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Chapter

Structural Insight into Regulation of the Proteasome Ub-Receptor Rpn10

Tal Keren-Kaplan, Ilan Attali, Olga Levin-Kravets, Oded Kleifeld, Shay Ben-Aroya and Gali Prag

Abstract

Ubiquitylation is a posttranslational modification that determines protein fate. The ubiquitin code is written by enzymatic cascades of E1 and E2 and E3 enzymes. Ubiquitylation can be edited or erased by deubiquitylating enzymes. Ub-receptors are proteins that read and decipher the ubiquitin codes into cellular response. They harbor a ubiquitin-binding domain and a response element. Interestingly, Ub-receptors are also regulated by ubiquitylation and deubiquitylation. However, until recently, the molecular details and the significance of this regulation remained enigmatic. Rpn10 is a Ub-receptor that shuttles ubiquitylated targets to the proteasome for degradation. Here we review recent data on Rpn10, with emphasis on its regulation by ubiquitylation.

Keywords: ubiquitin receptor, crystal structure, ubiquitylated ubiquitin receptor, regulation mechanisms, cargo shuttle

1. Introduction

Protein ubiquitylation functions as a cellular code to alter structure-function, localization, and interactions or as a destruction signal. The signal is decoded by several hundreds of ubiquitin receptors, proteins that carry a ubiquitin-binding domain(s) tethered to a response element. To precisely decode the numerous cellular ubiquitylation signals, ubiquitin receptors also carry element(s) that sense(s) the cellular context [1]. Intriguingly, ubiquitylation also regulates the function of ubiquitin receptors by their ubiquitylation.

The 26S proteasome is a multiprotein complex that degrades ubiquitylated proteins. Several proteasome Ub-receptors that mediate the recognition of ubiquitylated proteins were identified including proteasome subunits Rpn1 [2], Rpn10 [3], and Rpn13 [4] and shuttling factors Dsk2, Rad23, and Ddi1 that are not a proteasome subunit [5].

Rpn10 is one of several Ub-receptors that target ubiquitylated proteins destined for degradation by the 26S proteasome [3]. It contains a VWA (Von Willebrand factor type A domain) tethered to a ubiquitin-binding domain called UIM (ubiquitin-interacting motif). The VWA binds the proteasome, whereas the UIM binds ubiquitin non-covalently. Rpn10 is evolutionarily conserved with some species like human and plant having additional one or two UIM, respectively (**Figure 1**). Interestingly, deletion or silencing of Rpn10 in yeast and worm is dispensable for viability [3, 6]. However, in fruit fly and mice, Rpn10 deletion is lethal and has

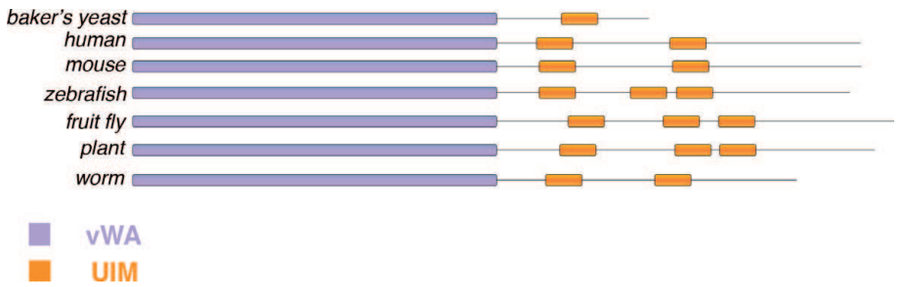


Figure 1.
Scheme of Rpn10 architecture and conservation. VWA, purple; UIM, orange.

deleterious phenotypes in plants [7–9]. Mice lacking only the UIM of Rpn10 are viable. Altogether, it is believed that in some organisms, the redundancy of proteasome Ub-receptors compensates for the lack of Rpn10.

