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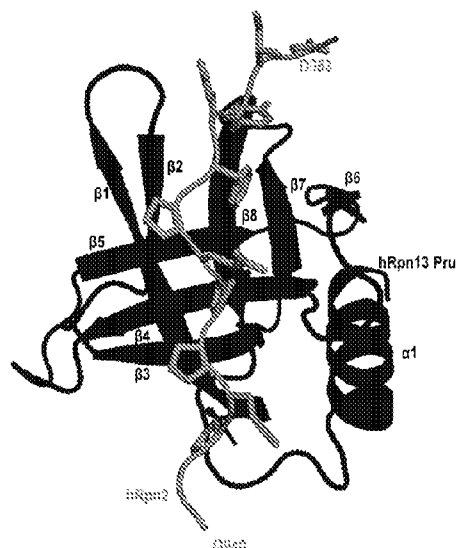
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[Continued on next page]

(54) Title: HUMAN RPN2 DERIVED PEPTIDES USEFUL FOR TREATING CANCER

FIGURE 8.

B



(57) Abstract: The disclosure includes peptides derived from the C-terminus of proteasome PC repeat protein hRpn2/PSMD1, which binds to hRpn13 Pru domain with high affinity. Amino acids F948, Y950, and I951 were identified as necessary for the interaction of hRpn2 with hRpn13. These peptides are useful for treating diseases and disorders which are modulated by proteolysis via the ubiquitin-proteasome pathway. Such diseases and disorders include a viral infection, a neurodegenerative disorder, an inflammatory disorder, or cancer, especially hematological cancer, HPV associated cancer, ovarian cancer, prostate cancer, breast cancer, gastric cancer, or colorectal cancer. The disclosure includes methods of treating cancer in a patient comprising administering a peptide of the disclosure to the patient. The peptide may be the only chemotherapeutic administered to the patient or may be administered together with an additional chemotherapeutic agent.



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HUMAN RPN2 DERIVED PEPTIDES USEFUL FOR TREATING CANCER

CROSS REFERENCE TO RELATED APPLICATION

[0001] This application claims priority of U.S. provisional application nos. 62/222,530, filed 23 September 2015, and 62/232,144, filed 24 September 2015, both of which are incorporated by reference in their entirety.

BACKGROUND

[0002] The ubiquitin-proteasome pathway is essential for regulated protein degradation in eukaryotes, enabling orderly cell cycle progression, clearance of misfolded proteins, numerous signaling mechanisms, and general protein homeostasis. Proteins are ubiquitinated by an enzymatic cascade and then recognized by ubiquitin receptors in the 26S proteasome, where ubiquitin is recycled and protein substrates degraded.

[0003] The proteasome has three major ubiquitin receptors Rpn10, Rpn13, and more recently identified Rpn1. Rpn13 has emerged as a therapeutic target for human cancers. Its ubiquitin-binding activity is confined to an N-terminal Pru (pleckstrin-like receptor for ubiquitin) domain that also docks it into the proteasome, while its C-terminal DEUBAD (DEUBiquitinase ADaptor) domain recruits deubiquitinating enzyme Uch37 to the proteasome. Bis-benzylidine piperidone derivatives that bind covalently to Rpn13 C88 cause the accumulation of polyubiquitinated proteins as well as ER stress-related apoptosis in various cancer cell lines, including bortezomib-resistant multiple myeloma lines.

[0004] 26S proteasome inhibitors, including carfilzomib and bortezomib, that act at the proteasome catalytic site are used to treat hematological cancers. However, resistance to these agents and their toxicity drives the need for new therapeutic strategies. Additionally the toxicity of carfilzomib and bortezomib limits their use for treatment of human cancers. Patients are not able to withstand the higher levels of these compounds needed to treat solid tumors. The toxicity of these compounds is hypothesized to be due to their direct and high level of inhibition of proteasome catalytic activity.

[0005] Rpn2 (26S Proteasome Regulatory Subunit 2) interacts with Rpn13 and activates Rpn13 for ubiquitin binding. Disruption of the Rpn2/ Rpn13 interaction inhibits proteolysis by the ubiquitin-proteasome pathway, but through a different mechanism than the approved proteasome inhibitors, carfilzomib and bortezomib. RA190 has previously been identified as a Rpn13 inhibitor efficacious against multiple myeloma and ovarian cancer xenografts. There is a demonstrated need for additional Rpn13 inhibitors for treating cancer, particularly solid tumors. This disclosure fulfills the need for additional Rpn13 inhibitors and has additional advantages.

SUMMARY

[0006] The disclosure includes a 38-amino acid peptide derived from the C-terminus of proteasome PC repeat protein hRpn2/PSMD1 which binds to hRpn13 Pru domain with 12 nM affinity. hRpn13-interacting amino acids in this 38-amino acid hRpn2 fragment, some of which are conserved among eukaryotes, were identified using NMR. hRpn2-derived peptides are shown to immunoprecipitate endogenous Rpn13 from 293T cells, and to displace it from the proteasome. These findings indicate that this region of hRpn2 is the primary binding site for hRpn13 in the proteasome. Amino acids F948, Y950, and I951 were identified as necessary for the interaction of hRpn2 with hRpn13. