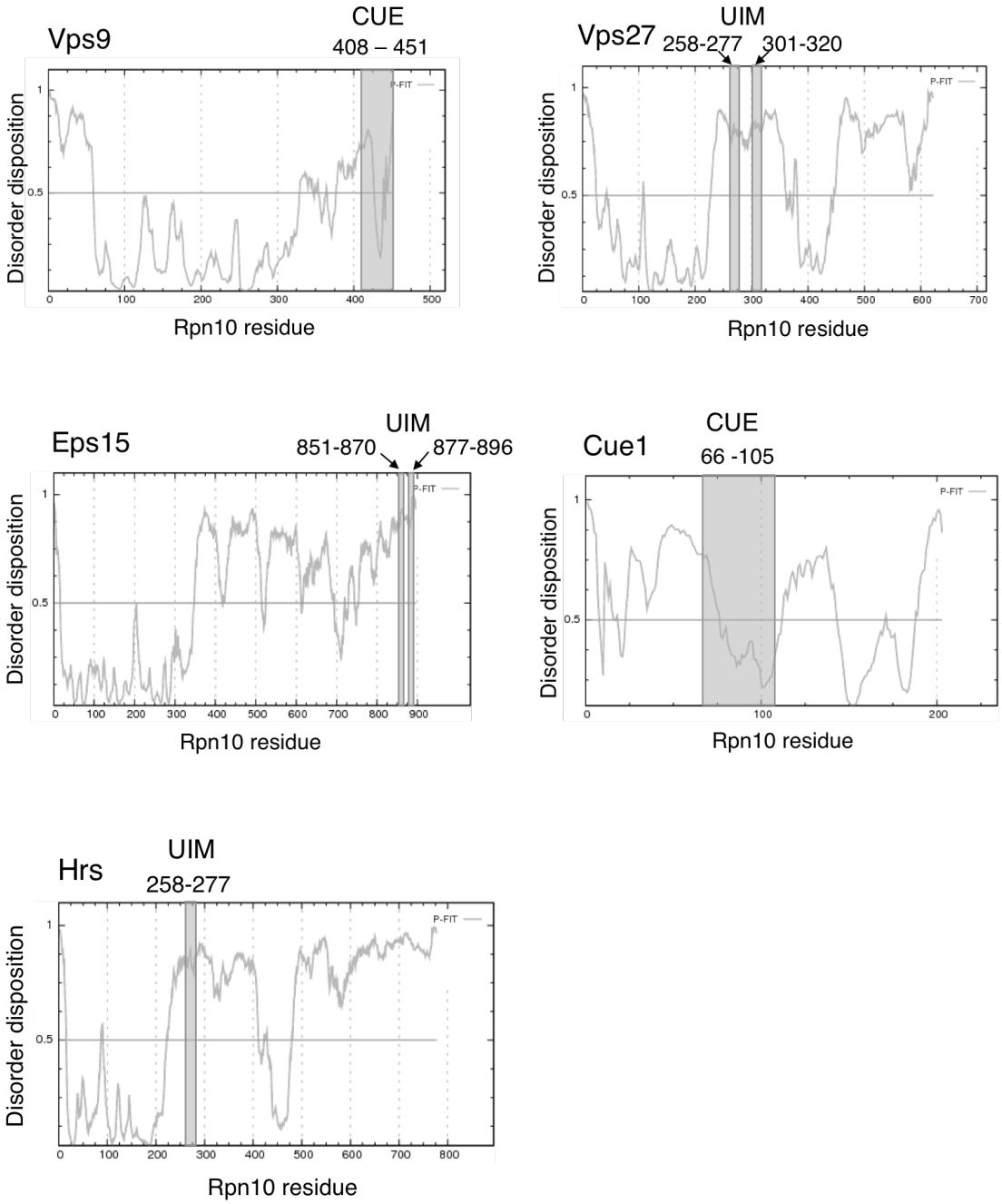


Figure 54. Disposition to disorder of other proteins that are monoubiquitinated. Plots showing the disposition to disorder of Eps15, Vps27, Vps9, Cue1 and Hrs. The rectangle areas correspond to the positions of the UIM and CUE domains.

We hypothesize that the presence of disordered regions could be a characteristic feature of monoubiquitination driven by the substrates. Further research will be required to validate this hypothesis.

CONCLUSIONS

The aim of my thesis research has been the study of the mechanism of Rpn10 monoubiquitination, using *Saccharomyces cerevisiae* as a model organism. The remarking conclusions are as follow:

- We have optimized the reaction of ubiquitination of Rpn10.
- We have obtained polyubiquitinated Rpn10 by adding a PPAY motif at the N-terminus of Rpn10 indicating that the monoubiquitination of Rpn10 does not result from an impaired polyubiquitination activity of Rsp5, but is determined by specific features of Rpn10.
- The UIM of Rpn10 is required to ubiquitinate Rpn10 and the PPAY motif does not guarantee the reaction.
- The balance of the activity of the Ubiquitin ligase, Rsp5, and the deubiquitinase, Ubp2, determines the final outcome of Rpn10 ubiquitination: Ubp2 outcompetes Rsp5 and completely deubiquitinates Rpn10 in vitro.
- The HECT domain of Rsp5 is sufficient to ubiquitinate Rpn10 *in vivo* and *in vitro*. $Rsp5^{HECT}$ proved to be more competent in ubiquitinating Rpn10 than Tom^{HECT}, another yeast HECT Ubiquitin ligase.
- $Rsp5^{HECT}$ is more efficient in monoubiquitinating Rpn10 than Rsp5 full-length.
- Increased levels of monoubiquitinated Rpn10 by Rsp5 produce a severe yeast growth defect.
- The VWA domain of Rpn10 is dispensable for Rpn10 ubiquitination and $Rpn10^{208\text{-}268}$ is the shortest sequence that undergoes ubiquitination. Additionally, we have shown that the region C-terminal to residue D254 is not needed to get ubiquitinated Rpn10.
- A disordered region in Rpn10, between the VWA and the UIM, plays a key role in promoting monoubiquitination by preventing Rpn10 polyubiquitination. The $Rpn10^{196\text{-}204A}$ mutant, that carries a block-substitution to alanines in this region, between amino acids S196 to G204, generates Rpn10 polyubiquitination and introduces, predictably, a helicoidal structure.