Rpn10 can be found in a proteasome bound form and in a free cytosolic form. Its association with the proteasome is therefore dynamic [10–12]. Experiments with yeast, fly, plant, and human cells collectively suggest that free Rpn10 molecules recognize and shuttle ubiquitylated targets to degradation in the proteasome [10, 12–14]. Excess Rpn10 can bind another receptor Dsk2 and restrict its association with the proteasome. This observation raised the hypothesis that the cytosolic Rpn10 pool possesses a regulatory role on proteasome function.

It has been demonstrated that non-covalent ubiquitin binding and intramolecular monoubiquitylation are coupled [15]. Moreover, monoubiquitin binding by ubiquitin receptors is regulated to avoid occupation of the ubiquitin-binding domain (UBD) by free ubiquitin [16]. The conjugated monoubiquitin might occupy the UBD to prevent the binding to ubiquitylated partners [18]. Therefore, cleavage of conjugated ubiquitin from the receptor would expose the UBD to bind a ubiquitylated cargo in a spatially and temporally mode.

In a seminal study, Crossas and co-workers demonstrated that Rpn10 is monoubiquitylated mostly on lysine 84 (K84) in vivo in yeast by the E3 ligase Rsp5 [17]. They showed that ubiquitylation has an inhibitory effect on the ability of Rpn10 to bind ubiquitylated substrates, suggesting that in the ubiquitylated from, the UIM is blocked by the conjugated ubiquitin. Rpn10 monoubiquitylation levels were reduced under cellular stress conditions where protein degradation was enhanced, thus supporting a connection between monoubiquitylation of Rpn10 and proteasome function. It was later shown that Rpn10 monoubiquitylation leads to its dissociation from the proteasome [11]. Ub-Rpn10 molecules are much less associated with the proteasome compared to *apo*-Rpn10. Proteasomes lacking Rpn10 were still functional suggesting that Rpn10 removal does not destabilize the proteasome [11, 17]. Several groups suggested that Rpn10 monoubiquitylation serves to decrease Rpn10-associated proteasome and increase Dsk2-associated proteasome. Elevation of Ub-Rpn10 decreases Dsk2 association with the proteasome, supporting a role for Rpn10 ubiquitylation as a way to fine-tune the substrates that reach the proteasome [10, 11]. Although Rpn10 loosely associates with the proteasomes, it remains an enigma how monoubiquitylation mediates the dissociation of Ub-Rpn10 from the proteasome.

In this chapter, we will review the purification process of Ub-Rpn10 for crystallization, determine the structure by X-ray diffraction, and present the structural models of Ub-Rpn10 as *apo* and in the context of the proteasome. Moreover, we will discuss a postulated mechanism of action derived from the structures and series of in vitro and in vivo experiments that corroborate this mechanism.

2. *E. coli*-based expression and purification system for ubiquitylated proteins

To obtain large quantity of ubiquitylated proteins for downstream biochemical and biophysical studies (including X-ray crystallography), we constructed an *E. coli*-based system that synthetically expresses a functional ubiquitylation apparatus [18]. The system consists of two or three compatible plasmids that express His_6-Ub, E1, E2, E3, and MBP fusion of protein target of interest for ubiquitylation (**Figure 2A**). We constructed the system in a polycistronic manner. In the case of Ub-Rpn10 purification, we specifically expressed His_6-Ub, UBA1 (E1), and Ubc4 (E2) from one plasmid and Rsp5 (E3) and MBP-Rpn10 from other two plasmids. As the ubiquitylated protein possesses both six-histidine- and maltose-binding protein tags, we used Ni^{2+} and amylose affinity chromatography columns to purify the modified protein. We then cleaved the affinity tags by His_6-tobacco etch virus (TEV) protease and removed tags and the protease using the same affinity columns. Final purification was accomplished by an additional ion-exchange chromatography steps. The expression system and purification process were found to be very efficient as we obtained milligrams of purified Ub-Rpn10 using this purification protocol (**Figure 2B**) [18, 19]. Biochemical and mass-spectrometry analysis clearly showed that Rpn10 is authentically monoubiquitylated at K84 in *E. coli* as was previously demonstrated to be modified originally in *Saccharomyces cerevisiae* [17].

