AN INTRINSICALLY DISORDERED REGION OF RPN10 PLAYS A KEY ROLE IN RESTRICTING UBIQUITIN CHAIN ELONGATION IN RPN10 MONOUBIQUITINATION

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Abstract

Despite being a common mechanism in eukaryotes, the process by which protein monoubiquitination is produced and regulated *in vivo* is not completely understood. We present here the analysis of the process of monoubiquitination of the proteasomal subunit Rpn10, involved in the recruitment of polyubiquitinated substrates. Rpn10 is monoubiquitinated *in vivo* by the Nedd4 enzyme Rsp5, and this modification impairs the interaction of Rpn10 with substrates, having a regulatory effect on proteasome function. Remarkably, a disordered region near the ubiquitin interacting motif of Rpn10 plays a role in the restriction of the polyubiquitin extension activity of Rsp5. Mutations in this disordered region promote ubiquitin chain extension of Rpn10. Thus, our work sheds light on the molecular basis and the functional relevance of a type of monoubiquitination that is driven by the substrate. Moreover, we uncover a putative role for disordered regions in modulating ubiquitin-protein ligation.

Introduction

A fundamental input of complexity in ubiquitin-regulated processes is the type of intermediate generated by covalently linked ubiquitin in the process of protein ubiquitination. Ubiquitination of proteins is produced through an enzymatic cascade involving ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin ligase (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 [1,2]. Ubiquitination can produce monoubiquitinated, multi-monoubiquitinated and polyubiquitinated proteins. Moreover, polyubiquitin chains can be generated by linkages at seven different lysine residues (K6, K11, K27, K29, K33, K48 and K63) as well as at the N-terminal methionine of ubiquitin, which produce topologically and functionally distinct ubiquitin chains [3–5]. Distinct types of ubiquitin linkages impose markedly different regulatory paths in modified proteins. Thus, lysine 48 and lysine 11 polyubiquitination target proteins to the proteasome. Lysine 63 polyubiquitination targets proteins to vacuolar transport and trafficking, but also exerts a non-degradative signal in diverse processes, such as DNA-damage response, ribosomal regulation and spliceosome function [3,6].

Monoubiquitination is a widespread mechanism of protein function regulation and signal transduction that is found in all eukaryotes. Usually, monoubiquitination changes the capacity of proteins to interact with partners, leading to either a gain or loss of interaction, thus acting as a rapid and versatile mechanism to regulate cellular processes. Monoubiquitination plays key roles in the regulation of the DNA damage response, histone function, gene expression, endocytosis, lipid droplet clustering, protein secretion and nuclear transport [7–11].

The proteasome is a multi-subunit complex involved in the turnover of a large fraction of cellular proteins. The proteasome is composed of a core particle (20S or CP) and a regulatory particle (19S or RP). While the CP is involved in the proteolytic digestion of substrates, the RP is involved in receiving polyubiquitinated protein substrates, processing and translocating them to the CP in an unfolded state [12]. Notably, the factors involved in the recruitment of substrates to the proteasome are highly conserved in evolution. Two mechanisms have been described until now. First, the proteasome contains three intrinsic receptors, Rpn10/S5a, Rpn13 and Dss1 [13–15]. Rpn10/S5a proteins bind polyubiquitin by means of ubiquitin interacting motifs (UIMs), Rpn13 subunits through a pleckstrin-like ubiquitin receptor and Dss1 through two ubiquitin-binding sites [13–15]. Second, a set of polyubiquitin receptors, including UBL-UBA proteins Rad23, Dsk2 and Ddi1, has been shown to interact transiently with the proteasome [16,17] and a substrate shuttling role has been proposed [18–24].

The receptor Rpn10/S5a has been shown to be involved in the proteasomal recruitment and degradation of multiple substrates in yeast and in mammals, such as cyclin B, Sic1, Gcn4, p53 and Mdm2 [25–28]. Recently, Rpn10 monoubiquitination has been defined as a novel level of proteasome pathway regulation in yeast and metazoans [29–31].

Rpn10 is mainly ubiquitinated at lysines K84, in the VWA domain, and K268, at the C terminus of the protein. Moreover, the UIM is required for the reaction of Rpn10 ubiquitination [23,30]. In a monoubiquitinated state, Rpn10 shows an inactive UIM, resulting in a decrease of Rpn10-mediated protein degradation by the proteasome [30]. Recently, it has been shown that human Rpn10 is also monoubiquitinated in the proteasome [32–34]. Furthermore, monoubiquitination of Drosophila Rpn10 blocks the Rpn10-Dsk2 interaction that is mediated by the Rpn10 UIM [35], underscoring the potential of Rpn10 monoubiquitination in the regulation of proteasome-mediated protein degradation.

Despite the fact that monoubiquitination plays a fundamental role in biology, the principles by which proteins are monoubiquitinated *in vivo* are not completely understood. Human Nedd4 ubiquitin ligase family members have been shown to promote monoubiquitination of ubiquitin binding domain (UBD)-containing proteins [36–38]. Moreover, a ‘fold-back’ model, based on a structural conformational change in the monoubiquitinated protein promoted by the UBD-ubiquitin interaction, has been proposed in several papers [30,36–38]. However, a correlation between structural properties and monoubiquitination of the substrate has never been established.

We characterize here the process of Rpn10 ubiquitination by Rsp5, NEDD4 ubiquitin-ligase family member, [30]. We have observed that an intrinsically disordered region flanking the UIM of Rpn10 acts as a downregulator of the ubiquitin-chain building activity of Rsp5, and, together with the UIM, promotes monoubiquitination. Moreover, mutants that affect the properties of the disordered region cause a change in the pattern of Rpn10 ubiquitination. Therefore, our data define the principles of a

substrate-driven monoubiquitination, a type of monoubiquitination, which is not controlled by properties of the enzymatic factors involved in the reaction, but instead, is regulated by intrinsic features of the substrate. Moreover, our results provide a link between protein unstructured regions and the regulation of ubiquitin ligase activity, defining a novel function for unstructured protein domains.

