The Proteasome Subunit Rpn8 Interacts with the Small Nucleolar RNA Protein (snoRNP) Assembly Protein Pih1 and Mediates Its Ubiquitin-independent Degradation in Saccharomyces cerevisiae*

Received for publication, November 2, 2015, and in revised form, March 25, 2016 Published, JBC Papers in Press, April 6, 2016, DOI 10.1074/jbc.M115.702043

Alexandr Paci, Peter X. H. Liu, Lingjie Zhang, and Rongmin Zhao

From the Department of Biological Sciences, University of Toronto, Toronto, Ontario M1C 1A4, Canada

Pih1 is a scaffold protein of the Rvb1-Rvb2-Tah1-Pih1 (R2TP) protein complex, which is conserved in fungi and animals. The chaperone-like activity of the R2TP complex has been implicated in the assembly of multiple protein complexes, such as the small nucleolar RNA protein complex. However, the mechanism of the R2TP complex activity in vivo and the assembly of the complex itself are still largely unknown. Pih1 is an unstable protein and tends to aggregate when expressed alone. The C-terminal fragment of Pih1 contains multiple destabilization factors and acts as a degron when fused to other proteins. In this study, we investigated Pih1 interactors and identified a specific interaction between Pih1 and the proteasome subunit Rpn8 in yeast Saccharomyces cerevisiae when HSP90 co-chaperone Tah1 is depleted. By analyzing truncation mutants, we identified that the C-terminal 30 amino acids of Rpn8 are sufficient for the binding to Pih1 C terminus. With in vitro and in vivo degradation assays, we showed that the Pih1 C-terminal fragment Pih1(282–344) is able to induce a ubiquitin-independent degradation of GFP. Additionally, we demonstrated that truncation of the Rpn8 C-terminal disordered region does not affect proteasome assembly but specifically inhibits the degradation of the GFP-Pih1(282–344) fusion protein in vivo and Pih1 in vitro. We propose that Pih1 is a ubiquitin-independent proteasome substrate, and the direct interaction with Rpn8 C terminus mediates its proteasomal degradation.

The R2TP2 complex, which is composed of Rvb1, Rvb2, Tah1, and Pih1, was first discovered in baking yeast (1) and is required for box C/D small nucleolar RNA protein (snoRNP) complex assembly and thus for the ribosomal RNA biogenesis (2). R2TP is a conserved protein complex and has also been identified in mammalian cells (3–5), fruit fly (6), plasmodium (7), and many other fungi species (8), whereas it is absent in higher plants (9). There are more than 70 different snoRNP complexes that are directly involved in ribosomal RNA processing, a critical step for ribosome assembly that requires a total of more than 200 assembly factors (10). The assembly of R2TP complex in vivo is also a highly dynamic process and is regulated by the molecular chaperone HSP90 and nutrition status (11). However, the mechanism by which R2TP controls box C/D snoRNP complex assembly is still largely unknown.

Pih1, the scaffold protein within the R2TP complex, mediates the interaction between Tah1 and Rvb1-Rvb2 and subsequently controls the biogenesis of box C/D snoRNP (2, 3). Tah1 recruits HSP90 and protects Pih1 from degradation in vivo (12, 13). Pih1 interacts directly with Nop58, one of the core protein subunits in box C/D snoRNP (11) and affects its stability (14). Pih1 also interacts with Rsa1, another snoRNP assembly factor through which the R2TP complex may interact with the snoRNP core protein Snu13 (15). Similar to the role of Tah1 in protecting Pih1, a small protein, Hit1, was also identified to interact with and protect Rsa1, thus revealing a complex regulatory network controlling snoRNP assembly in vivo (16). The human R2TP complex was also shown to aid in the assembly of RNA polymerase II complex (17). Human Pih1 homologue Pih1D1 interacts with phosphorylated Tel2 and recruits the R2TP complex in the assembly of PIKKs (phosphatidylinositol 3-kinase-related kinases) (18).

Yeast Pih1 contains a Pih1 domain in its N-terminal fragment (9). The atomic structures of the Pih1 domain have been solved for both yeast and human homologues (19, 20). The Pih1 domain adopts an unusual ββαβββα topology. The β-sheet forms an interface that specifically binds a phosphorylated DSDD motif in Tel2 (19). The length of Pih1 C-terminal fragment varies among species. The yeast Pih1 C-terminal fragment contains a CS domain, which also appears in other proteins, such as HSP90 co-chaperones p23 and Sgt1 (21, 22), and interacts with HSP90 directly (23). The Tah1 C-terminal fragment forms a constitutive interaction with the Pih1 CS domain through main chain interactions, in one way by forming β-strand to extend the β-sheet of the Pih1 CS domain and in the other way by bridging the edges of the two β-sheets of the CS domain (20). The tight interaction between Tah1 and Pih1 has also been observed when they are co-expressed in Escherichia coli (24). In contrast, expressing individual fragments is prob-

---

*References and The abbreviations used are: R2TP, Rvb1-Rvb2-Tah1-Pih1 complex; DHFR, dihydrofolate reductase; DHFR*, temperature-sensitive dihydrofolate reductase; snoRNP, small nucleolar RNA protein; Ni-NTA, nickel-nitritotri-acetic acid; R2P, Rvb1-Rvb2-Pih1; RP, regulatory particle; suc, succinyl; AMC, aminomethylcoumarin; Ub, ubiquitin; CS, CHORD-containing proteins and SGT1; MPN, Mpr1 and Pad1 N termini; UB4DHFR, Ub-Ub-Ub-Ub-DHFR fusion protein.

1 To whom correspondence should be addressed: Dept. of Biological Sciences, University of Toronto Scarborough, 1265 Military Trail, Toronto, Ontario M1C 1A4, Canada. Tel: 416-287-2740; Fax: 416-287-7676; E-mail: rzha0@utsc.utoronto.ca.

2 The abbreviations used are: R2TP, Rvb1-Rvb2-Tah1-Pih1 complex; DHFR, dihydrofolate reductase; DHFR*, temperature-sensitive dihydrofolate reductase; snoRNP, small nucleolar RNA protein; Ni-NTA, nickel-nitritotri-acetic acid; R2P, Rvb1-Rvb2-Pih1; RP, regulatory particle; suc, succinyl; AMC, aminomethylcoumarin; Ub, ubiquitin; CS, CHORD-containing proteins and SGT1; MPN, Mpr1 and Pad1 N termini; UB4DHFR, Ub-Ub-Ub-Ub-DHFR fusion protein.

3-kinase-related kinases) (18).
lematic, and Pih1 is prone to degradation in vivo in the absence of Tah1 (2).

In addition to a Pih1 domain and a CS domain, we previously showed that Pih1 also contains two intrinsically disordered regions that are essential for the binding to the Rvb1-Rvb2 AAA+ family DNA helicases (8). Intrinsically disordered regions are important features of a protein, and they often mediate protein-protein interaction or are involved in post-translational regulation of the protein activities (25). Additionally, we previously showed that the Pih1 extreme C-terminal fragment, Pih1(282–344), contains multiple destabilization elements sufficient to induce fast degradation of well folded proteins, such as GFP, in vivo (8, 12). Therefore, Pih1 is a multidomain scaffold protein responsible for binding different interacting partners, whereas the stability and turnover of Pih1 in vivo is probably controlled by a complex mechanism.

In this study, we further investigated the role of the Pih1 C-terminal fragment in the assembly of R2TP complex and identified the proteasome lid subunit Rpn8 as a Pih1 interacting partner. By in depth analysis of protein interactions using both in vivo and in vitro pull-down assays, we discovered a specific interaction between Pih1 and Rpn8 mediated by their C-terminal fragments. Tah1 and Rpn8 exclusively bind to the Pih1 C terminus. We also showed that the association of Pih1 to Rpn8 leads to proteasomal degradation of Pih1 in a ubiquitin-independent manner. Our study therefore not only provides mechanistic insights into the Pih1 cellular proteostasis but also reveals a novel binding site on the 26S proteasome that mediates ubiquitin-independent degradation of Pih1.

**Experimental Procedures**

**Plasmid Construction**—The plasmid pET22b-UB4DHFR-CytB-His₆ was constructed by amplifying the fusion protein coding sequence from pGEM3Z-UB4DHFR-Cytb-His₆ (26) and insertion into the Ndel/HindIII sites of pET22b. To construct the plasmid expressing DHFR-CytB-His₆, the DHFR-CytB-His₆ coding sequence in pET22b-UB4DHFR-CytB-His₆ was cleaved with Ncol and HindIII and inserted into pProEXHTb. The plasmids expressing GFP-Pih1(231–344) and GFP-Pih1(282–344) in E. coli and yeast were described previously (8). GFP-Pih1(282–344ΔK) expressed from p415GPD vector was constructed by replacing the Pih1(282–344) coding sequencing (EcoRI/HindIII) with a new EcoRI/HindIII fragment that was synthesized as oligonucleotides and annealed together, in which the codons for lysine were replaced by arginine codons. Plasmid p415GPD-UB-Arg-DHFR₄₈, which expresses the ubiquitin-dependent model substrate DHFR₄₈, was generated by amplifying Ub-Arg-DHFR₄₈ from pRS315-Kan-Ura3Pro-UB-Arg-DHFR₄₈ (a kind gift of Dr. Charlie Boone, University of Toronto) and inserting the fragment between the XbaI and HindIII sites of p415GPD. To construct GST fusion proteins for the Rpn8 C-terminal fragments, the Rpn8(291–338) and Rpn8(309–338) coding sequences were amplified and cloned into the Ncol/BamHI sites of pETM30 vector. The GST protein alone was expressed and purified from plasmid pGEX2TK. NusA-His₆-Pih1 fusion protein construct was made by cloning the Pih1 coding sequence into the Ncol/BamHI sites of pETM60. The construction of plasmids p414GPD-Tah1 and p414GPD-Pih1 was described previously (2). The expression and purification of proteins Pih1 and Pih1(1–230) were also described previously (12). All constructs were confirmed by sequencing.

**Yeast Strains**—PCR-based gene tagging was used to generate C-terminal 3×FLAG-tagged Rpn8, Rpn8(1–323), Rpn8(1–308), Rpn8(1–290), or Rpn1 in yeast W303 (MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15) and MYH501 (S288C, MATa his3-D200 leu2-3,112 ura3-52 lys2-801 trp1-1 gal2) (27). pFA6a-3×FLAGkanMX6 was used as a PCR template, and the PCR product was incorporated into the yeast genome through homologous recombination (28). Uba1 wild type-like strain (W303, MATa uba1::KANMX, [pRS313-UBA1], can1-100, leu2-3,-112, his3-11,-15, trp1-1, ura3-1, ade2-1) and the uba1-204 mutant strain (W303, MATa uba1::KANMX, [pRS313-uba1-204], can1-100, leu2-3,-112, his3-11,-15, trp1-1, ura3-1, ade2-1) were described previously (29). Yeast strain tah1Δ (S288C, MATa leu2 his3 lys2 can1::STE2pr-Sp_his5 tah1Δ::NAS) was also described previously (2). All strains used in this study are included in Table 1.

**In Vitro Pull-down Assays**—The 3×FLAG-tagged Pih1 complexes were purified using anti-FLAG antibody resin according to an established procedure (30). The 26S proteasome was purified using 3×FLAG-tagged Rpn8, C-terminally truncated Rpn8, Rpn1, or Pre1 in the presence of 5 mM ATP and ATP regeneration system according to the procedure described previously (31). To purify the 19S complex, similar procedures were applied but without including the ATP or ATP regeneration system. 20S proteasome was purified using FLAG-tagged Pre1 without including ATP and ATP regeneration system. The expression and purification of all other proteins were performed from E. coli BL21 (DE3) pRIL cells by using either Ni-NTA (Qiagen) or glutathione-agarose (Sigma) according to the manufacturer’s instructions. 3×FLAG-tagged, His₆-tagged, and GST fusion protein complexes were eluted using 3×FLAG peptide, 250 mM imidazole, and reduced glutathione, respectively.

**In Vitro Protein Degradation Assay**—50 nM 26S or 20S proteasomes and substrates, such as 80 nM UB4DHFR, 100 nM DHFR, or 150 nM Pih1, were combined in the following buffer: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 100 μM creatine phosphate, 4 units/ml creatine kinase, 5 mM MgCl₂, 1 mM ATP, and 10% glycerol. When proteasome inhibitors were used, 80 μM MG132 (Boston Biochem, I-130) and 50 μM lactacystin (Boston Biochem, I-114) at the final concentrations were included in the reaction mixture. The reaction was carried out at 30 °C, and the reaction was stopped by incubating the reaction aliquots in 2× SDS Laemmli buffer for 10 min at 95 °C.

**In Vivo Protein Stability Assay**—GFP-Pih1(282–344) or DHFR₄₈ was expressed from plasmid p415GPD under the constitutive GPD promoter (32) in vivo from the following strains: TetO₂-controlled RPn8, RPNI1, and RPT2 strains; uba1-204 mutant strain, or yeast strains that have the full-length Rpn8 (Rpn8(1–338)FLAG) or C-terminally truncated Rpn8 (Rpn8(1–308)FLAG, Rpn8(1–290)FLAG). For the different Rpn8 C-terminally truncated mutants, both W303 and S288C genetic background cells were used. The degradation was performed at either 30 or 37 °C as indicated after inhibition of the
protein translation using 50 μg/ml cycloheximide. Specifically, to analyze the protein stability in TetO7-controlled strains, the cellsharbing GFP-Pih1(231–344) or GFP-Pih1(282–344) construct were grown at 30 °C overnight and then re-inoculated into SD−Leu liquid media to a density of 0.05 at A600. The cultures were then grown with the addition of 10 μg/ml doxycycline until the latest stages, when the growth of respective cells was not significantly different from that of the untreated cells, whereas the expression of the controlled proteins has been significantly repressed. The remaining GFP fusion proteins or DHFR+ at different time points was monitored by immunoblotting using anti-GFP or anti-DHFR antibodies. The data were plotted and fit to first order decay kinetics using Origin.

Yeast Dilution Series Assay—Rpn8(1–338)FLAG, Rpn8(1–308)FLAG, and Rpn8(1–290)FLAG yeast cells in either W303 or S288C genetic background were grown overnight in YPD medium. The cells were diluted to an optical density of 2.0 measured at 600 nm. The cells were diluted 10-fold in a dilution series. 10 μl of each culture was inoculated onto YPD plates using a solid pin replicator (VP Scientific, Inc.), and cells were grown for 36 h.

Size Exclusion Chromatography—Size exclusion chromatography was used to separate complexes of the 26S proteasome, 20S proteasome, and 19S proteasome. Cell lysates from Rpn8(1–338)FLAG, Rpn8(1–308)FLAG, Rpn8(1–290)FLAG yeast (W303, MATα his3Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 gal2) was directly loaded into Superose 6 10/300 GL column attached to ÄKTA (GE Healthcare) FPLC system. The column was equilibrated with a low salt running buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 1 mM ATP, and 10% glycerol, 150 mM NaCl) or a high salt running buffer that included 1 M NaCl instead.

Antibodies—Anti-GFP antibody (G1544), anti-FLAG antibody (F1804), and anti-DHFR antibody (D1067) were purchased from Sigma. Rabbit anti-Rpn8 antibody was a kind gift of Dr. Daniel Finley (Harvard University). Anti-His₆ antibody was purchased from Cell Signaling Technology (catalog no. 2365). Monoclonal anti-Rpt1, anti-Pre10 antibodies were kind gifts of Dr. William Tansey (Vanderbilt University). Polyclonal anti-Tah1 and anti-Pih1 antibodies were raised and described previously (2). Polyclonal anti-GST antibody (G7781) was purchased from Sigma.

Results

Pih1 C-terminal Fragment Triggers Proteasome-dependent Fusion Protein Degradation—To understand the molecular mechanism of protein degradation induced by the Pih1 C-terminal fragment, we first examined the degradation of GFP-Pih1(231–344) in yeast cells that have RPN8, RPT2, or RPN11, three of the 26S proteasome regulatory particle genes, controlled under the TetO7 promoter (32). The choice of TetO7-controlled yeast strains allows us to switch off the expression of the controlled gene upon the addition of doxycycline (Fig. 1A).

Because Rpn8, Rpt2, and Rpn11 have very different half-lives (33), we monitored the growth of TetO7-controlled yeast strains after treatment with 10 μg/ml doxycycline. The degradation of GFP-Pih1(231–344) was then analyzed after 10-, 2-, and 10-h treatment, respectively, for the three strains. We observed that, at these time points, the cell density was not significantly affected, whereas the expression of the TetO7-controlled protein has already been significantly reduced, as shown by examining the integrity of purified 26S proteasome (Fig. 1B) or the relative amount of 26S proteasome in total cell lysate (Fig. 1C). As shown in Fig. 1, D−F, the degradation of GFP-Pih1(231–344) was slower in all cells when the TetO7-controlled subunits were repressed by application of doxycycline. Because GFP-Pih1(231–344) was not degraded as fast as GFP-Pih1(282–344), due to the binding to and protection by other partners, such as Tah1 (8), we tested the degradation of GFP-Pih1(282–344) in TetO7-RPN8 strains. As shown in Fig. 1G, GFP-Pih1(282–344) is degraded quickly in cells treated with DMSO only, whereas its degradation is significantly impaired with the RPN8 gene being repressed. These data suggest that in vivo degradation of both GFP-Pih1(231–344) and GFP-Pih1(282–344) is dependent on the 26S protea-

| TABLE 1 | Strains used in this study |
|---------|--------------------------|
| Name    | Background | Genotypes | References |
| W303A   | W303       | MATα leu2-3, 112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 | Saccharomyces Genome Database |
| AP01    | W303       | MATα leu2-3, 112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 RPN8::3FLAG::KAN | This study |
| AP02    | W303       | MATα leu2-3, 112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 RPN8::3FLAG::KAN | This study |
| AP03    | W303       | MATα leu2-3, 112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 RPN8::3FLAG::KAN | This study |
| AP04    | W303       | MATα leu2-3, 112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 RPN8::3FLAG::KAN | This study |
| MHY1501 | S288C      | MATα his3 Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 gal2 | Ref 27 |
| AP05    | S288C      | MATα his3 Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 gal2 RPN8::3FLAG::KAN | This study |
| AP06    | S288C      | MATα his3 Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 gal2 RPN8::3FLAG::KAN | This study |
| AP07    | S288C      | MATα his3 Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 gal2 RPN8::3FLAG::KAN | This study |
| AP08    | S288C      | MATα his3 Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 gal2 RPN8::3FLAG::KAN | This study |
| RJD234  | W303       | MATα can1-100 leu2-3,112 his3-11,15 trp1-1 ade2-1 tub1-1 ubf1-1::KANMX [pRS313-ubf1-204] | Ref 29 |
| R0067   | W303       | MATα leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 his2Δ::LEU2 hsp82Δ::LEU2 | Ref 2 |
| R0068   | W303       | MATα leu2-3,112 trp1-1 can1-100 ura3-1 his3-11,15 hsp82Δ::LEU2 hsp82Δ::LEU2 | Ref 2 |
| TH3852  | S288C      | MATα his3-1 leu2-0 met15-0 URA3-CMV-tTA rpt2-tetO7::KAN | Ref 32 |
| TH5276  | S288C      | MATα his3-1 leu2-0 met15-0 URA3-CMV-tTA rpn8-tetO7::KAN | Ref 32 |
| TH5970  | S288C      | MATα his3-1 leu2-0 met15-0 URA3-CMV-tTA rpn11-tetO7::KAN | Ref 32 |
| BB01    | S288C      | MATα his3-1 leu2-0 met15-0 URA3-CMV-tTA rpn2-tetO7::KAN PRE1−3FLAG::HIS | This study |
| BB02    | S288C      | MATα his3-1 leu2-0 met15-0 URA3-CMV-tTA rpn8-tetO7::KAN PRE1−3FLAG::HIS | This study |
| BB03    | S288C      | MATα his3-1 leu2-0 met15-0 URA3-CMV-tTA rpn11-tetO7::KAN PRE1−3FLAG::HIS | This study |
some, and Pih1(282–344) induces a much faster degradation of GFP, also in agreement with our previous observations (8).

To further understand whether the protein degradation triggered by the Pih1 C-terminal fragment is specific to the 26S proteasome, we purified the 26S proteasome as well as the 20S proteasome (Fig. 2A shows example subunit profiles and the activity of the 26S proteasomes) and used them to degrade full-length Pih1, which was expressed and purified from E. coli. Interestingly, although the purified Pih1 was not polyubiquitinated, it was degraded by 26S proteasome in vitro. The degradation was inhibited by the specific inhibitors MG132 and lacinystin (Fig. 2B, top), whereas the 20S core particle did not degrade Pih1 well (Fig. 2B, bottom). Additionally, the degradation of Pih1 seems to be mainly attributed to the C-terminal fragment, because Pih1(1–230) was not degraded as efficiently as the full-length Pih1 by 26S proteasome (Fig. 2B, top). As controls, the purified 26S proteasome was able to degrade a model ubiquitin fusion substrate UB4DHFR (26) well, but not the non-ubiquitinated DHFR (Fig. 2C).

FIGURE 1. Repression of the proteasomal subunits inhibited Pih1 fusion protein degradation in vivo. A, dilution assay to show growth inhibition by doxycycline (DOX; 10 μg/ml) of the three strains that have RPN8, RPT2, or RPN11 controlled by the TetO7 promoter. The cells were grown for 36 h. B, SDS-PAGE and Coomassie Blue staining of proteasomes purified using FLAG-tagged Pre1 from cells with RPN8, RPT2, or RPN11 controlled by the TetO7 promoter and carrying p415GPD-GFP-Pih1(231–344) in the presence or absence of doxycycline. The cells were treated with doxycycline for 10, 2, and 10 h, respectively. The purification was performed with an ATP regeneration system. C, 4% native PAGE of total cell lysates prepared from cells treated as in B was run, and then in-gel peptidase activity assay using succ-LLVY-AMC was performed with 0.05% SDS included in the substrate solution. D–G, yeast cells carrying p415GPD-GFP-Pih1(231–344) and with RPN8 (D), RPT2 (E), or RPN11 (F) controlled by the TetO7 promoter or carrying p415GPD-GFP-Pih1(282–344) and with RPN8 controlled by the TetO7 promoter (G) were treated with DMSO or 10 μg/ml doxycycline and then with 100 μg/ml cycloheximide to inhibit protein translation. Equal amounts of cells were taken at different time points to examine the expression level of GFP fusion protein by immunoblotting using anti-GFP antibody. The proteasome core particle subunit Pre1 was FLAG-tagged, and the expression level of Pre1 was examined using anti-FLAG antibody as a control. The times treated with doxycycline are indicated in parentheses.
**Rpn8 Interacts and Targets Pih1 for Proteasomal Degradation**

To confirm the mass spectrometry results, we analyzed R2TP and R2P complexes with immunoblotting. As shown in Fig. 3B, the Rpn8 signal is much more pronounced in the R2P complex purified from *tah1Δ* cells. Reintroducing Tah1 to the *tah1Δ* cells significantly reduced the association of Rpn8 with the R2P complex, whereas introducing an empty plasmid did not have any effect. Because Tah1 specifically binds to the C terminus of Pih1 (8, 12), these data suggest that Tah1 might compete with Rpn8 for binding to the R2P complex and that Rpn8-Rpn9 may bind to the C-terminal fragment of Pih1.

Rpn8 and Rpn9 form a stable interaction when they are part of the proteasome lid with at least five other subunits (34, 35). Our mass spectrometry analysis indicated that Rpn8 and Rpn9 were both associated with the R2P complex (Fig. 3A). To understand which subunit interacts directly with Pih1, we cloned, expressed, and purified individual Rpn8 and Rpn9 as well as a NusAHis6Pih1 fusion protein to increase the stability and solubility of Pih1 (36). By using an *in vitro* pull-down assay, we observed that NusAHis6Pih1 was able to interact with purified Rpn8, but not Rpn9 (Fig. 3C), suggesting that Rpn8, not Rpn9, directly interacts with Pih1. A model was proposed and shown in Fig. 3D to indicate the binding between Rpn8 and the R2P complex.

**The C-terminal Fragment Pih1(282–344) Is Sufficient for Rpn8 Interaction through Rpn8 C Terminus**—Because the Pih1 C-terminal fragment contains two intrinsically disordered regions and multiple degrons, we tested which region of the Pih1 C-terminal fragment is required for binding to Rpn8. Interestingly, His6-tagged GFP fusion protein pull-down assays showed that both Pih1(231–344) (Fig. 4A) and Pih1(282–344) (Fig. 4B) were able to bind to Rpn8. These data suggest that the mechanism of Pih1/Rpn8 binding is different from the one between Pih1 and Tah1, because Tah1 binds to the CS domain of Pih1 (20), and the fragment Pih1(282–344) alone is not able to bind Tah1 (8).

Rpn8 contains an MPN domain and forms a stable dimer with Rpn11, and the MPN domains in both Rpn8 and Rpn11 are required for substrate deubiquitination during proteasomal degradation (37). No atomic structure is currently available for any full-length proteasome lid subunit, but it was reported that all lid subunits have helical structures at their C termini, and the formation of a helical bundle drives the assembly of the lid subcomplex (38). Rpn8 contains a relatively long C-terminal intrinsically disordered region (Fig. 5A). To understand how Rpn8 binds to Pih1 and furthermore the physiological function of this interaction, we constructed two Rpn8 C-terminal truncation mutants, Rpn8(1–308) and Rpn8(1–290), which both also have a 3×FLAG tag at their C termini. Affinity purification of the 26S proteasome indicated that Rpn8(1–308) exists with other proteasome subunits with a roughly 1:1 stoichiometry, a profile similar to that of the one purified using the FLAG-tagged full-length Rpn8 (Fig. 5B). However, affinity chromatography using FLAG-tagged truncation mutant Rpn8(1–290) predominantly co-purified subunits Rpn5 and Rpn9 and other subunits, such as Rpn3, Rpn6, Rpn7, Rpn11, and Rpn12, were also co-purified but at a much lesser extent, as shown by the overall coverage of the peptides analyzed using tandem mass spectrometry (Table 2). These data suggest that Rpn8(1–290) does not associate with other subunits in the lid complex as stably as the full-length Rpn8 or Rpn8(1–308), and the C-ter-

---

**FIGURE 2. In vitro degradation of Pih1 by the 26S proteasome.** A, 26S proteasomes purified by affinity chromatography using FLAG-tagged Rpn1 (6.2 µg) or Rpn8 (5.1 µg) and analyzed by SDS-PAGE and Coomassie Brilliant Blue staining (left) or 4% native PAGE and in-gel peptidase activity assay using suc-LLVY-AMC (right). B, *in vitro* degradation of full-length Pih1 (Pih1[1–344]) or the Pih1 N-terminal domain Pih1(1–230) using 26S proteasome purified with FLAG-tagged Rpn1 or 20S proteasome purified with FLAG-tagged Pre1. Pih1(1–344) and Pih1(1–230) were expressed and purified from *E. coli*. The reaction mixtures were incubated without (−inhibitors) or with (+inhibitors) the presence of MG132 and lactacystin. The remaining substrate proteins were examined by immunoblotting using anti-Pih1 antibody. Anti-Pre10 and anti-FLAG immunoblots were used as internal controls for equal loading. C, *in vitro* degradation of model substrate UB4DHFR and DHFR (schematic diagram shown at the top) using purified 26S proteasome. Anti-His6 antibody was used to detect the remaining substrates. Both UB4DHFR and DHFR were His6-tagged and purified from *E. coli*. It should be noted that the ubiquitin moiety in the fusion protein is not cleavable by deubiquitination enzymes, such as Rpn11. FLAG-tagged Rpn1 served as a loading control.
minal disordered region Rpn8(309–338) is not required for the proteasome lid assembly.

To further understand the role of the Rpn8 C-terminal fragment, we fractionated the total cell lysates from yeast cells expressing different C-terminal truncation mutants by size exclusion chromatography. As shown in Fig. 5C, under the low salt condition with ATP, which preserves the 26S proteasome, the elution profile of the Rpn8(1–308)-containing complex, as detected by anti-Rpn8 antibody, is very similar to that of the non-truncated Rpn8(1–338)-containing complex. The Rpn8(1–290)-containing complex is eluted slightly differently; there seem to be more low molecular weight Rpn8(1–290) complexes. In the high salt elution condition, which promotes the disassociation of the 19S lid from the base, the Rpn8(1–308)-containing lid complex resembles more closely the wild type lid complex. In the control experiment, the elution profile of the proteasome base complex, as detected by the anti-Rpt1 antibody, did not show any difference in the two Rpn8 truncation mutants under either the low salt or the high salt condition. These data imply that the relative amount of the 26S proteasome in vivo or the assembly of the 26S proteasome might be slightly compromised in the Rpn8(1–290) strain.

Nonetheless, neither the strain with Rpn8(1–290) nor the one with Rpn8(1–308) showed any growth defect under normal growth conditions at 30 °C. We therefore tested the temperature sensitivity of Rpn8 truncation mutants at higher temperature. As shown in Fig. 6A, we only observed minor temperature sensitivity for Rpn8(1–290) cells and no obvious growth defect for Rpn8(1–308) cells in the W303 genetic background. Interestingly, the temperature sensitivity of Rpn8(1–290) is more pronounced in S288C cells (Fig. 6B). With the application of canavanine to induce proteolysis stress, we also observed slight temperature sensitivity in the Rpn8(1–308) strain (Fig. 6B, bottom). These data indicate that the Rpn8 C-terminal disordered region is not required for proteasome assembly but rather is needed for optimal growth under certain stress conditions.