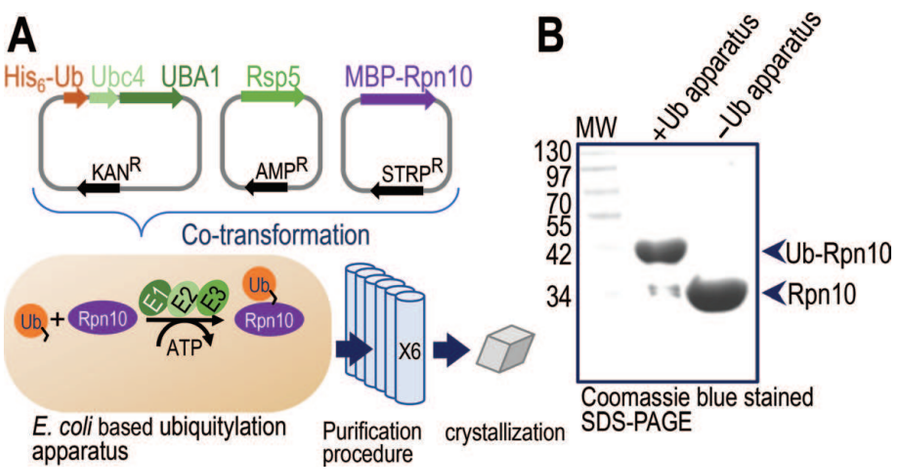


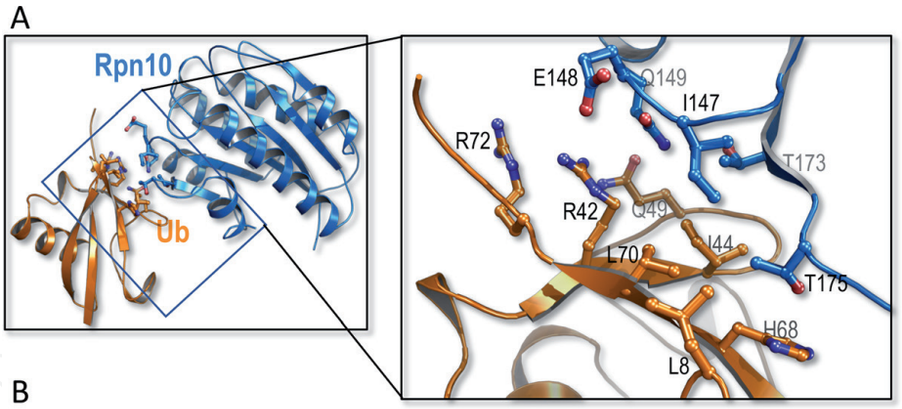
Figure 2.
Expression and purification of ubiquitylated Rpn10. (A) Scheme of the expression and purification of Ub-Rpn10 and (B) Coomassie-stained SDS-PAGE showing the purified sample of Ub-Rpn10 and apo Rpn10 for comparison.

3. Structure of Ub-Rpn10

The structure of Ub-Rpn10 highlighted two key findings [20]: (i) it revealed that in addition to the UIM, the VWA domain may also function as a UBD, and (ii) it allowed assessment of the Ub-Rpn10 structure in the context of the structure of the proteasome. We will now elaborate on the findings from the structure.