Conclusions

- The region S196-G204 has an effect on cell viability by producing a decrease of growth in cells at 37ºC when mutated to alanines.
- Polyubiquitination of $Rpn10^{196\text{-}204A}$ *in vivo* could not be detected.
- Mutations in the region between the VWA and the UIM1 in S5a change the ubiquitination pattern.

  Our findings agree well with the fold-back model, although future experiments are needed to confirm the introduction of an α-helix in the region S196-G204.

MATERIALS AND METHODS

Assays of ubiquitination *in vitro*

Rpn10 *in vitro* ubiquitination reactions (50 µL) contained 50 nM of GST-Rpn10 or versions of Rpn10, 87 nM of human activating E1, 5,7 µM of GST-Ubc4, 50nM of GST-Rsp5 or 6HIS-Rsp5^{HECT} and 35,3 µM of Ub or at the indicated concentrations. Reactions for Figure 3A contained 0.87 µg (150 nM) of GST-Rsp5. Different types of ubiquitins were used: recombinant wild-type, Ub K63-only, Ub K48-only, Ub K63R, Ub K48R, Ub K0 (BostonBiochem) and methylated Ub (Enzo Life Sciences). Rpn10 ubiquitination *in vitro* reactions were also performed using Tom1^{HECT} and Hul4^{HECT} at the indicated concentrations.

in vitro S5a ubiquitination reactions (50 µL) contained 500 nM of GST-S5a or GST-S5a mutants, 17,4 nM or 174 nM of human activating E1, 45 nM or 0.45 µM of his-UbcH5b (BostonBiochem), 20 nM of GST-Nedd4, and 35,3 µM of Ubiquitin.

Reactions were carried out in 100 mM Tris-HCl (pH 7.4), 200 mM NaCl, 10 mM ATP, 10mM $MgCl_2$ and 1 mM DTT buffer at 30ºC (for Rpn10 reactions) and at 37 ºC (for S5a reactions) and were incubated for 3 hours or for the indicated times. Reactions were stopped by the addition of reducing SDS-PAGE loading buffer and analysed by western blot against Rpn10.

The auto-ubiquitination assays of Rsp5^{HECT}, Tom1^{HECT} and Hul4^{HECT} were carried out in the same buffer as the ubiquitination reactions and contained 1 µg of human activating E1, 2 µg of GST-Ubc4, 2 µg of ligase, 0.5 µg of Ub and 10mM ATP in 25,4 µl final volume. Reactions were incubated at 37ºC for 30 minutes. Reactions were stopped by the addition of reducing SDS-PAGE loading buffer and analysed by western blot against Ubiquitin.

Assays of deubiquitination *in vitro*

Deubiquitination assays were performed in the presence of 100 mM Tris-HCl (pH 7.4), 200 mM NaCl, 10 mM ATP, 10mM $MgCl_2$ and 1 mM DTT. Different amounts of GST-Ubp2 (10 nM, 25 nM and 50 nM) were added either once the ubiquitination reaction was finished or at the beginning of the ubiquitination reaction. Reactions were incubated at 30ºC for different times (15 min, 45 min and 120 min or 2h, 4h and 6h). Reactions were stopped by the addition of reducing SDS-PAGE loading buffer and analysed by western blot against Rpn10.

Ubiquitin binding experiments

Rpn10$^{195\text{-}268}$, Rpn10$^{211\text{-}268}$, Rpn10$^{195\text{-}268\ UIM}$, Rpn10$^{195\text{-}254}$ and Rpn10$^{195\text{-}239}$ were purified as GST-fusion proteins on glutathione-sepharose beads and same amounts of proteins were used in binding assays. Equal amounts of input, 1.5 µg of either K48-linked or K63-linked poly-Ub chains were incubated with Rpn10 fragments (in Figure 31 and 33) overnight at 4ºC in the presence of binding buffer containing 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 100 mg.ml^{-1} BSA. Beads were washed with 10 volumes of this buffer supplemented with 150 mM NaCl. Bound proteins were eluted by boiling 5 min in 2x reducing SDS-PAGE loading buffer, separated by SDS-PAGE and immunoblotted with an anti-Ubiquitin antibody. The UIM from RNF114 (Figure 31) was used as a positive control and the GST protein and the beads alone as negative controls.