Intrinsically disordered regions are often involved in protein-protein interactions (25, 39). We therefore investigated whether the Rpn8 C-terminal disordered region binds directly to the Pih1 C terminus. Both Rpn8(291–338) and Rpn8(309–338) were fused to the C terminus of GST, and in vitro pull-down analyses showed that Pih1(282–344) interacts with both Rpn8(291–338) and Rpn8(309–338) (Fig. 6C), suggesting that the Rpn8 C-terminal disordered region is sufficient to bind Pih1.
Rpn8 Interacts and Targets Pih1 for Proteasomal Degradation

between Rpn8-Rpn9 and Pih1 in the absence of Tah1 (Fig. 3A), presumably due to the exposure of the Pih1 C terminus. Identification of proteins within the Pih1 complex by mass spectrometry did not identify Rpn8 either (data not shown). Additionally, Pih1 does not seem to be a proteasome-associated protein or involved in proteasome assembly, because the 26S proteasome and its associated proteins have been extensively analyzed in yeast (40), and Pih1 has not been identified as a robust proteasome-associated protein. Because Pih1 serves as a scaffold protein in the R2TP complex, which has multiple functions in vivo (9), we propose that such an interaction triggers proteasomal degradation of Pih1 in a ubiquitin-independent manner.

To elucidate the possible ubiquitin-independent mechanism of Pih1 degradation, we took advantage of the fast degradation of GFP-Pih1(282–344) in vivo (Fig. 1) and analyzed its degradation when the cellular polyubiquitination pathway is compromised. We introduced GFP-Pih1(282–344) into a uba1-204 strain that is defective in the polyubiquitination pathway at 37 °C because of the temperature sensitivity of the E1 ubiquitin activation enzyme (29). As shown in Fig. 7, A and B, degradation of DHFR\(^\text{36}\), which is expressed from a Ub-R-DHFR\(^\text{36}\) cassette and a well documented ubiquitin-dependent proteasome substrate (41), is completely inhibited. The degradation of GFP-Pih1(282–344), however, is only slightly affected under the same condition (Fig. 7, A and C). This result suggests that degradation of GFP-Pih1(282–344) is primarily ubiquitin-independent.

There are five lysine residues within the Pih1(282–344) fragment to which the polyubiquitin chain could theoretically attach. To further explore the ubiquitin-independent degradation of Pih1, we mutated and replaced all five lysine residues within Pih1(282–344) to arginine, resulting in a Pih1(282–344\(\Delta K\)) fragment. Interestingly, Pih1(282–344\(\Delta K\)) induced a very similar, if not faster, degradation of GFP (Fig. 7D). Although these data cannot rule out the possibility that there are polyubiquitination sites on the Pih1(282–344) fragment, they indicate that no polyubiquitination site on the Pih1(282–344) fragment is required for the fast degradation of GFP-Pih1(282–344).

To further understand the mechanism by which Pih1 directly binds and gets degraded by the proteasome, we examined the direct interaction between Pih1 and the 19S particle. We analyzed the association of Pih1 in purified 19S complex or 26S proteasome by immunoblotting from normally grown yeast cells, but no significant binding of Pih1 was observed (data not shown). Nonetheless, we observed Pih1 association with the 19S complex after expressing extra Pih1 protein from a plasmid and using either Rpn8 or Rpn1 as bait to purify the 19S complex (Fig. 7E). The Pih1 association with 19S complex was significantly reduced if the Rpn8 C terminus was truncated (Rpn8(1–308)FLAG in Fig. 7E). Because Rpn1 is a base subunit and does not seem to form any assembly intermediate with Rpn8 (42), these data imply that Pih1 indeed interacts with the intact 19S complex, and the Rpn8 C-terminal fragment Rpn8(309–338) is involved in this interaction. However, the weak interaction or high degradation processivity in vivo does not allow more in depth studies on the direct interaction under normal conditions.

Truncation of Rpn8 C Terminus Inhibited Pih1(282–344)-mediated GFP Degradation in Vivo and Pih1 Degradation in Vitro—Because the C terminus of Rpn8 is required for binding to Pih1(282–344), we tested whether deletion of the Rpn8 C-terminal disordered region inhibits the in vivo degradation of GFP-Pih1(282–344). As shown in Fig. 8A and the quantitative analyses shown in Fig. 8C, the degradation of GFP-Pih1(282–344) is slower in the Rpn8(1–290) strain but not in the Rpn8(1–308) strain when compared with that in the wild type W303 strain. Interestingly, the degradation of DHFR\(^\text{36}\), the ubiquitin-dependent model substrate, is unaffected in the Rpn8(1–290) mutant strain (Fig. 8, A and D). It is likely that ubiquitin-dependent proteasomal degradation activity is not significantly affected in the mutant, whereas the ubiquitin-independent degradation of GFP-Pih1(282–344), specifically through the binding to the Rpn8 C terminus, is slightly impaired. Because truncation of the Rpn8 C terminus shows differential temperature sensitivity depending on the genetic background (Fig. 6), we reexamined the degradation of GFP-Pih1(282–344) in S288C strain in which the Rpn8 C-terminal disordered region Rpn8(309–338) was deleted. Interestingly, we observed a significant inhibition of GFP-Pih1(282–344) degradation when Rpn8(309–338) was truncated (Fig. 8, B and E), whereas the degradation of ubiquitin-dependent substrate DHFR\(^\text{36}\) was not significantly affected (Fig. 8, B and F).

To further confirm the role of the Rpn8 C-terminal fragment in binding and mediating the ubiquitin-independent degradation of Pih1, we purified the 26S proteasome from the yeast strain that expressed the C-terminally truncated and FLAG-
tagged Rpn8 and used the purified proteasomes to degrade Pih1 protein in vitro. Equivalent amounts of the 26S proteasomes with either wild type Rpn8 or Rpn8(1–308), as shown by examining the protein levels of Rpt1 and Pre10 (Fig. 9A), have similar peptidase activity in degrading the fluorogenic substrate SUC-LLVY-AMC (Fig. 9B) and ubiquitinated DHFR (Fig. 9C and E). However, the purified 26S proteasome with Rpn8(1–308) degrades Pih1 protein at a much slower rate compared with the 26S proteasome containing wild type Rpn8 (Fig. 9D and F), thus supporting the in vivo degradation data (Fig. 8) and further establishing the role of Rpn8 C-terminal fragment in directly mediating Pih1 degradation.