4. The VWA domain of Rpn10 is a *bona fide* UBD

The structure shows that the covalently bound Ub-moiety at K84 interacts with the neighboring VWA molecule in a non-covalent manner (**Figure 3**). The interaction interface centered at the famous ubiquitin I44 patch. This observation suggests that binding of ubiquitin by the VWA might be biologically important. In silico algorithm that screens for potential UBDs corroborated this finding [21]. Indeed,



Protein type	K_D (SPR Analyses) [μM]	Ubiquitylation dependent bacterial growth efficiency [Normalized]
Rpn10 Full length	274±13.3	0.981
UIM	184±29.5	1.00
vWA	330±38.0	0.469
vWA-Q149A	613+100	0.273
Ub I44E	Not done	0.258
vWA Q149R	Not done	0.197
Ub H68A	Not done	0.067
vWA T173,175R	1138±233	0.024
vWA I147E	No binding	No growth
Ub L8E,I44E,V70D	No binding	No growth

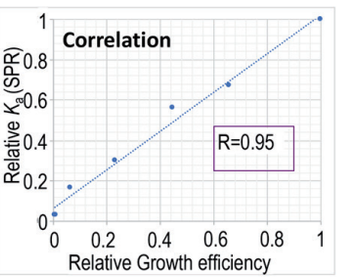


Figure 3.
A non-covalent binding interface between ubiquitin and Rpn10: (A) shows the major residues forming the interaction network and (B) shows the affinity values as measured by SPR (BIACORE) and quantitative growth efficiency as measured from bacterial spots. Correlation between the measurements is shown (right).

surface plasmon resonance experiments showed that VWA binds wild-type ubiquitin. Moreover, ubiquitin mutants at the interaction interface presented significant lower affinity. Similarly, structural-based mutations at the ubiquitin-binding patch on VWA significantly reduced or abrogated the interaction (**Figure 3B**). In an orthogonal study, we harnessed a bacterial genetic selection system for ubiquitylation to quantify the effect of these mutants on *E. coli* growth. In this system *E. coli* cells co-express split antibiotic protein tethered to ubiquitin and the VWA domain along with functional ubiquitylation cascade. The non-covalent interaction of ubiquitin with the VWA domain promotes the ubiquitylation of the latter, results in a functional assembly of the reporter, which give rise to bacterial growth under selective conditions (i.e., in the presence of antibiotic). The data obtained using this system also indicated that the VWA domain binds ubiquitin. Moreover, structural-based mutants abrogated the ubiquitylation and the growth. **Figure 3B** demonstrates the strong correlation between the two orthogonal studies. Altogether, these data indicate that the Rpn10-vWA domain is a *bona fide* ubiquitin-binding domain.

5. Ub-Rpn10 clashes with proteasome subunit Rpn9

The second finding raised from superpositioning of the Ub-Rpn10 with the proteasome cryo-EM structures [22–25]. This operation revealed that whereas the structures of the VWA domain from the two complexes are perfectly aligned, the conjugated ubiquitin collides with the proteasome adjacent subunit Rpn9. This suggests that Ub-Rpn10 cannot reside on the proteasome. The hypothesis was evaluated by three different methodologies: first, biochemical experiments with purified proteasome lacking Rpn10, supplemented with purified Rpn10 and enzymes mix of

its ubiquitylation cascade. These experiments showed that while Rpn10 can associate with the proteasome, Ub-Rpn10 cannot [20]. Second, pulldown experiments show that Rpn9 binds Rpn10 but not Ub-Rpn10. Third, in orthogonal study in vivo in yeast together with the group of Ben Aroya, we demonstrated that native chromosomal expression of Rpn10 harboring the K84R mutation, which cannot undergo ubiquitylation at this site, tightly binds the proteasome subunit Rpn9. However, wild-type Rpn10 that can undergo ubiquitylation at K84 shows no interaction with Rpn9. Together, these experiments demonstrate that Rpn10 but not Ub-Rpn10 interact with Rpn9.

The structure, the biochemical, the biophysical, and the genetic experiments therefore support a model where upon ubiquitylation, Ub-Rpn10 dissociates from the proteasome, allowing a new molecule of Rpn10 to bind (**Figure 5**). Crosas and co-workers independently showed that ubiquitylation of Rpn10 leads to reduction of Rpn10-associated proteasomes, supporting our observation [11].