Yeast methods and media

Strain transformations were performed following standard techniques (Rose et al, 1990). YPD medium consisted of 1% yeast extract, 2% Bacto-Peptone, and 2% dextrose. Synthetic media consisted of 0.7% Yeast Nitrogen Base supplemented with amino acids, adenine and uracil as described (Rose et al, 1990), 2% dextrose (SDC) or, if necessary, 1% galactose (SGRC). For plasmid selection, synthetic media lacking uracil, leucine or tryptophan or the lack of two of these amino acids were prepared. Cultures were grown at 30ºC or at the indicated temperature; *rsp5–1 ubp2*Δ strain was induced at 35ºC for 7 hours (Figure 27C). Samples taken from growing cultures were normalized by optical density at 600 nm using Eppendorf Biophotometer plus (Eppendorf). Spot assays were prepared after 4 hours induction with galactose in liquid and agitation at 30ºC. Cells in Figure 26E were induced in liquid at 30ºC. After that the OD_{600} of each cell culture was adjusted to 0.04, and then spotted in 5-fold serial dilutions onto plates with (SGRC) or without (SDC) galactose before incubation at 22ºC. Cells in Figure 41A were grown in liquid at 37ºC and spots were grown at 30ºC or 37ºC in SDC plates. Images of colony spot assays were taken using GeneGenius Bioimaging System (Syngene). Detailed description of genotypes of strains is provided below.

TABLE 1

Yeast strains used in this study

Strain	Genotype	Source
SUB62	*MATa lys2-801 leu2-3, 2-112 ura3-52, his3- Δ200 trp1-1*	Finley Lab
11d	*Rpn10::natMX, Rad23::KanMX4 (Based on Sub62)*	
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf
S67	*Wild-type (based on BY4741)*	
S72	*Rpn10::KanR (based on BY4741*	
S33	*Rpn4::KanR (based on BY4741)*	
FY56	*MATa ura3-52, his4-912-R5, lys2Δ 128*	Beaudenon Lab

Analysis of Rpn10 in yeast

Yeast wild-type, *rpn10*Δ, *rpn10*Δ*rad23*Δ, *rsp5–1 ubp2*Δ and *ubp2*Δ strains were grown under normal conditions and an equivalent number of cells were taken after 4 hours of induction or at the indicated time points (Figures 26, 27, 42 and 43). Cells were harvested, resuspended with buffer composed of 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, pelleted again and resuspended in Laemmli loading buffer. Cells were then lysed by vortexing 1 minute and boiling 2 minutes, twice, then vortexing 1 minute and boiling 4 minutes. Supernatants were resolved by SDS-PAGE and analysed by immunoblotting against Rpn10, Pgk1 and Hemagglutinin.

*ubp2*Δ knockout

To create the double mutant *rpn10*Δ *ubp2*Δ, linear DNA substrate was made by PCR amplification of a cassette encoding the kanamycin gene (pFAGKANmx) using bi-partite primers. These primers consisted of (from 5'→3') 50 bases of homology to Ubp2, where the cassette is to be inserted, followed by 25 bases to prime the kanamycin cassette. DNA was next transformed into yeast cells and cells

were plated in YPD medium. Two days later, we replica plated the lawn on selective medium (YPD+G418). Once the new colonies were grown, some were used as DNA template to run a PCR to confirm the incorporation of the kanamycin gene.

Immunoprecipitation

*rpn10*Δ (S72) strain carrying pRS425 Rpn10 or pRS425 $Rpn10^{K84,268R}$ and $^{HA}Rsp5^{HECT}$ galactose-inducible plasmid was grown in glucose and galactose for 4 hours. Cells were harvested and resuspended in Lysis buffer (50 mM Tris-HCl pH 7.4, 1 mM EDTA, 100 mM NaCl and 1x concentration of protease complete inhibitor cocktail EDTA free (GE Healthcare)). The disruption of yeast cells was done by agitation with glass beads (0.4-0.5 mm, Sigma). 8 cycles of vortexing (60 sec) were interspersed with cycles of cooling on ice to avoid overheating of the cell suspension.

Protein concentration was measured by Bradford and same amounts of the four whole cell extracts (875 µg) as well as purified Rpn10 were incubated with 1,5 µl of Rpn10 antibody at 4ºC overnight. IgG beads (35 µl 50% slurry/binding) were next added for 2 hours at 4ºC. Beads were washed with 10 bed volumes with lysis buffer. Bound proteins were eluted by boiling 5 minutes in 2x reducing SDS-PAGE loading buffer, separated by SDS-PAGE and immunoblotted with an anti-Ubiquitin and anti-Rpn10 antibodies.

Assays of deubiquitination *in vivo*

For *in vivo* deubiquitinating activities, *rpn10*Δ (S72) strain carrying pRS425 Rpn10 and $^{HA}Rsp5^{HECT}$ galactose-inducible plasmid was grown in galactose for 4 hours. 234 µg of whole cell extract were incubated with 600 nM of GST-Ubp2 in a buffer containing 50 mM Tris-HCl pH 7.4, 1mM EDTA and 200 mM NaCl at 30ºC. Same amounts of whole cell extract were incubated at 30ºC without Ubp2. Time points were taken at time 0, 15, 30 and 45 minutes after starting the incubation. Reactions were stopped by the addition of SDS-PAGE loading buffer and analysed by western blot against Rpn10 (Figure 26C).

Size-exclusion chromatography

*rpn10*Δ (S72) strain carrying Rpn10 or Rpn10$^{196\text{-}204A}$ was cultured (500 ml) and cells were harvested, resuspended in a 2-fold volume of 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 150 mM NaCl buffer and lysed using a cell disrupter (Constant Cell Disruptor Systems). Lysate was centrifuged twice at 10,000xg in cold for 40 min. The supernatant was recovered and centrifugation was repeated three times. Final lysate was filtered through a 0.45 µm filter before applying it to a Superose 6 gel filtration chromatography (GE Healthcare). The eluted fractions were analysed by western blot against Rpn10 antibody.