RESULTS

Slow growth defect caused by increased levels of monoubiquitinated Rpn10

In a previous work, it was shown that Rsp5 monoubiquitinates Rpn10 and that monoubiquitinated Rpn10 is inactive as a polyubiquitin receptor, causing a decrease in proteasome activity, thus constituting a mechanism of polyubiquitin receptor regulation [30]. Moreover, the levels of Rpn10 monoubiquitination are tightly controlled *in vivo*, and high levels of Rpn10-Ub (Rpn10 including C-terminally fused ubiquitin) cause growth deficiency in yeast [30]. 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 [39–42]. Rpn10 contains a UIM that is essential for its Rsp5-dependent monoubiquitination and lacks PPXY motifs [30]. Therefore, we generated a Rsp5 construct ($Rsp5^{HECT}$), encompassing residues 420 to 809 that include the catalytic HECT domain but not the WW motifs, and tested the activity of this $Rsp5^{HECT}$ in reactions of Rpn10 monoubiquitination. We observed that full length Rsp5 and $Rsp5^{HECT}$ exhibited similar activities in this reaction, indicating that the HECT domain is sufficient for Rpn10 monoubiquitination (Figure 1A). Moreover, we prepared the HECT version of the ubiquitin ligase Tom1 ($Tom1^{HECT}$) to compare the activity of this form to that of $Rsp5^{HECT}$. 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 autoubiquitination (Figure 1B), $Rsp5^{HECT}$ was much more competent in ubiquitinating Rpn10 (Figure 1C). Our finding that the HECT domain of Rsp5 alone is sufficient for monoubiquitination of Rpn10 gave us the opportunity to analyze *in vivo* the impact of Rsp5-dependent Rpn10 monoubiquitination without affecting the regulation of numerous PPXY-dependent protein substrates *in vivo* [39,41,43–45].

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. With this approach, we observed that a fraction of Rpn10 was efficiently monoubiquitinated after several hours of galactose treatment and $Rsp5^{HECT}$ induction (Figure 1D). We next tested the effect of increased Rpn10 monoubiquitination on yeast growth. When we induced Rpn10 monoubiquitination by over-expressing $Rsp5^{HECT}$ (Figure 1E, lane 7), we could observe that a severe slow-growth phenotype was produced at 22ºC (Figure 1F). Notably, both Rpn10 monoubiquitination and the deleterious phenotype were

completely dependent on Rsp5^{HECT} activity, since the catalytically inactive mutant Rsp5^{HECTC777A} abrogated the effect (Figure 1E, lane 10; and 1F, rows 1 and 2). To test the specificity of the phenotype caused by Rsp5^{HECT} induction, we prepared the Rpn10^{K84,268R} mutant, involving the two lysine residues targeted for ubiquitination [30]. Remarkably, when we performed the induction assay in the presence of this mutant, monoubiquitination was totally impaired (Figure 1E, lane 8), and a partial rescue of the slow growth phenotype was observed (Figure 1F, row 3), indicating the functional impact of Rsp5-induced Rpn10 monoubiquitination on yeast growth.

Minimal sequence requirements for Rpn10 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 [46,47]. The UIM-containing sequence is found in the context of an alpha-helix that is flanked by highly flexible linkers [48–50]. Moreover, the C-terminal end contains lysine residues that are modified by ubiquitin, as shown in *S. cerevisiae* and *D. melanogaster* [30,51]. With this information, we designed a Rpn10 version, Rpn10$^{195-268}$, which contains the ubiquitin-modified lysine residue K268 [30], lacks the VWA domain, and only includes the C-terminal arm of Rpn10. We observed that this version of Rpn10 was efficiently monoubiquitinated by Rsp5 (Figure 2A), showing that the VWA domain is dispensable for the reaction of monoubiquitination. Indeed, the fragment Rpn10$^{195-268}$, lacking the VWA domain but containing the UIM, behaved as a very good substrate of Rsp5, generating prominent mono- and di-ubiquitinated forms (Figures 2A and B) in a reaction dependent on the presence of the E3 (Figure 2B). Therefore, the reaction of Rpn10 monoubiquitination can be produced in a Rpn10 fragment of 73 amino acid residues, containing flexible linkers with high disposition to disorder, only interrupted by a helicoidal structure that contains the UIM (Supplementary Figure 1; [48,50,52]).

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 3A), 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 (Figures 3B).

As observed for other NEDD4 enzyme substrates [36,38], the UIM is necessary for the reaction of Rpn10 ubiquitination catalyzed by Rsp5 [30]. Therefore, the decreased monoubiquitination of Rpn10$^{211-268}$ (Figure 3B, lane 3) 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$^{195-268UIM}$ (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 Rpn10$^{195\text{-}268UIM}$ did not bind polyubiquitin (Figure 3C). These results indicated that there is no loss of ubiquitin binding of the Rpn10$^{211\text{-}268}$ mutant, and suggested a role for the region between amino acids D208 and A211 in the monoubiquitination of Rpn10.

Next, 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 3A), as substrates in monoubiquitination reactions. We observed that Rpn10$^{195\text{-}261}$ and Rpn10$^{195\text{-}254}$ were efficiently monoubiquitinated (Figure 3D, lanes 2 and 3), whereas Rpn10$^{195\text{-}239}$ was not (Figure 3D, lane 4). 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 3E). Therefore, the loss of monoubiquitination of the Rpn10$^{195\text{-}239}$ fragment could be due to a dysfunctional UIM. Moreover, the proximity of the lysine residue to the ubiquitin interacting surface in the Rpn10$^{195\text{-}239}$ fragment (see Figure 3A), could be an additional cause of the absence of monoubiquitination.

Together, these results suggest that the region in Rpn10 between positions D208 and A211 plays a role in the process of monoubiquitination that is independent of the ubiquitin-binding capacity of the UIM.

The unstructured linker between the VWA and the UIM of Rpn10 plays a role in monoubiquitination

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 4A). We deleted positions D208, S209 and D210 (Rpn10$^{DSD208\text{-}210\Delta}$), and we checked this form in an ubiquitination reaction. We observed that this deletion had an effect on the reaction, producing an oligoubiquitin/polyubiquitin signal, not observed when using ubiquitin K0 that cannot form poly-ubiquitin chains (Figures 4B and C). The C-terminal arm of Rpn10 proteins is highly prone to show a disordered structure (Supplementary Figure 1), only interrupted by a helicoidal structure that contains the UIM [48,50,52]. Positions D208, S209 and D210 are part of an unstructured linker between the alpha helix 6 of the VWA domain and the UIM-containing alpha helix 7 (Figure 4D; [48,53]).

Notably, this linker contains a Glycine-rich region from positions G195 to G207 (Figure 4D). We generated mutants with a block substitution of the region S196-G204 by alanine (Rpn10$^{196\text{-}204A}$) or serine residues (Rpn10$^{196\text{-}204S}$) in the context of full-length Rpn10 (Figure 4A). Additionally, we generated point mutations at two different conserved positions, M199 and G203 to alanine or serine (Rpn10^{M199A,G203A}, and Rpn10^{M199S,G203S}) (Figure 4A). Subsequently, these mutants were tested in Rsp5-dependent ubiquitination reactions. We observed that the Rpn10$^{196\text{-}204A}$ mutant underwent an increase in polyubiquitin extension, as compared to full-length wild-type Rpn10 (Figures 4E, compare lanes 1 and 2;

and 5A). The Rpn10^{M199A,G203A}, Rpn10$^{196-204S}$ and Rpn10^{M199S,G203S} mutants showed an intermediate effect (Figure 4E, lanes 3-5), which appears to be similar to the one observed with Rpn10$^{DSD208-210\Delta}$. We further evaluated the ubiquitination of Rpn10$^{196-204A}$ by performing a reaction with ubiquitin 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$^{196-204A}$ are not the result of more extensive monoubiquitination but of a true increase in ubiquitin chain elongation (Figures 5B and C). Importantly, this polyubiquitin extension 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 5A). Considering that Rpn10$^{196-204A}$ was the substrate to undergo the strongest polyubiquitination, we conclude that the disordered region between amino acids S196 to G204 (see Figure 4A and D) plays a role in preventing polyubiquitin extension in Rpn10, although residues D208-D210 also contribute.

Does the conformation of the disordered linker play a role in preventing chain extension during the process of Rpn10 monoubiquitination? The 'fold-back' model [30,36–38] proposes that, in UIM-containing proteins that undergo monoubiquitination, the capacity of the UIM region to associate with the monoubiquitinated surface would avoid the process of polyubiquitin extension. In Rpn10, our data suggests that the properties of the linker between the VWA and the UIM would facilitate this 'fold-back' interaction. Furthermore, mutations such as Rpn10$^{196-204A}$, which change the properties of the linker, likely decreasing its flexibility (Supplementary Figure 2A and B), elicit a process of polyubiquitin formation. In order to evaluate the effect of mutations in the linker region on its capacity to fold towards the VWA, we performed measurements of fluorescence anisotropy of Rpn10 WT and Rpn10$^{196-204A}$ mutant. To prepare these assays, we took advantage of the fact that Rpn10 does not contain tryptophan residues. Thus, we placed a tryptophan residue in the position 205 of the sequence (mutation S205W) in both Rpn10^{WT} and Rpn10$^{196-204A}$ forms, to generate the Rpn10^{S205W} and the Rpn10$^{196-204A-S205W}$ versions of Rpn10 (Figure 5D). These versions would provide us information of the C-terminal region of the linker, near the mutated segment. Fluorescence anisotropy provides a measure of the capacity of the tryptophan residue to change its orientation in the short time between light excitation and fluorescence emission. If the side chain containing the tryptophan is locked with respect to the neighbor domain, the reorientation will be that of a slowly tumbling domain. On the contrary, if the linker is not tethered to the domain, reorientation will be much faster and a strong decrease in anisotropy will be observed. The anisotropy of the Rpn10^{S205W} construct was 0,069±0,0024, significantly higher than the one obtained with the Rpn10$^{196-204A-S205W}$ form (0,058±0,0027). Measurements were repeated a total of 30 times in four independent samples. These results suggest that the C-terminal arm of Rpn10 interacts with the VWA domain through the S196-G204

region, since the interaction is abrogated by the replacement of residues S196-G204 by alanines. To our view, these differences could explain that distinct ubiquitination output of the two forms.

Do the mutations in the unstructured linker that lead to polybuquitination have an effect on Rpn10 function *in vivo*? To address this point we analyzed the turnover of the proteasome substrate CPY*, an unstable mutant form of CPY, in cell cultures. We first evaluated the influence of Rpn10 in CPY* degradation rates. We observed that in wild type cells CPY* showed a half-life of less than 15 minutes, and that in the absence of Rpn10, degradation was slightly delayed (Figure 6A). Next, we compared the CPY* turnover in *rpn10*Δ cells transformed with either empty vector, wild-type Rpn10 or the Rpn10 mutant showing the highest level of polyubiqutination, $Rpn10^{196\text{-}204A}$. We could observe that, whereas wild type Rpn10 notably rescued CPY* degradation rates, the $Rpn10^{196\text{-}204A}$ mutant only had a partial effect (Figure 6B). This suggests that aberrant polyubiquitination of Rpn10 affects its function.

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* [29,30], and that the Rpn10 orthologue in humans (S5a) has been shown to be mono- and diubiquitinated in the proteasome [40], 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 7A; lanes 4 and 7). Moreover, we analyzed point mutations at positions M196 and G200, and a substitution of the G193-G200 segment ($S5a^{M196A,G200A}$ and $S5a^{193\text{-}200A}$ mutants, respectively), corresponding to Rpn10 mutants $Rpn10^{M199A,G203A}$ and $Rpn10^{196\text{-}204A}$, respectively. 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 7A, lanes 4 and 7), $S5a^{M196A,G200A}$ and $S5a^{193\text{-}200A}$ showed increased polyubiquitination at high concentrations of E2 (Figure 7A, compare lanes 5 and 6 with lanes 8 and 9). Therefore, analogously to the results observed in Rpn10, the region flanking the UIM in S5a has an influence on the ubiquitination pattern.

DISCUSSION

The complexity of the ubiquitin 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 [4,54]. In the present work we have uncovered a link between the capacity of the proteasomal receptor Rpn10 to undergo monoubiquitination and the presence of an unstructured region in the Rpn10 protein.

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. Notably, by expressing $Rsp5^{HECT}$ in cells we have been able to produce a slow-growth phenotype, which is dependent on the monoubiquitination of Rpn10 (Figure 1E and F). This result is in agreement with that observed by expressing a Rpn10-ubiquitin chimera, in a previous work [30]. 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 [30], 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 [55]. We envision inactivation of proteasome receptors as a potential biomedical approach for the inhibition of proteasome activity.

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 [56]. 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 [57]. 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 [7–9]. Another interesting model suggests that the activity of deubiquitinating enzymes (DUBs) processes polyubiquitinated proteins to produce monoubiquitination [58]. Moreover, the process of monoubiquitination can be induced by an external protein cofactor that modulates enzyme processivity [59]. Figure 7B (sections A-D) contains different possible models of monoubiquitination, focused 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 7B, section E and 7C). Our data, based on Rpn10, suggests that an intrinsically disordered linker

between the last alpha helix of the VWA domain (helix 6) and the alpha helix that contains the UIM (helix 7; see Figure 4D) plays a role in the process of monoubiquitination. Mutations that change the properties of this region cause a change in the processivity of Rsp5, the ubiquitin-protein ligase involved in the reaction of ubiquitination, promoting polyubiquitination (Figures 4 and 5).

The polyubiquitin chain extension observed in $Rpn10^{196\text{-}204A}$ is probably due to a change in the capacity of the C-terminal region of Rpn10 to fold towards the globular VWA domain, as suggested by the values of fluorescence anisotropy. In the $Rpn10^{196\text{-}204A}$ mutant, the linker could exhibit less capacity to interact with the VWA domain, decoupling the VWA and the UIM. A similar scaffolding effect of neighbor domains has been observed in other intrinsically disordered linkers [60]. These results represent the first reported experimental evidence, at the structural level, substantiating the 'fold-back' model. Our findings do not completely exclude a change in the interaction with Rsp5 as an alternative explanation of the distinct ubiquitination outputs observed in our mutants. However, the fact that in the catalysis, Rpn10 interacts with auto-polyubiquitinated Rsp5 loaded with a ubiquitin moiety in its active site (Figure 1B) [30,42] , makes this alternative hypothesis more unlikely. We have observed that truncations of the linker do not change the capacity of the UIM to interact with polyubiquitin chains (K48 and K63) (Figures 3C and E).