6. Ub-Rpn10 modifies Dsk2 interactions at the proteasome

At the same time we determined and analyzed the structure of Ub-Rpn10, Crosas and his co-workers found that monoubiquitylation of Rpn10 dissociates Ub-Rpn10 from the proteasome [11]. Moreover, they found that the proteasome shuttle Dsk2 interacts with Rpn10 but this interaction is precluded by the ubiquitylation of Rpn10. They examined the localization of Dsk2 in a model of constitutively ubiquitylated Rpn10 in which Ub is fused to Rpn10 (Ub-Rpn10). They demonstrated that Dsk2 no longer interacts with Ub-Rpn10 but instead it associates the proteasome by interaction with Rpn1. Their data indicate that under these conditions, more Dsk2 is associated with the proteasome suggesting that ubiquitylation of Rpn10 also regulates the interaction of Dsk2 with the proteasome.

7. Conclusions

It is now becoming clear that ubiquitylation signal goes beyond degradation and serves as a regulation mechanism for protein-protein interaction. Recent study in our laboratory demonstrated that a similar mechanism also regulates the activity of HECT E3 ligases [26] and other Ub-receptors (unpublished data). We, therefore, postulate that other cellular Ub-receptors and perhaps shuttling factors are regulated by coupled monoubiquitylation.

It is convincing to see that different studies conducted by several laboratories found the same data in which monoubiquitylation of Rpn10 at K84 induces dissociation of the receptor from the proteasome. While each laboratory examined different outcomes, it seems that there is no contradiction between the models, but they actually provide a more complete and comprehensive view on the regulation mechanism of Rpn10 and proteasome function. It would be interesting to explore the mechanism that determines the timing of Rpn10 ubiquitylation on the proteasome. Moreover, it is yet to be explored if and when deubiquitylation promotes the recycle of Rpn10-dependent substrate degradation.

Acknowledgements

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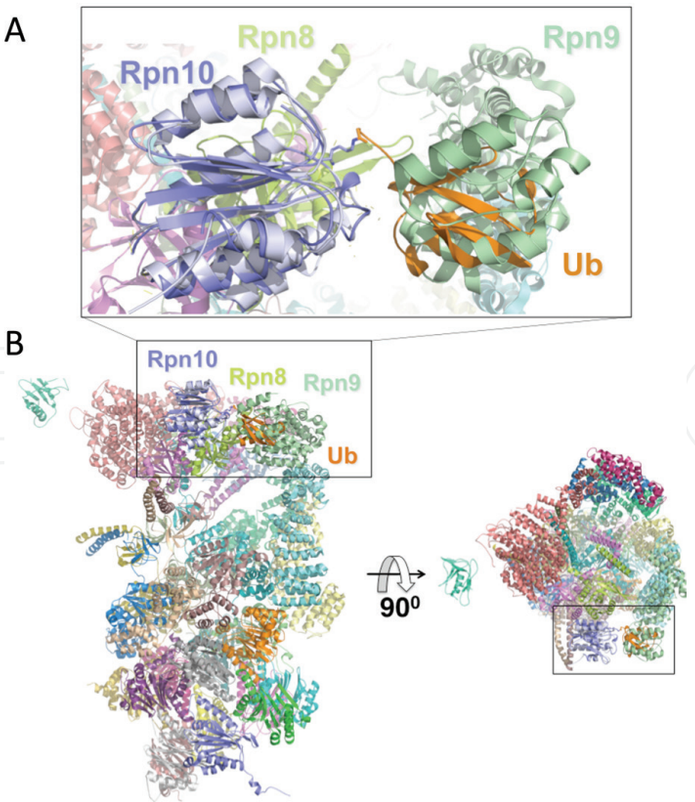

Figure 4.
Structure of Ub-Rpn10 at the proteasome context. Superimposition of the Ub-Rpn10 structure with the proteasome complex cryo-EM structure. (A) Zoom into the interaction interface between Rpn10, Rpn9, and Rpn8. The Ub-moiety [from Ub-Rpn10 molecule (colored in orange)] clearly clashes with Rpn9 subunit (light green). There is no interaction between the Ub-moiety and subunit Rpn8. (B) A view in context of the proteasome.