Protein stability by cycloheximide chase assay

Comparison of CPY* stability has been achieved by the protein inhibitor, clycoheximide. Degradation of HA-tagged CPY* was compared in wild-type (S67) and *rpn10*Δ (S72) strains carrying empty vector pRS424, pRS424 Rpn10 or pRS424 Rpn10$^{196\text{-}204A}$. Overnight cultures of the indicated strains were adjusted to an equal OD_{600} and allowed to grow in the exponential phase for 4 h at 30°C. Cultures were again normalized by OD_{600}, and 200 µg/ml cycloheximide were added (Figure 44). Equal number of cells was taken at indicated time points after cycloheximide treatment. Samples were pelleted and resuspended in 1x Laemmli loading buffer and were analysed by western blot against HA and Pgk1 for quantification.

Prediction of unstructured regions

To carry out the prediction of unstructured regions we used a meta-predictor computer program called PONDR-FIT (Predictors of Natural Disordered Regions) (Xue *et al*, 2010). The meta-predictor uses an amino acid sequence as the input and gives structure (order) or disorder as the output by combining the prediction of different software's predictions. Access to PONDR-FIT is available at www.disprot.org.

Prediction of secondary structures

To predict the secondary structure of Rpn10 and Rpn10$^{196\text{-}204A}$, we used MeDoR (Metaserver of Disorder) that includes a Secondary Structure prediction that makes use of the StrBioLib java library (LGPL), which is the basis for the Pred2ary program. The library can be found from its official web page http://www.strbio.org. MeDoR also helps to identify protein disorder by providing a graphical interface with a unified view of the output of multiple disorder predictors.

Expression and purification of GST-fusion proteins in *E.coli*

In *E.coli*, glutathione S-transferase fusion vectors (pGEX-4T-3) were used to express and purify the following proteins: Rpn10, ^{PPAY}Rpn10, Rpn10$^{40\text{-}268}$, Rpn10$^{208\text{-}268}$, ^{PPAY}Rpn10$^{208\text{-}268}$, Rpn10$^{208\text{-}268\ UIM}$, ^{PPAY}Rpn10$^{208\text{-}268\ UIM}$, Rpn10$^{195\text{-}268}$, ^{PPAY}Rpn10$^{195\text{-}268}$, Rpn10$^{195\text{-}268\ UIM}$, ^{PPAY}Rpn10$^{195\text{-}268\ UIM}$, Rpn10$^{211\text{-}268}$, ^{PPAY}Rpn10$^{211\text{-}268}$, Rpn10$^{195\text{-}268\ K268R}$, ^{PPAY}Rpn10$^{195\text{-}268\ K268R}$, Rpn10$^{208\text{-}268\ K268R}$, ^{PPAY}Rpn10$^{208\text{-}267\ K268R}$, Rpn10$^{211\text{-}268\ K268R}$, ^{PPAY}Rpn10$^{211\text{-}268\ K268R}$, Rpn10$^{DSD208\text{-}210\Delta}$, Rpn10$^{196\text{-}204A}$, Rpn10$^{M199A\text{-}G203A}$, Rpn10$^{196\text{-}204S}$, Rpn10 $^{M199S\text{-}G203S}$, Rpn10$^{195\text{-}239}$, Rpn10$^{195\text{-}254}$, Rpn10$^{195\text{-}261}$, Nedd4, S5a, S5a$^{193\text{-}201A}$, S5a $^{M196A\text{-}G200A}$, Rsp5, Tom1^{HECT}, Hul4^{HECT} and Ubc4. Bacterial cultures (500 mL) were grown to an OD_{600} of 0.7, induced with 500 µM isopropylthiogalactoside (IPTG) for 15 h at 20ºC, resuspended with 2 volumes of 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 1x concentration of protease complete inhibitor cocktail EDTA free (GE Healthcare) buffer and lysed using either a cell disrupter (Constant Cell Disruptor Systems) or a sonicator (Vibra-Cell VCX 750, Sonics). The supernatant was mixed with Glutathione (GSH) Sepharose 4B beads at a ratio of 1 ml of 50% beads slurry to 500 mL of initial culture size. The mixture was incubated at 4ºC rolling for 1 h. Beads were washed with 50 bed volumes of the previous lysis buffer supplemented with 150 mM NaCl. Proteins were eluted either with SDS loading buffer or with a reduced glutathione buffer of 50 mM Tris-HCl pH 8.8, 1 mM EDTA and 35 mM reduced glutathione. When necessary (Figures 14 and 28C, D and E), GST-UbRpn10L85 and GST-Rpn10$^{195\text{-}268}$ were digested with biotinylated thrombin (Novagen) (1U enzyme for 10 µg target). Digestions were incubated overnight at 4ºC. The efficiency of cleavage was

determined by SDS-PAGE analysis. Thrombin was removed with benzamidine beads (Amersham Biosciences) according to the manufacturer's instructions.
Additional purification steps were added to increase the purity of UbRpn10L85. UbRpn10L85 was concentrated and 500 µl were loaded to a size-exclusion chromatography column Superdex 200 (10/300). To obtain higher purity, a monoQ high resolution ion exchange chromatography was used with the next buffers:
Buffer A (50mM Tris-HCl pH 7.6, 50mM NaCl, 1mM DTT, 1mM EDTA) and Buffer B (50mM Tris-HCl pH 7.6, 50mM NaCl, 1mM DTT, 1mM EDTA) with a linear ionic strength gradient of 0-40% elution buffer in 20 CV.