The control of the activity of substrate receptors such as 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 [30] and with the ubiquilin type protein Dsk2 [31]. Remarkably, Rpn10 monoubiquitination has been found in distant groups such as yeast, drosophila and humans [30–34], suggesting that this level of regulation is evolutionarily conserved and functionally relevant. Moreover, Rpn10 can be regulated by its proteasome association status, existing in both proteasome-bound and proteasome-unbound forms *in vivo* [29,30,61,62]. 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 [31,61]. 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 [62].

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 [32]. 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 an intramolecular interaction with the ubiquitin moiety linked to the substrate [30,36–38]. Although Rpn10 is mostly monoubiquitinated at one position [30], three more lysines can be modified with a ubiquitin molecule (multi-monoubiquitination) suggesting that the interaction between the UIM and the substrate-bound ubiquitin 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 favored and, consequently, the UIM is no longer accessible and no chain extension can be built in Rpn10 by Rsp5. Analogously, in a previous study, the UIM of the transcription factor Met4 was found to both restrict chain elongation on Met4 and prevent the recognition and proteolysis of ubiquitinated Met4 by the proteasome [63]. 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 fold-back conformation and, thus, preventing ubiquitin chain extension, has never been established.

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 (Supplementary Figure 3A) suggests a conserved mechanism facilitating monoubiquitination. 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* [36–38]. We observed that these proteins show high disposition to disorder in regions flanking the UIM (Supplementary Figure 3B). 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.

MATERIALS AND METHODS

Plasmids – Rpn10 and Rpn10 mutants were made in the *Escherichia coli* expression vector pGEX-4T-3 and cloned in pRS425 to be expressed from their own promoter in *S. cerevisiae* (strain S72). Rpn10$^{195\text{-}268\ UIM}$ had residues 228 to 232 mutated to asparagines. Rpn10$^{M199A\text{-}G203A}$ and Rpn10 $^{M199S\text{-}G203S}$ had M199 and G203 Ala or Ser substitutions, respectively. Rpn10$^{196\text{-}204A}$ and Rpn10$^{196\text{-}204S}$ had from residues S196 to G204 Ala or Ser block substitutions, respectively. Rpn10$^{DSD208\text{-}210\Delta}$ had residues D208 to D210 deleted.

Rpn10^{K84,268R} mutant had residues K84 and K268 mutated to arginines. S5a and S5a mutants were cloned in pGEX-4T-3 vector. S5a$^{M196A-G200A}$ had M196 and G200 Ala substitutions. S5a$^{193-200A}$ had from residues 193 to 200 Ala block substitutions. Rsp5^{HECT} was cloned in a pET28a vector to be expressed in *E. coli*. ^{HA}Rsp5^{HECT} and ^{HA}Rsp5^{HECTC777A} were cloned in a pYES plasmid to be overexpressed under control of the GAL1 promoter. The UIM of Rnf114 was cloned in a pGEX-4T-3. Tom1^{HECT} was made in the *Escherichia coli* expression vector pGEX-4T-3.

Expression and purification of GST-fusion proteins in E.coli– Glutathione S-transferase fusion vectors (pGEX-4T-3) were used to express and purify as follows: bacterial cultures (500 mL) were grown to an OD_{600} of 0.7, induced with 500 µM isopropylthiogalactoside (IPTG) for 15 h at 20 ºC, 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 (Figure 2), GST-Rpn10$^{195-268}$ was digested with biotinylated thrombin (Novagen) (1U enzyme for 10 µg target). The reaction was incubated overnight at 4 ºC. The efficiency of cleavage was determined by SDS-PAGE analysis. Thrombin was removed with benzamidine beads (Amersham Biosciences) according to the manufacturer's instructions.

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, 1 ml of Ni-NTA beads was 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.

Assays of Rpn10 ubiquitination in vitro – Rpn10 *in vitro* ubiquitination reactions (50 µL) contained 50 nM of GST-Rpn10 or versions of Rpn10, 174 nM of human activating E1, 5.7 µM of GST-Ubc4, 50nM of GST-Rsp5 or 6his-Rsp5^{HECT}, 37.5 µM of ubiquitin, except for reactions in Figure 2 that contained 150 nM GST-Rsp5. Reactions were incubated for 3 hours or at the indicated times. Different types of ubiquitins were used: recombinant wild-type, ubiquitin K63 only, ubiquitin K48 only, ubiquitin K0

(BostonBiochem) and methylated ubiquitin (Enzo Life Sciences). Rpn10 ubiquitination *in vitro* reactions were also performed using Tom1^{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 6his-UbcH5b (BostonBiochem), 20 nM of GST-Nedd4, and 37.5 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 ° C (for S5a reactions) for 3 hours. Reactions were stopped by the addition of reducing SDS-PAGE loading buffer and analyzed by western blotting 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 the Rpn10 fragments (in Figure 3C and E) 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 3C) was used as a positive control and the GST protein or the beads alone as negative controls.

Yeast Methods and Media – The strains used in this study are listed in Table 1. Strain transformations were performed following standard techniques [64]. 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 [64], 2% dextrose (SDC) or, if necessary, 1% galactose (SGRC). For plasmid selection, synthetic media lacking uracil or leucine were prepared. Samples taken from growing cultures were normalized by optical density at 600 nm using Eppendorf Biophotometer plus (Eppendorf). Spot assay was prepared after 4 hours induction with galactose in liquid and agitation at 30ºC (Figure 1F). 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. Images of colony spot assays were taken using GeneGenius Bioimaging System (Syngene).

TABLE 1

Yeast strains used in this study

Strain	Genotype	Source
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf
S72	*Rpn10::KanR*	

	(based on BY4741	
FY56	*MATa ura3-52, his4-912-R5, lys2Δ 128*	Beaudenon Lab

Analysis of Rpn10 in yeast – Yeast wild-type and *rpn10*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 (Figure 1). Cells were harvested, resuspended with buffer composed of 50 mM Tris-HCl (pH 7.8), 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 analyzed by immunoblotting against Rpn10 and Hemagglutinin.

Fluorescence Anisotropy measurements – Serine 205 of both Rpn10 and Rpn10$^{196\text{-}204A}$ was mutated to a tryptophan, generating Rpn10^{S205W} and Rpn10$^{196\text{-}204A\text{-}S205W}$, respectively. The two proteins were expressed in pGEX plasmids and the N-terminal GST tag was cleaved. Fluorescence measurements were done at 4ºC in a PTI fluorimeter. Tryptophan fluorescence was excited with plane polarized light at 290 nm, to minimize tyrosine excitation and we measured the fluorescence anisotropy by simultaneously recording the polarized emission at 347 nm in parallel and perpendicular planes using two detectors.