Discussion

The 26S proteasome is a macromolecular structure specialized to degrade misfolded or non-functional proteins that are marked by polyubiquitin chains. The 19S regulatory particle (RP) contains the ubiquitin chain recognition subunits Rpn10 and Rpn13 (43, 44), and the base of the RP unfolds and threads the substrate into the 20S core particle for degradation. The
MAY 27, 2016 • VOLUME 291 • NUMBER 22
JOURNAL OF BIOLOGICAL CHEMISTRY

Rpn8 Interacts and Targets Pih1 for Proteasomal Degradation

polyubiquitin chain is cleaved by Rpn11 (31) or other associated deubiquitin enzymes (45). In addition to recognition by RP, efficient degradation also requires a disordered initiation signal (46, 47). In this study, we showed that Pih1(282–344) fragment binds to Rpn8 C-terminal fragment (Figs. 4 and 6C) and is sufficient to trigger GFP degradation in a ubiquitin-independent manner (Figs. 7 and 8). This suggests that Pih1(282–344) functions as an autonomous degron, thus providing another example of a ubiquitin-independent protein substrate. Ubiquitin-independent proteasomal degradation has been observed for many proteins, such as ODC (L-ornithine decarboxylase) (48, 49), thymidylate synthase (50–52), and the yeast transcription factor Rpn4 (53, 54). The common feature of proteins degraded in a ubiquitin-independent manner is the presence of a disordered region that serves as a degron to both tether and initiate the unfolding (55–58). However, how the proteasome recognizes the disordered region is poorly understood. It has also been proposed that a disordered region with an adjacent helical structure in thymidylate synthase is required for the recognition by the 26S proteasome (50). In addition to the 20S proteasome, which is able to degrade a significant amount of proteins with intrinsically disordered regions independent of ubiquitin (59), the 19S RP has also been shown to be required for the ubiquitin-independent degradation of tumor suppressor proteins (60) as well as NOXA, a sensor of the proteasome integrity (61). Additionally, some proteins can be degraded by both the 26S and 20S proteasome. However, the exact subunit that is required for and/or directly associated with the binding of ubiquitin-independent substrates is still unclear. By using siRNA knockdown, it has been reported that the regulatory particle base subunits Rpt5 and Rpt6 are required for the ubiquitin-independent degradation of topoisomerase IIβ (62) and the Fran1 in tumor cells (63), whereas knockdown of the lid subunits Rpn11, Rpn2, and Rpn10 did not inhibit the degradation. This suggests that the unfolding process is required to facilitate both ubiquitin-dependent and -independent protein degradation, although these studies did not reveal the underlying mechanism of substrate recognition.

We identified the proteasome lid subunit Rpn8 as an interactor for Pih1, and the interaction is mediated through their C termini (Figs. 4 and 6C). In particular, we showed that C-terminal truncation mutant Rpn8(1–308) did not significantly impair the assembly of 26S proteasome (Figs. 5B and 9A) or the degradation of ubiquitin-dependent substrate both in vivo (Fig. 8) and in vitro (Fig. 9). Instead, the Rpn8(1–308) truncation significantly inhibited the degradation of GFP-Pih1(282–344) in vivo (Fig. 8) and Pih1 in vitro (Fig. 9), suggesting a role for the Rpn8 C-terminal fragment in the tethering and ubiquitin-independent degradation of Pih1. Taken together with the observation that purified 26S proteasome is able to degrade non-ubiquitinated Pih1 and the degradation being inhibited by the proteasome-specific inhibitors MG132 and lactacystin (Fig. 2), we propose that the Rpn8 C-terminal disordered region,
Rpn8((309–338)), acts as a receptor for Pih1 in the ubiquitin-
independent pathway, especially when the HSP90 co-chaperone Tah1 is absent (Fig. 3D). The Rpn8-Rpn11 complex is in close proximity to the gate of the AAA+/H11001 family unfoldase of the proteasome base, to facilitate the deubiquitination of substrates (42). The C terminus of Rpn8 is disordered, and its precise location is still unknown. It is plausible that Rpn8((309–338)) is in close proximity to the gate of the unfoldase, such that it could bind and direct ubiquitin-independent proteasomal substrates. It should be noted that deletion of Rpn8((308–338)) reduced the interaction between Pih1 and 19S complex, but the interaction is not completely abolished (Fig. 7E). It is clear that tethering of Rpn8 Interacts and Targets Pih1 for Proteasomal Degradation

![Image](image-url)
Pih1 to the 19S complex through Rpn8 C terminus is probably only one of the mechanisms to degrade Pih1 in vivo. As also shown in Figs. 8 and 9, even with deletion of the Rpn8 C terminus, degradation of GFP-Pih1(282–344) was slowed down but not completely blocked. It would be interesting to further investigate the other pathways by which Pih1 is degraded in vivo. Additionally, it would be worth investigating the binding interface between Pih1 and the 19S complex with other techniques, such as using chemical cross-linkers that may better reveal weak interactions.

Yeast cells expressing Rpn8(1–308) or Rpn8(1–290) grew as well as wild type cells under permissive temperature (Fig. 6, A and B, left), although the integrity of the 26S proteasome was not well preserved in Rpn8(1–290) cells. This is probably due to the fact that 26S proteasome is very abundant within the cells, and the growth defect is only visible when normal cellular pro-
tein homeostasis is challenged. In our study, cells expressing Rpn8(1–290) showed a severe growth defect at 37 °C, whereas cells expressing Rpn8(1–308) only showed slight temperature sensitivity at 37 °C when the protein degradation machinery is stressed by canavanine (Fig. 6). If the Rpn8 C-terminal fragment is a common receptor for multiple ubiquitin-independent substrates, it will provide only a supplementary, rather than a major, role in degrading abnormal proteins. However, we cannot rule out the possibility that ubiquitin-independent protein degradation via interaction with the Rpn8 C terminus might play a significant role in specific cell types or growth conditions. For example, most known proteins that undergo a ubiquitin-independent degradation pathway play critical regulatory roles, and many of them are oncoproteins or oncosuppressor proteins that are specifically degraded in fast dividing cancer cells (64, 65). The high energy demand of cancer cells may partially limit the energy-consuming polyubiquitination pathway and promote ubiquitin-independent degradation of oncoproteins and/or oncosuppressor proteins. Particularly, we did not observe temperature sensitivity for the Rpn8(1–308) mutant in the W303 strain but saw the temperature sensitivity for the Rpn8(1–308) mutant in S288C (Fig. 6). The commonly used S288C haploid cells contain an abnormally low amount of cytochrome c and assimilate several carbon sources at reduced rates (66), and some have a defect in mitochondria respiratory function due to the his3-Δ200 allele (67). Moreover, a previous study showed that C-terminal truncation of Rpn11 caused proteasome instability and mitochondrial morphology defects, and overexpression of Rpn8 corrected the proteasome instability defect but not the mitochondrial malfunction (68). Taken all
together, we propose that the Rpn8 C terminus might harbor a specific role that is more pronounced when the nutrition or energy status of the cells is challenged.

In agreement with the possible energy/nutrition status-regulated function of Rpn8, it was recently reported that the chaperone-like activity of R2TP in regulating the snoRNP complex assembly is modulated by the cell growth stage and the nutrition status (11), and the R2TP complex is even proposed to have functions in tumorigenesis (69). When yeast cells are in the fast growing stage, R2TP is mainly localized in the nucleus to regulate snoRNP assembly. Under nutrient limitation, it is located more in the cytoplasm. Similar to the R2TP complex, the majority of the 26S proteasome is also located in the nucleus (70). The biogenesis of the 26S proteasome starts with the assembly of subcomplexes within the cytoplasm. The subcomplexes are then transported separately to the nucleus, where they are assembled to the 26S proteasome in a modular manner (71). Similar to the growth stage-regulated subcellular localization of R2TP complex, the proteasome subcomplexes are also mainly trapped within the cytoplasm at stationary growth stage and transported to the nucleus during active cell proliferation (72). This implies that the cellular protein homeostasis, ribosome biogenesis, and cell growth are well coordinated and tightly regulated. In this study, we demonstrated the direct interaction between Pih1 and Rpn8 and showed that yeast cells expressing C-terminally truncated Rpn8 are temperature-sensitive, especially in cells prone to respiratory deficiencies. This study therefore provides a novel link that may coordinate the different cellular regulatory pathways and it deserves further investigation in the future.