Expression and purification of 6his-Rsp5^{HECT} in *E.coli*

pET28a was used to express and purify Rsp5^{HECT}. The protocol for its induction and lysis was the same used for GST-proteins. The lysis buffer contained 50 mM Tris-HCl pH 7.4, 10mM imidazole, 1x concentration of protease complete inhibitor cocktail EDTA free (GE Healthcare), 150mM NaCl and 10% glycerol. The supernatant was mixed with Ni-NTA beads previously equilibrated with 10mM imidazole. For 50 mL of culture, 200 µl of Ni-NTA beads were used. The mixture was incubated at 4 ºC rolling for 1 h and then washed first with 10 bed volumes of 50 mM Tris-HCl pH 7.4, 25 mM imidazole, 150 mM NaCl and 10% glycerol and finally with 10 bed volumes of the same buffer including 500 mM NaCl. Rsp5^{HECT} bound to the resin was eluted by competition with imidazole following a gradient-step imidazole elution ranging from 50 mM to 500 mM imidazole including 500 mM NaCl and 10% glycerol.

Electrophoresis and immunoblot analysis

In Figures 26A, B and C, 27, 41B, 42, 43 and 44, proteins were resolved by SDS-PAGE 12% polyacrylamide gels with Tris-Glycine buffer. The rest of the proteins were resolved by 4-12% gradient SDS-PAGE with Tris-Glycine buffer (Invitrogen). For immunoblots, proteins were transferred to polyvinylidene difluoride membranes, which were then blocked, and incubated with antibodies using TBST buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween 20) with 5% w/v nonfat powdered milk, and washed with TBST and with H_2O.

Antibodies

Polyclonal antibodies to Rpn10 were raised in rabbits against full length protein and second bleed antiserum was used (Isasa *et al*, 2010). Anti-S5a antibody was produced in rabbit and obtained from Enzo Life Sciences. Anti-hemagglutinin antibody produced in rabbit was obtained from Sigma-Aldrich. Anti-Ubiquitin (P4D1) is a mouse monoclonal antibody obtained from Santa Cruz Biotechnology. The pgk1 monoclonal antibody produced in mouse was obtained from Life Technologies. Anti-rabbit IgG Horseradish Peroxidase-linked whole antibody from Donkey was obtained from GE Healthcare. Goat anti-mouse IgG (H+L), peroxidase Conjugated antibody was obtained from Thermo Scientific.

Plasmid constructions

Plasmid and oligonucleotide primers detailed information is included below.

All single and multiple nucleotide mutations were performed in one round and using Quick Change Site Directed Mutagenesis kit (Stratagene). 1% agarose gels in TAE (20mM Tris-Acetate, 0,5mM EDTA) were used to analyse DNA fragments for cloning.

Constructions regarding the purification of Rpn10 mutant proteins were made in the *Escherichia coli* expression vector pGEX-4T-3 by PCR strategies. All of them were cloned into *EcoRI* and *SalI* cleavage sites except for UbRpn10L85.

UbRpn10L85 (pPIL 106) construct was created in three steps. First, we obtained Rpn10L85 from Rpn10 wild-type (pMIC26) by PCR amplification (primers used were Rpn10L85 forward and reverse). Next, we cloned Rpn10L85 into *Kpn1* and *EcoR1* sites of pGEX Ub WT. Finally, we mutagenized the stop codon of Ub and the second triplet of kpn1 to obtain three glycines in a row using UbRpn10L85GGG primers.

^{PPAY}Rpn10 construct (pPIL132) was derived from Rpn10 wild-type (pMIC26) by PCR amplification (primers used were *R10M1PPAY Fw* and *R10K268 Rev*) and cloned into *Sal1* and *EcoR1* sites of pGEX-4T-3. ^{PPAY}Rpn10 included a sequence between the GST and the N-terminus of the protein encoding for the PPAY motif: CCTCCTGCATAC.

For N-terminal-deleted constructions, pPIL133, pPIL137, pPIL141 and pPIL143 5'-amplification oligonucleotides were designed to create a new start codon and *R10K40 Fw, R10D208 Fw, R10 G195 fw, R10 A211 fw* primers were used, respectively. All of them were derived from pMIC26 and the reverse primer used for all of them was *R10K268 Rev*.

^{PPAY}Rpn10$^{195\text{-}268}$ and ^{PPAY}Rpn10$^{211\text{-}268}$ were derived from Rpn10 by PCR amplification (primers used were *R10G195PPAY Fw* and *R10K268 Rev* and *R10A211PPAY Fw* and *R10K268 Rev,* respectively) and cloned into *Sal1* and *EcoR1* sites of pGEX-4T-3.

For the C-terminal-deleted constructions, pPIL195, pPIL206 and pPIL207 the 3'-amplification oligonucleotides were designed to add a lysine just before the stop codon and were derived from pPIL141, using primers *R10G195 E239+K fw, R10G195D254+K Fw* and *R10G195Q261+K Fw,* respectively together with *R10K268 Rev*.

Rpn10^{UIM} (pPIL169), Rpn10$^{195\text{-}268\ UIM}$ (pPIL171), ^{PPAY}Rpn10$^{195\text{-}268\ UIM}$ (pPIL172), Rpn10$^{208\text{-}268\ UIM}$ (pPIL167) and ^{PPAY}Rpn10$^{208\text{-}268\ UIM}$ (pPIL168) were derived from pMIC26, pPIL141, pPIL141, pPIL137 and pPIL137, respectively. Residues from 228 to 232 were mutated to asparagines by multiple nucleotide mutagenesis using primers *R10 uim Fw* and its reverse complement.