Antibodies – Polyclonal antibodies to Rpn10 were raised in rabbits against full-length protein and second bleed antiserum was used [30]. 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. Anti-pgk1 antibody produced in mouse was obtained from Invitrogen. 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.

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) [65]. 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.

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FOOTNOTES

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FIGURE LEGENDS

FIGURE 1. Slow growth phenotype caused by Rpn10 monoubiquitination *in vivo*

A Reactions of ubiquitination of Rpn10 incubated with Rsp5 full-length (F) or Rps5^{HECT} (H). Long and short exposures of the film are shown.

B Autoubiquitination reaction of Rps5^{HECT} and Tom1^{HECT} using Ub WT.

C Rpn10 ubiquitination assays using $Rsp5^{HECT}$ or $Tom1^{HECT}$ at different concentrations analyzed by western blotting using an Rpn10 antibody.

D 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.

E *rpn10*Δ strain (S72) carrying plasmids $Rpn10^{WT}$, $Rpn10^{K84,268R}$, from its own promoter, or the empty plasmid and either the $^{HA}Rsp5^{HECT}$ or $^{HA}Rsp5^{HECTC777A}$ 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.

F The same cultures shown in panel E were diluted to an OD_{600} = 0.04, spotted in 5-fold serial dilutions and grown at 22ºC, as shown.

FIGURE 2. Monoubiquitination is efficiently catalyzed on the C-terminal end of Rpn10

A Ubiquitination reaction of the $Rpn10^{195-268}$ fragment using wild-type (left) and ubiquitin mutant - without lysine residues (K0) (right) incubated with Rsp5 full-length.

B $Rpn10^{195-268}$ ubiquitination reaction in which Uba1, Ubc4, Rsp5 full-length and ubiquitin were added as indicated.

FIGURE 3. Rpn10 truncation analysis shows the indispensable regions of Rpn10 to undergo monoubiquitination

A Schematical representation of the C terminal fragments of Rpn10 used for ubiquitination reactions and binding assays showing the UIM within the alpha helix.

B Ubiquitination reactions of equimolar amounts of $Rpn10^{195-268}$, $Rpn10^{208-268}$ and $Rpn10^{211-268}$ using different concentrations of Rsp5 full-length.

C Binding assay of K63-only polyubiquitin chains and K48-only 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. 1/20 of the input used per binding was loaded. Bound material was eluted and analyzed by Ubiquitin western blotting. Lower panel shows similar amounts of the proteins bound to the beads in a Coomassie blue staining.

D Ubiquitination reactions of $Rpn10^{195-268}$, $Rpn10^{195-261}$, $Rpn10^{195-254}$ and $Rpn10^{195-239}$ incubated with Rsp5 full-length.

E Binding assay of GST fusion proteins Rpn10$^{195-268}$, Rpn10$^{195-254}$ and Rpn10$^{195-239}$, immobilized in beads to K63-only polyubiquitin chains (input). 1/20 of the input used per binding was loaded. Bound material was eluted and analyzed by Ubiquitin western blotting. Right panel, Coomassie of the amounts of fusion proteins used in each assay.

FIGURE 4. Altering the unstructured linker flanking the UIM promotes polyubiquitination

A Diagram of the Rpn10$^{195-268}$ and full-length versions of Rpn10 including the mutations analyzed.

B Time course *in vitro* ubiquitination assay of Rpn10 and Rpn10$^{DSD208-210\Delta}$ incubated with Rsp5 full-length. Points at indicated times were taken and analyzed by Rpn10 western blotting. Asterisk, unspecific band. Long and short exposures of the film are shown.

C Time course *in vitro* ubiquitination reaction of Rpn10 $^{DSD208-210\Delta}$ using wild-type ubiquitin or Ub K0 and Rsp5 full-length. Points at indicated times were taken and analyzed by Rpn10 western blotting.

D Residues from E183 to L234 are shown in *Saccharomyces cerevisiae*. First and last boxes correspond to the residues within the alpha helix 6 of the VWA domain and the UIM-containing alpha 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 methionine 199 and glycine 203 that we mutated.

E Ubiquitination reactions of Rpn10^{WT}, Rpn10$^{196-204A}$, Rpn10$^{M199A-G203A}$, Rpn10$^{196-204S}$ and Rpn10$^{M199S-G203S}$ using Rsp5 full-length.

FIGURE 5. Rpn10$^{196-204A}$ mutant: effect on ubiquitination

A Time course *in vitro* ubiquitination assay of Rpn10 and Rpn10$^{196-204A}$ incubated with Rsp5 full-length. Points at indicated times were taken and analyzed by Rpn10 western blotting. Asterisk, unspecific band.

B Time course *in vitro* ubiquitination reaction of Rpn10$^{196-204A}$ using wild-type ubiquitin and Ub K0 and Rsp5 full-length. Points at indicated times were taken and analyzed by Rpn10 western blotting.

C Ubiquitination reaction of Rpn10$^{196-204A}$ mutant using wild-type and methylated ubiquitin and Rsp5 full-length. Asterisks, unspecific bands.

D Schematical representation of Rpn10^{S205W} and the Rpn10$^{196-204A-S205W}$ versions of Rpn10.

FIGURE 6. Effect of Rpn10$^{196-204A}$ mutant in CPY* turnover

A Degradation assays using cycloheximide were carried out in wild-type and *rpn10*Δ cells expressing CPY*.

B Degradation assays in *rpn10*Δ cells carrying Rpn10 wild-type, empty and Rpn10$^{196\text{-}204A}$ vectors and expressing CPY*. Pgk1 inmunoblots were used as loading controls.

FIGURE 7. Mutations of the unstructured region before the UIM1 of S5a cause a change in the process of ubiquitination. Models of monoubiquitination.

A Ubiquitination reaction of S5a, S5a$^{193\text{-}201A}$ and S5a$^{M196A\text{-}G200A}$ carried out for 3 hours at 37ºC. Indicated amounts of UbcH5b were used.

B 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.

C Model representing the involvement of the characterized linker between the VWA domain and the UIM in Rsp5-dependent Rpn10 monoubiquitination and showing a putative role of the flexibility of the linker in a fold-back disposition.