**Author Contributions**—R. Z. conceived and coordinated the study and wrote the paper. A. P. and R. Z. designed, performed, and analyzed the experiments shown in Figs. 1–7 and 9. P. X. H. L. designed, performed, and analyzed the experiments shown in Fig. 8. L. Z. provided technical assistance and contributed to the preparation of Fig. 6C. All authors reviewed the results and approved the final version of the manuscript.

**Acknowledgments**—We thank Abelyn Lim for help with the degradation assays and Banujan Balachandran for help with constructing some of the FLAG-tagged strains. We also thank Nathaniel Anderson for help with the purification of protein complexes and yeast strain construction.

**References**

1. Zhao, R., Davey, M., Hsu, Y. C., Kaplanek, P., Tong, A., Parsons, A. B., Krokan, N., Cagnely, G., Mai, D., Greenblatt, J., Boone, C., Emili, A., and Houry, W. A. (2008) Navigating the chaperone network: an integrative map of physical and genetic interactions mediated by the hsp90 chaperone. Cell 120, 715–727

2. Zhao, R., Kakihara, Y., Gribun, A., Huen, J., Yang, G., Khanna, M., Costanzo, M., Brost, R. L., Boone, C., Hughes, T. R., Yip, C. M., and Houry, W. A. (2005) Navigating the chaperone network: an integrative map of physical and genetic interactions mediated by the hsp90 chaperone. Cell 120, 563–578

3. Boulon, S., Marmier-Gourrier, N., Pradet-Balade, B., Wurth, L., Verheugen, C., Jády, B. E., Rothé, B., Pescia, C., Robert, M. C., Kiss, T., Bardon, B., Krol, A., Brantl, C., Allmann, C., Bertrand, E., and Charpentier, B. (2008) The Hsp90 chaperone controls the biogenesis of L7Ae RNPs through conserved machinery. J. Cell Biol. 180, 579–595

4. Dong, F., Shibahara, K., Botilde, Y., Nabheshima, R., Asai, Y., Fukumoto, A., Hasegawa, T., Matsuo, M., Takeda, H., Shiratori, H., Nakamura, T., and Hamada, H. (2014) Pih1d3 is required for cytoplasmic preassembly of axonemal dynein in mouse sperm. J. Cell Biol. 204, 203–213

5. Ponsuksili, S., Murani, E., Trakooljul, N., Scherwin, M., and Wimmers, K. (2014) Discovery of candidate genes for muscle traits based on GWAS supported by eQTL-analysis. Int. J. Biol. Sci. 10, 327–337

6. Benbaouche He, N., Ilipoulos, I., Török, I., Marhold, J., Henri, J., Kajava, A. V., Farkas, R., Kempf, T., Schnöller, M., Meyer, P., Kiss, I., Bertrand, E., Mechler, B. M., and Pradet-Balade, B. (2014) Drosophila Spag is the homolog of RNA polymerase II-associated protein 3 (RPAP3) and recruits the heat shock proteins 70 and 90 (Hsp70 and Hsp90) during the assembly of cellular machineries. J. Biol. Chem. 289, 6236–6247

7. Ahmad, M., Afrin, F., and Tuteja, R. (2013) Identification of R2TP complex of Leishmania donovani and Plasmodium falciparum using genome wide in-silico analysis. Commun. Integr. Biol. 6, e26005

8. Paci, A., Liu, X. H., Huang, H., Lim, A., Houry, W. A., and Zhao, R. (2012) The stability of the small nucleolar ribonucleoprotein (snoRNP) assembly protein Pih1 in Saccharomyces cerevisiae is modulated by its C terminus. J. Biol. Chem. 287, 43205–43214

9. Kakihara, Y., and Houry, W. A. (2012) The R2TP complex: discovery and functions. Biochim. Biophys. Acta 1823, 101–107

10. Woolford, J. L., Jr., and Baerga, S. I. (2013) Ribosome biogenesis in the yeast Saccharomyces cerevisiae. Genetics 195, 643–661

11. Kakihara, Y., Macknevych, T., Zhao, L., Tang, W., and Houry, W. A. (2014) Nutritional status modulates box C/D snoRNP biogenesis by regulated subcellular relocalization of the R2TP complex. Genome Biol. 15, 404

12. Jiménez, B., Ugwu, F., Zhao, R., Ortiz, L., Macknevych, T., Pineda-Lucena, A., and Houry, W. A. (2012) The structure of the minimal tetratricopeptide repeat domain protein Tah1 reveals the mechanism of its interaction with Pih1 and Hsp90. J. Biol. Chem. 287, 5698–5709

13. Back, R., Dominguez, C., Rothé, B., Bobo, C., Beaufils, C., Moréra, S., Meyer, P., Charpentier, B., Brantl, C., Allain, F. H., and Manivel, X. (2013) High-resolution structural analysis shows how Tah1 tethers Hsp90 to the R2TP complex. Structure 21, 1834–1847

14. Prieto, M. B., Georg, R. C., Gonzales-Zubiate, F. A., Luz, J. S., and Oliveira, C. C. (2015) Nop17 is a key R2TP factor for the assembly and maturation of box C/D snoRNP complex. BMC Mol. Biol. 16, 7

15. Rothé, B., Back, R., Quintermier, M., Bizarro, J., Robert, M. C., Bland, M., Romier, C., Manivel, X., Charpentier, B., Bertrand, E., and Brantl, C. (2014) Characterization of the interaction between protein Snurp15/15.5K and the Rsa1p/NUFIP factor and demonstration of its functional importance for snoRNP assembly. Nucleic Acids Res. 42, 2015–2036

16. Rothé, B., Saliou, J. M., Quintermier, M., Back, R., Tiotiu, D., Jacquier, C., Loegler, C., Schlottar, F., Péna, V., Eckert, K., Moréra, S., Dorsselaer, A. V., Brantl, C., Massenet, S., Sanglier-Cianferani, S., Manivel, X., and Charpentier, B. (2014) Protein Hit1, a novel box C/D snoRNP assembly factor, controls cellular concentration of the scaffolding protein Rsa1 by direct interaction. Nucleic Acids Res. 42, 10731–10747

17. Boulon, S., Pradet-Balade, B., Verheugen, C., Molle, D., Boireau, S., Georgieva, M., Azzag, K., Robert, M. C., Ahmad, Y., Neel, H., Lamond, A. I., and Bertrand, E. (2010) Hsp90 and its R2TP/Prefoldin-like co-chaperone are involved in the cytoplasmic assembly of RNA polymerase II. Mol. Cell 39, 912–924

18. Horéjši, Z., Takai, H., Adelman, C. A., Collis, S. J., Flynn, H., Maslen, S., Skehel, J. M., de Lange, T., and Boulton, S. J. (2010) CK2 phospho-dependent binding of R2TP complex to Tel2 is essential for mTOR and SMG1 stability. Mol. Cell 39, 839–850

19. Horéjši, Z., Stach, L., Flower, T. G., Joshi, D., Flynn, H., Skehel, J. M., O’Reilly, N. J., Ogodrowicz, R. W., Smerdon, S. J., and Boulton, S. J. (2014) Phosphorylation-dependent PIH1D1 interactions define substrate specificity of the R2TP co-chaperone complex. Cell Rep. 7, 19–26

20. Pal, M., Morgan, M., Phelps, S. E., Roe, S. M., Parry-Morris, S., Downs, J. A., Polier, S., Pearl, L. H., and Prodromou, C. (2014) Structural basis for phosphorylation-dependent recruitment of Tel2 to Hsp90 by Pih1. Structure 22, 805–818
Rpn8 Interacts and Targets Pih1 for Proteasomal Degradation