Fragments of Rpn10 with the 268 lysine mutated to an arginine, pPIL161, pPIL162, pPIL163, pPIL164, pPIL165 and pPIL166 were derived from pPIL141, pPIL142, pPIL137, pPIL138, pPIL143 and pPIL144, respectively and mutated by single nucleotide mutagenesis using primers *R10k268r* fw and its reverse complement.

Mutants within Rpn10 full length, pPIL194, pPIL205, pPIL210, pPIL211, and pPIL220 were obtained through mutagenesis using pMIC26 as a template. Residues from 196 to 204 were mutated either to alanines in pPIL194 or to serines in pPIL210 (primers *R10 link Ala* and *R10 link Ser fw* and their reverse complement, respectively). Residues M199 and G203 were mutated to alanines (primers *R10 MG Ala fw* and its reverse complement for pPIL205) or to serines (primers *R10 mg Ser fw* and its reverse complement for pPIL211). Residues from

208 to 210 were deleted in pPIL220 using primers *R10G195Δ208-210 Fw* and its reverse complement.

S5a (pPIL193) was kindly provided by Dr. F. Hanaoka and was used as a template for constructing pPIL208 and pPIL209. We followed the same strategy described for plasmids pPIL194 and pPIL205 using primers *S5a link Ala fw* and *S5a m-g to Ala fw* and their reverse complement, respectively.

pRS424 Rpn10 (pMIC93) was kindly provided by Dr. H. Fu and used as a template for constructing $Rpn10^{DSD208-210\Delta}$ (pPIL222) and $Rpn10^{196-204A}$ (pPIL225) following the same strategy described for plasmids pPIL220 and pPIL194, respectively. pRS424 contains a TRP gene marker.

Nedd4 (pPIL202) was kindly provided by B. Schulman.

$Tom1^{HECT}$ (pPIL200) and $Hul4^{HECT}$ (pPIL201) were derived from genomic DNA by PCR using primers *Tom1 Hect FW* and *Tom1 Rv* and *Hul4 Hect Fw* and *Hul4 Rv*, respectively and cloned into *BamH1* and *Xho1* sites of pGEX-4T-3.

Rsp5 was derived from pMIC81 and then cloned into *Nde1* and *EcoR1* sites of pET28a generating plasmid pPIL152 using primers *Rsp5 Fw nde1* and *Rsp5 Rv ecoR1*.

$Rsp5^{HECT}$ (pPIL157) was derived from pPIL152 by PCR amplification using primers *Rsp5 L420 Nde1* Fw and *Rsp5 Rv ecoR1.*

$^{HA}Rsp5^{HECT}$ (pPIL214) was derived from pPIL157 and cloned into *BamH1* and *EcoR1* sites of pYES2 using primers *HA-Rsp5 HECT Fw,* that included the sequence encoding for hemagglutinin (TATCCATATGATGTTCCAGATTATGCT), and *Rsp5 Rv ecoR1.*

$^{HA}Rsp5^{HECT\ C777A}$ (pPIL218) was derived from pPIL214 by single nucleotide mutagenesis using *Rsp5 c777a fw* and its reverse complement, being the cysteine 777 mutated to an alanine. pYES2 contains a URA3 marker gene.

pRS425 empty (pBC238) was provided by Dr. J. Vilardell.

Rpn10, together with the promoter of Rpn10 was derived from pRS424 and cloned into SacII site of pRS425 (which contains a LEU2 marker gene) resulting in pALI239.

pRS425 $Rpn10^{K84,268R}$ (pPIL245) was derived from pRS425 Rpn10 (pALI239) by two rounds of single nucleotide mutagenesis using *R10 K84R fw* and *R10 K268R fw* and their respective reverse complements, being the lysines 84 and 268 mutated to alanines.

UIM (pMJB1) corresponds to the UIM of Rnf114 from the amino acids 183 to 228 and was provided by Dr. M. Biljmakers.

Both DNA strands of cloned and mutated fragments in all plasmids described in this study were verified as correct by DNA sequence analysis using the 48-capillary *ABI 3730 DNA Analyzer* for capillary electrophoresis and fluorescent dye terminator detection.

A list with the plasmids and primers used is included in the Appendix section.

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APPENDIX

Rpn10 sequences

Rpn10 WT

```
        10          20          30          40          50
MVLEATVLVI DNSEYSRNGD FPRTRFEAQI DSVEFIFQAK RNSNPENTVG
        60          70          80          90         100
LISGAGANPR VLSTFTAEFG KILAGLHDTQ IEGKLHMATA LQIAQLTLKH
       110         120         130         140         150
RQNKVQHQRI VAFVCSPISD SRDELIRLAK TLKKNNVAVD IINFGEIEQN
       160         170         180         190         200
TELLDEFIAA VNNPQEETSH LLTVTPGPRL LYENIASSPI ILEEGSSGMG
       210         220         230         240         250
AFGGSGGDSD ANGTFMDFGV DPSMDPELAM ALRLSMEEEQ QRQERLRQQQ
       260      268
QQQDQPEQSE QPEQHQDK
```

Rpn10$^{195\text{-}268}$ sequence

```
195
GSSGMGAFGGSGGDSDANGTFMDFGVDPSMDPELAMALRLSMEEEQ
QRQERLRQQQQQDQPEQSEQPEQHQDK
```