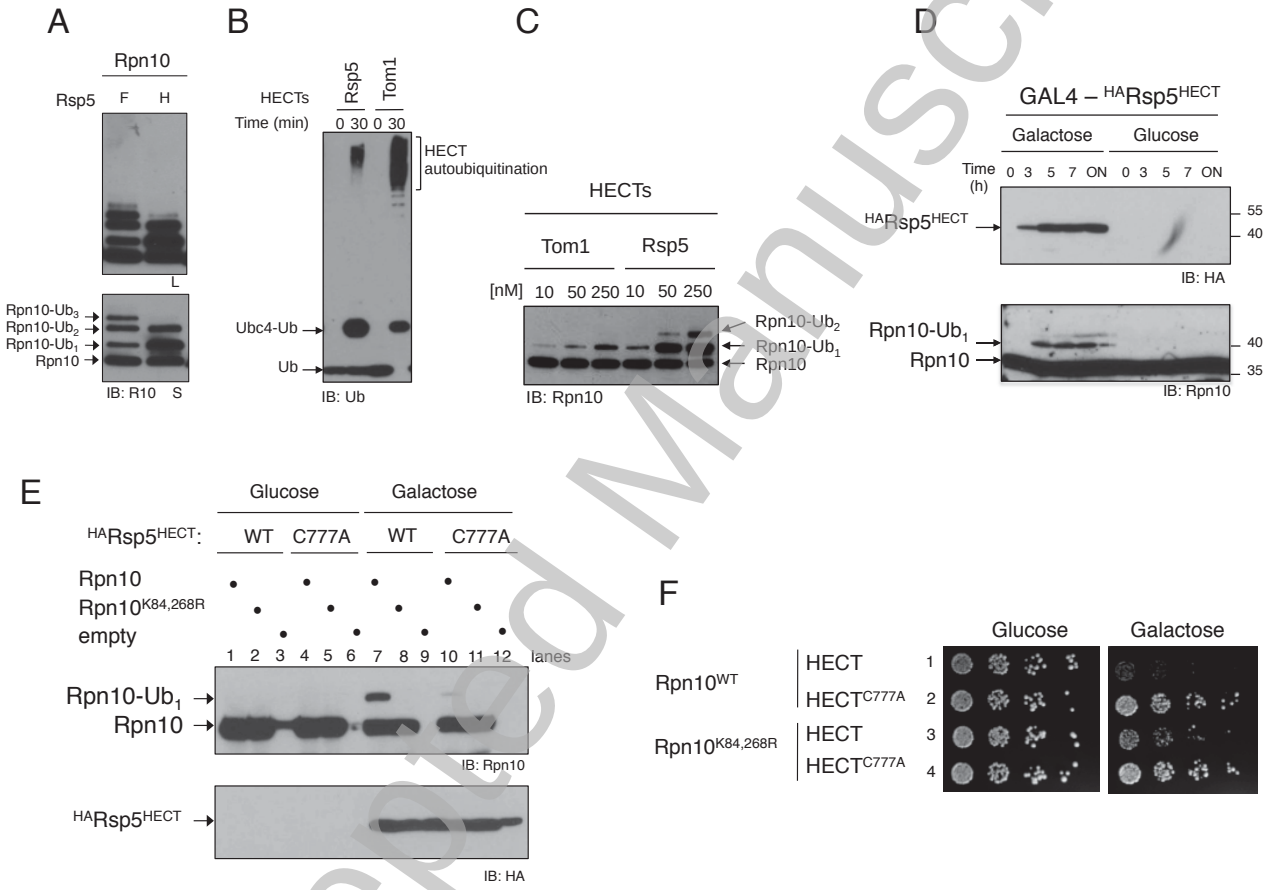

FIGURE 1

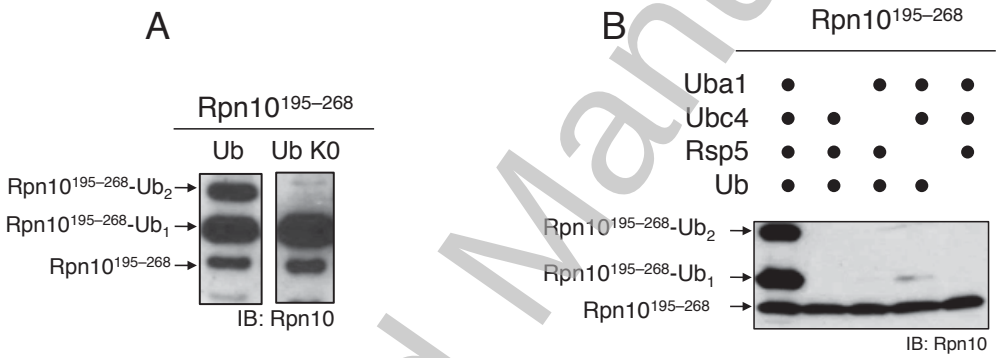

FIGURE 2