21. Zhang, M., Boté, M., Li, K., Kadota, Y., Panaretou, B., Prodomou, C., Shirasu, K., and Pearl, L. H. (2008) Structural and functional coupling of Hsp90- and Sgr1-centred multi-protein complexes. *EMBO J.* 27, 2789–2798

22. Ali, M. M., Roe, S. M., Vaughan, C. K., Meyer, P., Panaretou, B., Piper, P. W., Prodomou, C., and Pearl, L. H. (2006) Crystal structure of an Hsp90-nucleotide-p23/Sha1 closed chaperone complex. *Nature* 440, 1013–1017

23. Quinternet, M., Rothé, B., Barbier, M., Bobo, C., Salou, J. M., Jacquemin, C., Back, R., Chagot, M. E., Cianférani, S., Meyer, P., Branlant, C., Charpentier, B., and Manival, X. (2015) Structure/function of protein-protein interactions developed by the yeast Pih1 platform protein and its partners in box C/D snoRNP assembly. *J. Mol. Biol.* 427, 2816–2839

24. Eckert, K., Salou, J. M., Monlezun, L., Vigouroux, A., Atmane, N., Cailla, C., Quevillon-Chéruel, S., Madiona, K.,Nicea, M., Lazereg, S., Van Dorsselaer, A., Sanglier-Cinèré, S., Meyer, P., and Moréra, S. (2010) The Pih1-Tah1 chaperone complex inhibits Hsp90 molecular chaperone ATPase activity. *J. Biol. Chem.* 285, 31304–31312

25. Mészáros, B., Simon, I., and Dosztányi, Z. (2011) The expanding view of protein-protein interactions: complexes involving intrinsically disordered proteins. *Phys. Biol.* 8, 035003

26. Prakash, S., Inobe, T., Hatch, A. J., and Matouschek, A. (2009) Substrate selection by the proteasome during degradation of protein complexes. *Nat. Chem. Biol.* 5, 29–36

27. Chen, P., Johnson, P., Sommer, T., Jentsch, S., and Hochstrasser, M. (1993) Multiple ubiquitin-conjugating enzymes participate in the *in vivo* degradation of the yeast MAT α2 repressor. *Cell* 74, 357–369

28. Longtie, M. S., McKenzie, A. 3rd, Demarini, D. J., Shah, N. G., Wach, A., Bracht, A.,Philippens, P., and Pringle, J. R. (1998) Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast* 14, 953–961

29. Ghaobosi, N., and Deshaies, R. J. (2007) A conditional yeast E1 mutant blocks the ubiquitin-proteasome pathway and reveals a role for ubiquitin conjugates in targeting Rad23 to the proteasome. *Mol. Biol. Cell* 18, 1953–1963

30. Shen, X. (2004) Preparation and analysis of the INO80 complex. *Methods Enzymol.* 377, 401–412

31. Verma, R., Aravind, L., Oania, R., McDonald, W. H., Yates, J. R., 3rd, Koonin, E. V., and Deshaies, R. J. (2002) Role of Rpn11 metalloprotease in ubiquitination and degradation by the 26S proteasome. *Science* 298, 611–615

32. Maimone, S., Davierwala, A. P., Haynes, J., Moffat, J., Peng, W. T., Zhang, W., Yang, P., Pootoolal, J., Chua, G., Lopez, A., Trochesset, M., Morse, D., Krogan, N. J., Hiley, S. L., Li, Z., et al. (2004) Exploration of essential gene functions via titratable promoter alleles. *Cell* 118, 31–44

33. Belle, A., Tanay, A., Bitincka, L., Shamir, R., and O’Shea, E. K. (2006) Quantification of protein half-lives in the budding yeast proteome. *Proc. Natl. Acad. Sci. U.S.A.* 103, 13004–13009

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

35. Lasker, K., Förster, F., Bohn, S., Walzthoeni, T., Villa, E., Unverdorben, P., Beck, F., Abebersol, R., Sali, A., and Baumeister, W. (2012) Molecular architecture of the 26S proteasome holocomplex determined by an integrative approach. *Proc. Natl. Acad. Sci. U.S.A.* 109, 1380–1387

36. Davis, G. D., Elisee, C., Newham, D. M., and Harrison, R. G. (1999) New fusion protein systems designed to give soluble expression in *Escherichia coli*. *Biotechnol. Bioeng.* 65, 382–388

37. Worden, E. J., Padovan, C., and Martin, A. (2014) Structure of the Rpn11-Rpn8 dimer reveals mechanisms of substrate deubiquitination during proteasomal degradation. *Nat. Struct. Mol. Biol.* 21, 220–227

38. Estrin, E., Lopez-Blanco, J. R., Chacón, P., and Martin, A. (2013) Formation of an intricate helical bundle dictates the assembly of the 26S proteasome lid. *Structure* 21, 1624–1635

39. Tompa, P. (2002) Intrinsically unstructured proteins. *Trends Biochem. Sci.* 27, 527–533

40. Verma, R., Chen, S., Feldman, R., Schieltz, D., Yates, J., Dohmen, J., and Deshaies, R. J. (2000) Proteasomal proteomics: identification of nucleus-
a novel ubiquitin-independent proteasome pathway when RNA polymerase II encounters a protein roadblock. *Mol. Cell Biol.* **33**, 4008–4016

63. Pakay, J. L., Diesch, J., Gilan, O., Yip, Y. Y., Sayan, E., Kolch, W., Mariadason, J. M., Hannan, R. D., Tulchinsky, E., and Dhillon, A. S. (2012) A 19S proteasomal subunit cooperates with an ERK MAPK-regulated degron to regulate accumulation of Fra-1 in tumour cells. *Oncogene* **31**, 1817–1824

64. Stintzing, S., and Lenz, H. J. (2014) Molecular pathways: turning proteasomal proteindegradation into a unique treatment approach. *Clin. Cancer Res.* **20**, 3064–3070

65. Shen, M., Schmitt, S., Buac, D., and Dou, Q. P. (2013) Targeting the ubiquitin-proteasome system for cancer therapy. *Expert Opin. Ther. Targets* **17**, 1091–1108

66. Sherman, F. (1991) Getting started with yeast. *Methods Enzymol.* **194**, 3–21

67. Young, M. J., and Court, D. A. (2008) Effects of the S288c genetic background and common auxotrophic markers on mitochondrial DNA function in *Saccharomyces cerevisiae*. *Yeast* **25**, 903–912

68. Rinaldi, T., Pick, E., Gambadoro, A., Zilli, S., Maytal-Kivity, V., Frontali, L., and Glickman, M. H. (2004) Participation of the proteasomal lid subunit Rpn11 in mitochondrial morphology and function is mapped to a distinct C-terminal domain. *Biochem. J.* **381**, 275–285

69. Kakihara, Y., and Saeki, M. (2014) The R2TP chaperone complex: its involvement in snoRNP assembly and tumorigenesis. *Biomol. Concepts* **5**, 513–520

70. Huh, W. K., Falvo, J. V., Gerke, L. C., Carroll, A. S., Howson, R. W., Weissman, J. S., and O’Shea, E. K. (2003) Global analysis of protein localization in budding yeast. *Nature* **425**, 686–691

71. Gu, Z. C., and Enenkel, C. (2014) Proteasome assembly. *Cell Mol. Life Sci.* **71**, 4729–4745

72. Enenkel, C. (2014) Nuclear transport of yeast proteasomes. *Biomolecules* **4**, 940–955