Rpn10$^{208\text{-}268}$ sequence

```
208
DSDANGTFMDFGVDPSMDPELAMALRLSMEEEQQRQERLRQQQQQDQPEQSE
QPEQHQDK
```

Appendix

Rpn10$^{211\text{-}268}$ sequence

```
211
ANGTFMDFGVDPSMDPELAMALRLSMEEEQQRQERLRQQQQQDQPEQSEQPEQHQ
DK
```

Rpn10$^{196\text{-}204A}$ sequence

```
        10          20          30          40          50
MVLEATVLVI DNSEYSRNGD FPRTRFEAQI DSVEFIFQAK RNSNPENTVG
        60          70          80          90         100
LISGAGANPR VLSTFTAEFG KILAGLHDTQ IEGKLHMATA LQIAQLTLKH
       110         120         130         140         150
RQNKVQHQRI VAFVCSPISD SRDELIRLAK TLKKNNVAVD IINFGEIEQN
       160         170         180         190         200
TELLDEFIAA VNNPQEETSH LLTVTPGPRL LYENIASSPI ILEEGAAAAA
       210         220         230         240         250
AAAASGGDSD ANGTFMDFGV DPSMDPELAM ALRLSMEEEQ QRQERLRQQQ
       260      268
QQQDQPEQSE QPEQHQDK
```

Plasmids used in the present study

Plasmid	Details	Reference
pPIL106	GST-UbRpn10L85 (pGEX-4T-3)	This study
pPIL132	GST-^{PPAY}Rpn10 (pGEX-4T-3)	This study
pPIL133	GST-Rpn10$^{40\text{-}268}$ (pGEX-4T-3)	This study
pPIL137	GST-Rpn10$^{208\text{-}268}$ (pGEX-4T-3)	This study
pPIL141	GST-Rpn10$^{195\text{-}268}$ (pGEX-4T-3)	This study
pPIL142	GST-^{PPAY}Rpn10$^{195\text{-}268}$ (pGEX-4T-3)	This study
pPIL143	GST-Rpn10$^{211\text{-}268}$ (pGEX-4T-3)	This study
pPIL144	GST-^{PPAY}Rpn10$^{211\text{-}268}$ (pGEX-4T-3)	This study
pPIL152	6HIS- Rsp5 (pET28a)	This study
pPIL157	6HIS- Rsp5^{HECT} (pET28a)	This study
pPIL161	GST-Rpn10$^{195\text{-}268\ K268R}$ (pGEX-4T-3)	This study
pPIL162	GST-^{PPAY}Rpn10$^{195\text{-}268\ K268R}$ (pGEX-4T-3)	This study
pPIL163	GST-Rpn10$^{208\text{-}268\ K268R}$ (pGEX-4T-3)	This study
pPIL164	GST-^{PPAY}Rpn10$^{208\text{-}267\ K268R}$ (pGEX-4T-3)	This study
pPIL165	GST-Rpn10$^{211\text{-}268\ K268R}$ (pGEX-4T-3)	This study
pPIL166	GST-^{PPAY}Rpn10$^{211\text{-}268\ K268R}$ (pGEX-4T-3)	This study
pPIL167	GST-Rpn10$^{208\text{-}268\ UIM}$ (pGEX-4T-3)	This study
pPIL168	GST-^{PPAY}Rpn10$^{208\text{-}268\ UIM}$ (pGEX-4T-3)	This study
pPIL169	GST-Rpn10^{UIM} (pGEX-4T-3)	This study
pPIL171	GST-Rpn10$^{195\text{-}268\ UIM}$ (pGEX-4T-3)	This study
pPIL172	GST-^{PPAY}Rpn10$^{195\text{-}268\ UIM}$ (pGEX-4T-3)	This study
pPIL193	GST-S5a (pGEX2T(+))	Provided by F. Hanaoka
pPIL194	GST- Rpn10$^{196\text{-}204A}$ (pGEX-4T-3)	This study
pPIL195	GST- Rpn10$^{195\text{-}239}$ (pGEX-4T-3)	This study
pPIL200	GST- Tom1^{HECT} (pGEX-4T-3)	This study
pPIL201	GST- Hul4^{HECT} (pGEX-4T-3)	This study
pPIL202	GST- Nedd4 (pGEX)	Provided by B. Schulman
pPIL205	GST- Rpn10$^{M199A\text{-}G203A}$ (pGEX-4T-3)	This study
pPIL206	GST- Rpn10$^{195\text{-}254}$ (pGEX-4T-3)	This study
pPIL207	GST- Rpn10$^{195\text{-}261}$ (pGEX-4T-3)	This study
pPIL208	GST- S5a$^{193\text{-}201A}$ (pGEX-4T-3)	This study
pPIL209	GST- S5a $^{M196A\text{-}G200A}$ (pGEX-4T-3)	This study
pPIL210	GST- Rpn10$^{196\text{-}204S}$ (pGEX-4T-3)	This study

Appendix

pPIL211	GST- Rpn10 $^{M199S-G203S}$ (pGEX-4T-3)	This study
pPIL214	^{HA}Rsp5^{HECT} (pYES2)	This study
pPIL218	^{HA}Rsp5$^{HECT\ C777A}$ (pYES2)	This study
pPIL220	GST- Rpn10$^{DSD208-210\Delta}$ (pGEX-4T-3)	This study
pPIL222	Rpn10$^{DSD208-210\Delta}$ (pRS424)	This study
pPIL225	Rpn10$^{196-204A}$ (pRS424)	This study
pBC238	pRS425	Provided by J. Vilardell
pALI239	Rpn10 (pRS425)	This study
pPIL245	Rpn10^{K84,268R} (pRS425)	This study
pMIC26	GST- Rpn10 (pGEX-4T-3)	Crosas *et al,* 2006
pMIC81	Rsp5 (pGEX)	Provided by J. Huibregtse and S. Polo
pMIC93	Rpn10 (pRS424)	Provided by H. Fu
pMBJ1	GST-UIM (pGEX-5X-2)	Capon *et al,* 2008

Oligonucleotide primers used in the present study

Only forward sequences of mutagenic primers are shown.