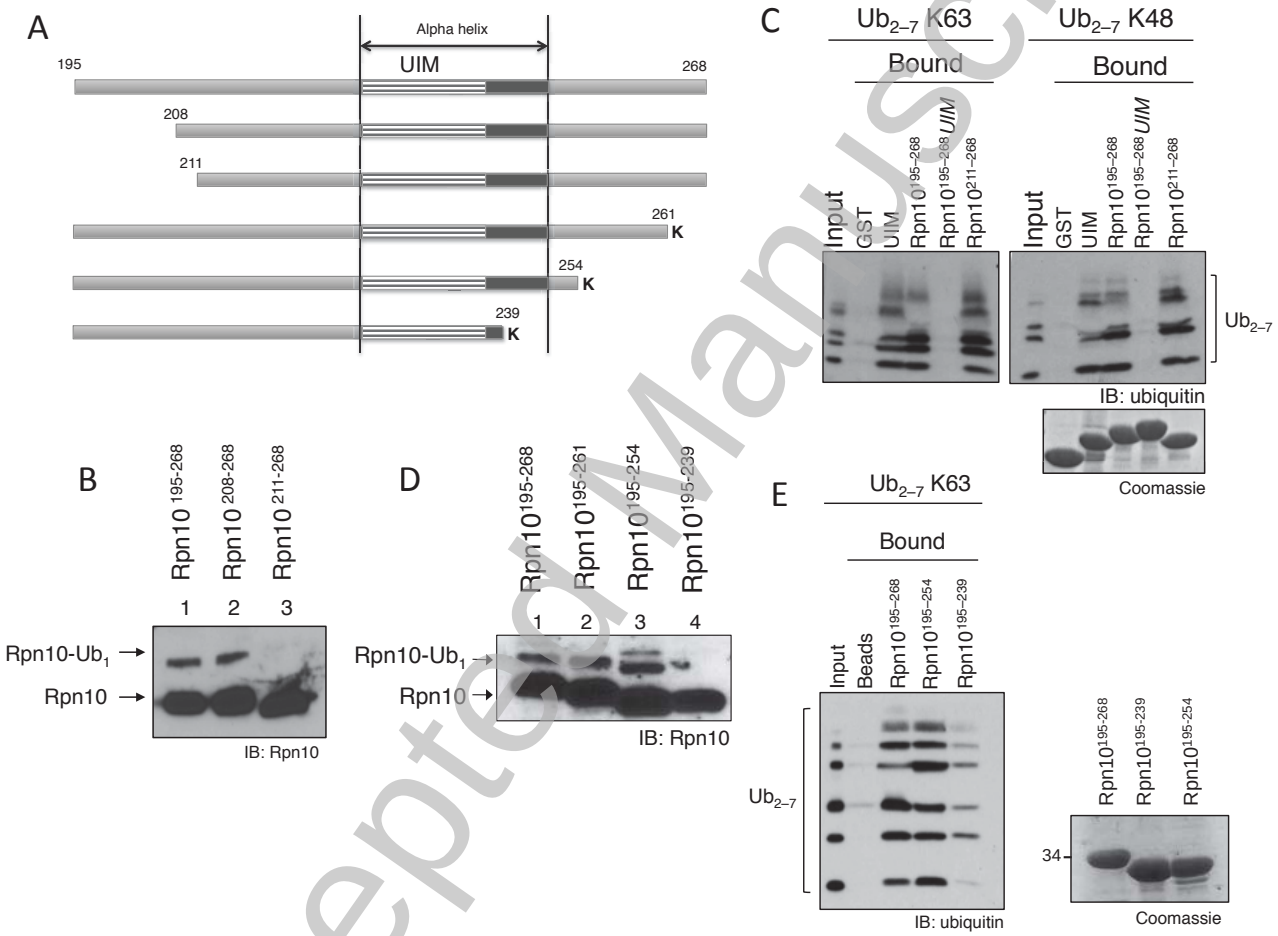

FIGURE 3

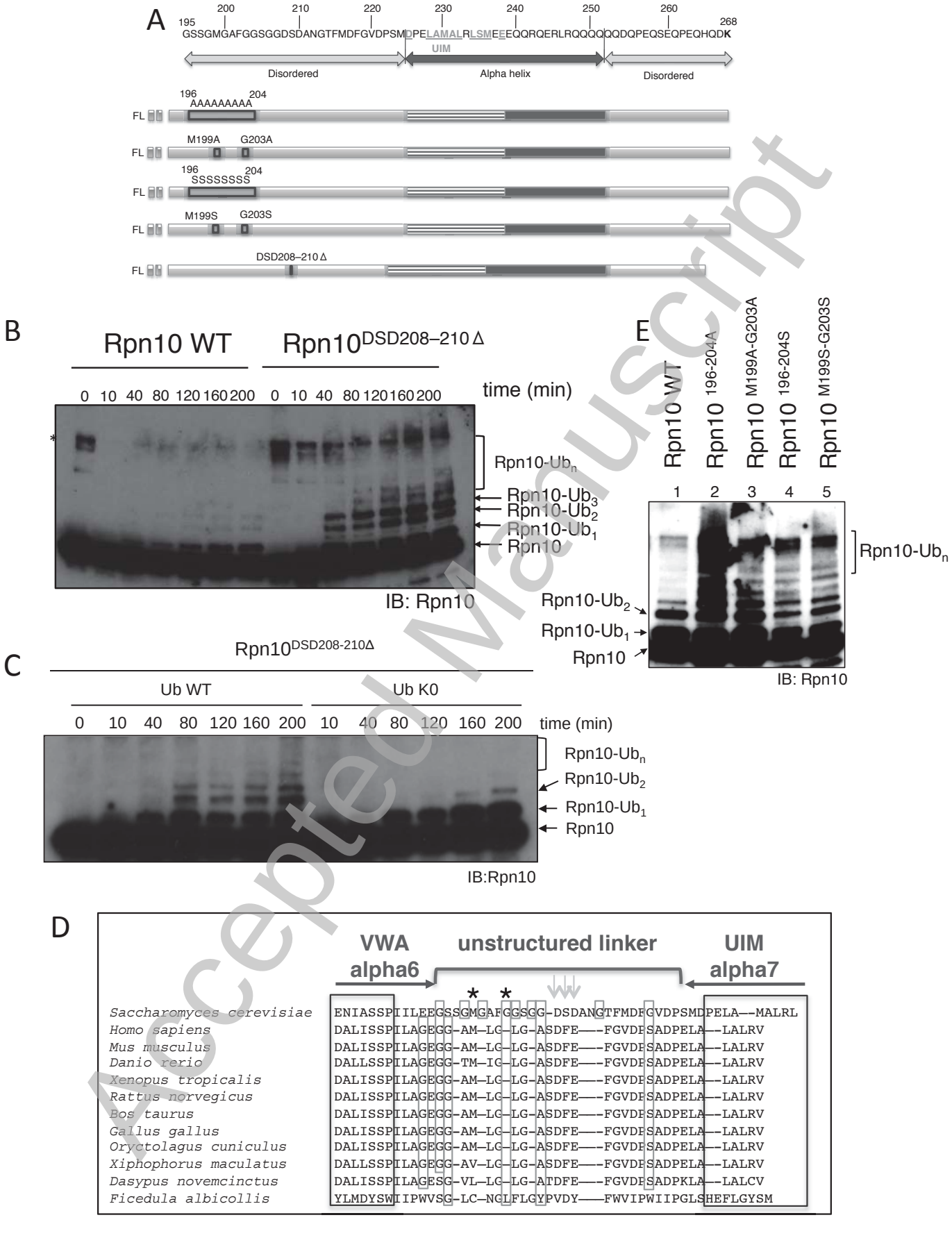

FIGURE 4

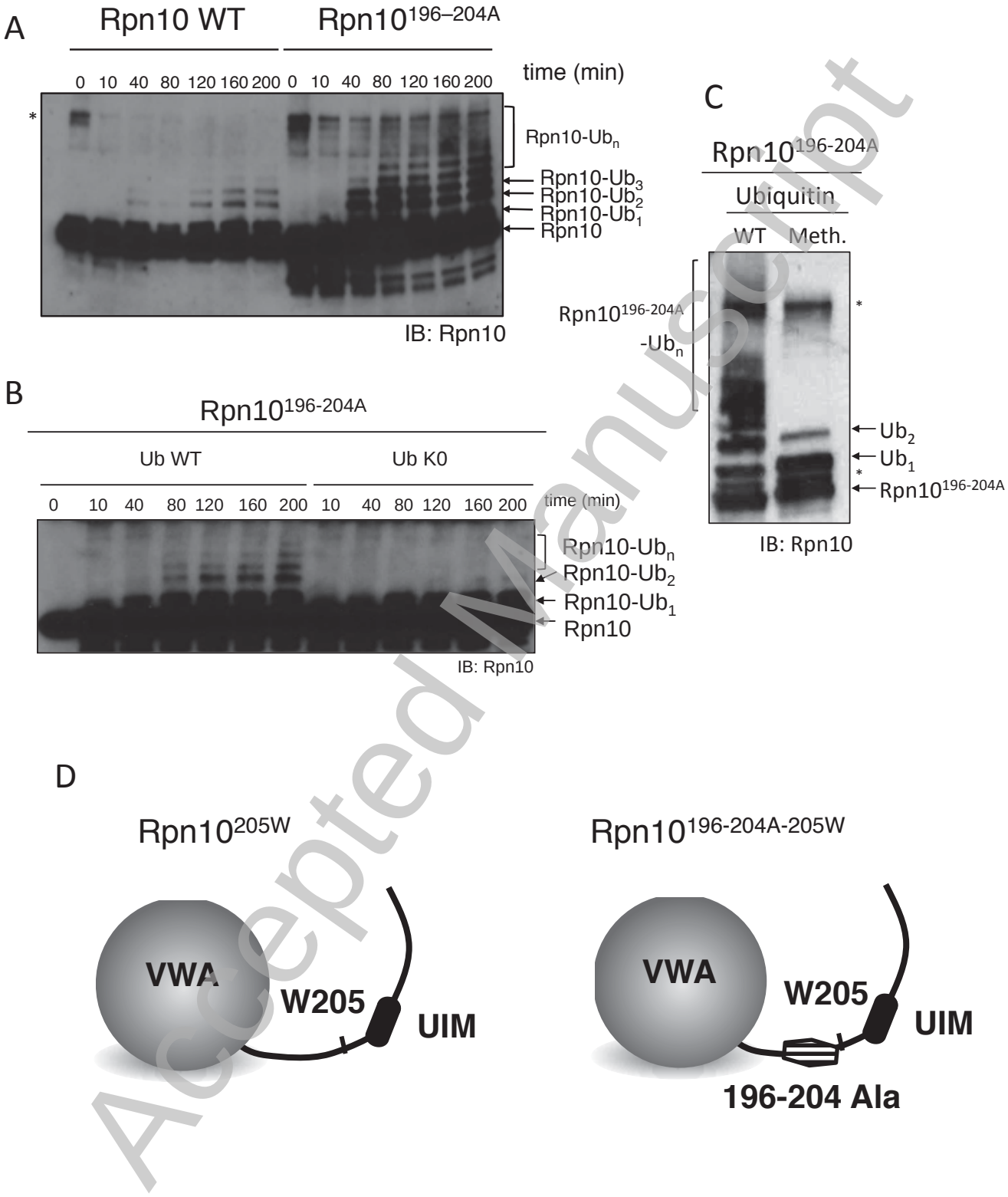