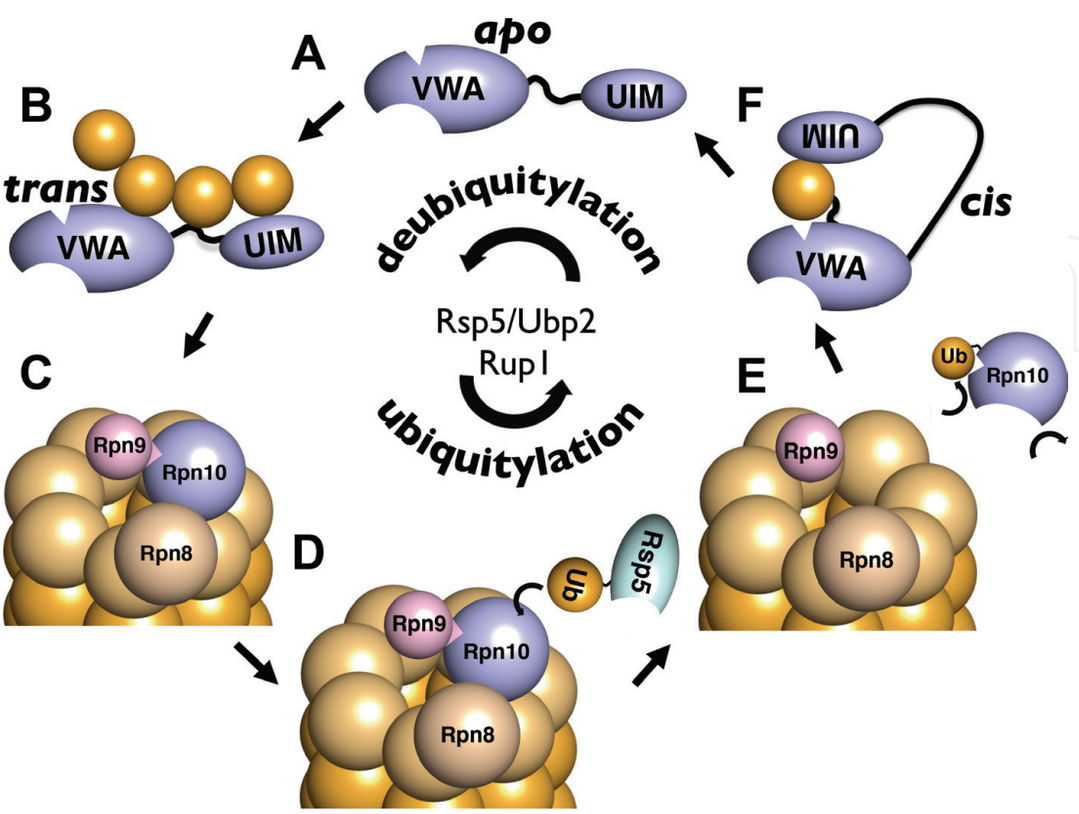

Figure 5.
A model for the regulation of Rpn10 by monoubiquitylation.

of Ub-Rpn10 was performed at the ID14-4 beamline at ESRF, Grenoble, France. The research entailed in this chapter was funded by grants from the Israel Science Foundation 1695/08 and 464/11 to G.P.; from the EC FP7 Marie Curie International Reintegration Grant (PIRG03-GA-2008-231079) to G.P.; and from the Israeli Ministry of Health (5108) to G.P. **Figures 2B**, **4** and **5** were adapted from [27].

Conflict of interest

The authors declare no conflict of interest.

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