Name	Sequence
R10L85 Fw	CGTACGGTACCCTGCATATGGCCACT
R10L85 Rev	CGGTAGAATTCCTATTTGTCTTGGTGT
UbR10L85 Fw	TAAGACTAAGAGGTGGTGGAGGTGG
R10M1PPAY fw	GACTGAATTCCCCTCCTGCATACATG
R10K40 Fw	GACTGAATTCCAAGAGAAACAGCAATCCTGAGAATACAG
R10D208 Fw	GACTGAATTCCTGGGATTCCGATGCCAATGGCACATTTATG
R10K268 Rev	CGGTAGTCGACCTATCTGTCTTGGTGTTGTTC
R10 G195 fw	GACTGAATTCCTGGGGATCCTCCGGTATGGGCGCCTTTGGT
R10 A211 fw	GACTGAATTCCTGGGCCAATGGCACATTTATGGACTTCGG
R10G195ppay fw	GACTGAATTCCTGGCCTCCTGCATACGGATCCTCCGGTATGG
R10D208ppay Fw	GACTGAATTCCTGGCCTCCTGCATACGATTCCGATGCCAATGG
R10A211ppay fw	GACTGAATTCCTGGCCTCCTGCATACGCCAATGGCACATTTAT
R10 uim Fw	CATCAATGGACCCAGAAAACAACAACAACAACCGTCTGTCTAT
R10k268r fw (pRS424)	CAACACCAAGACAGATAGGTCGACTCGAGCGGC
R10 link Ala fw	CATAATTCTCGAAGAAGGAGCAGCAGCAGCAGCAGCAGCAGC AGCATCTGGCGGTGATTCCGATG
R10G195E239+K fw	CTGTCTATGGAAGAAGAGAAATAAAGACAGGAAAGGTTAAG
R10 MG Ala fw	GAAGAAGGATCCTCCGGTGCGGGCGCCTTTGCTGGGTCTGG CGGTGATTC
R10G195D254+K - Fw	GCAGCAACAACAACAAGATAAATAAGAGCAGTCTGAACAGCCT GAAC
R10G195Q261+K Fw	CAGCCTGAGCAGTCTGAACAGAAATAACAACACCAAGACAAAT AG
R10 link Ser fw	GAAGAAGGATCCTCCTCTTCGTCCTCCTCTTCTTCGTCTGGCG
R10 mg Ser fw	GAAGGATCCTCCGGTTCGGGCGCCTTTTCTGGGTCTGGCGGT
R10G195Δ208-210 Fw	CTTTGGTGGGTCTGGCGGTGCCAATGGCACATTTATGGAC
S5a link Ala fw	CGATTTTGGCTGGTGAAGCTGCTGCCGCGGCGGCTGCTGCTG CCAGTGACTTTGAATTTG
S5a mg Ala fw	GTGAAGGTGGTGCCGCGCTGGGTCTTGCTGCCAGTGACTTTG
Ubp2-Kan Fw	GTGGTAATTAAAAAGAAAGCTTTTGTTCAAGGTTAAGAAGGTAT AAGGAAGACATGGAGGCCCAGAATACCCTCC
Kan-Ubp2 Rv	GGTACTTATGGCAATAGTGACATTTTACATAAACTCTTCATTGA CTAAGACAGTATAGCGACCAGCATTCACATACG
Rsp5 Rv-ecoR1	CGGTAGAATTCTCATTCTTGACCAAACCCTATGG
Rsp5 Fw nde1	TACGTACGCATATGCCTTCATCCATATCCGTCAAG
Rsp5 L420 -Nde1 Fw	TACGTACGCATATGCTTCCATCATCGCTAGACC

Appendix

HA-Rsp5^{HECT} Fw	CGAGGGATCCATGTATCCATATGATGTTCCAGATTATGCTCTTC CATCATCGCTAGACC
Rsp5 c777a fw	GCCAAAATCTCACACAGCTTTTAACAGAGTTGATTTG
Tom1 Hect FW	CGT ACGGATCCATGAGTGGTCCTTTCGCATTATTG
Tom1 Rv	CGG TAC TCG AGT CAG GCA AGA CCA AAC CCT TCA TGC
Hul4 Hect Fw	CGTACGGATCCATGGAACATGAAGCTGAACAGGC
Hul4 Rv	CGGTACTCGAGTTAGCGGAACCCGTAACCTTCAG
HA-Tom1 HECT Fw (pYES)	CGAGAAGCTTATGTATCCATATGATGTTCCAGATTATGCTAGTG GTCCTTTCGCATTATTG
Tom1 Rv (Xba)	CGGTATCTAGATCAGGCAAGACCAAACCCTTCATGC
HA-Hul HECT Fw hindiii (pYES)	CGAGAAGCTTATGTATCCATATGATGTTCCAGATTATGCTGAAC ATGAAGCTGAACAGGC
Hul4^{HECT} Rv	CGGTATCTAGATCATTAGCGGAACCCGTAACCTTCAG
R10 K268R fw (pRS425)	AGCCTGAACAACACCAAGACAAATAGTAGTGTACAAAAATATCT ATATGGGTCAT
R10 K84R fw (pRS425)	CGCAGATCGAGGGTAGGCTGCATATGGCCAC