FIGURE 5

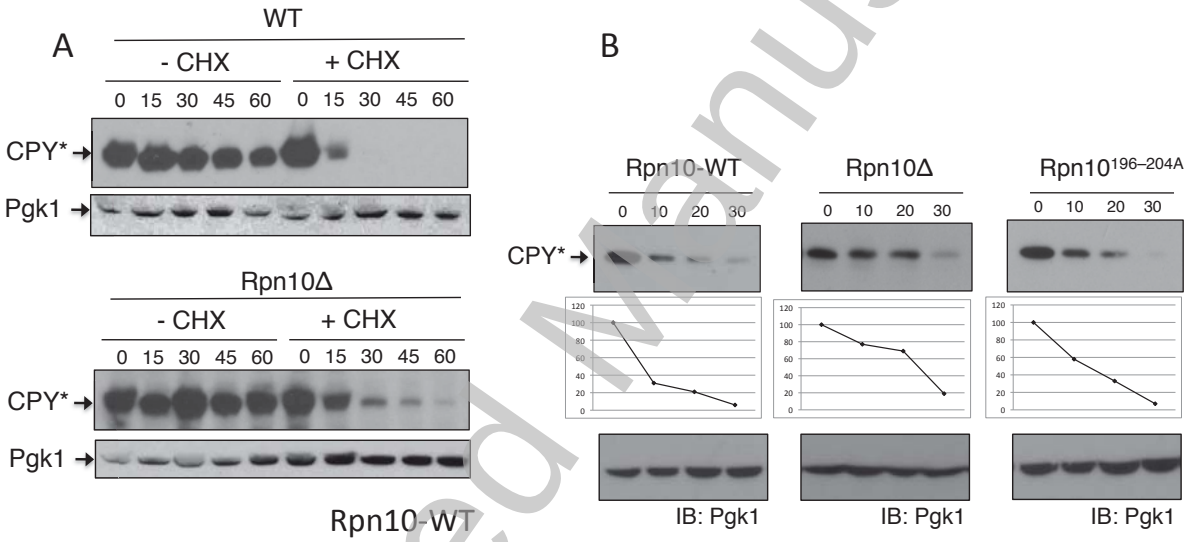

FIGURE 6

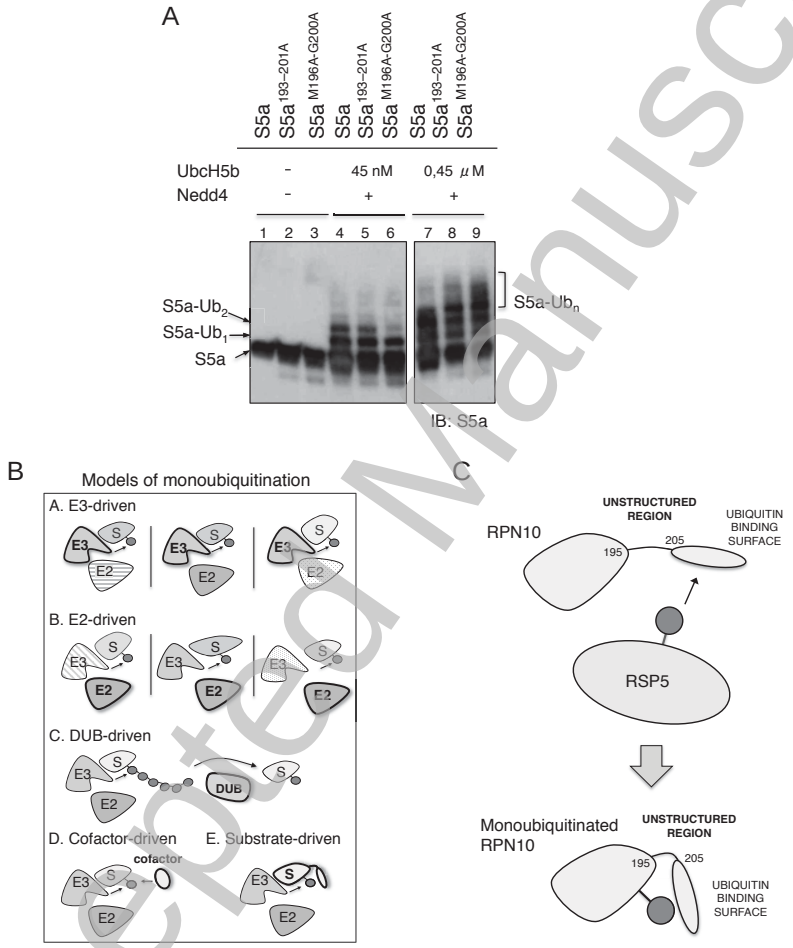

FIGURE 7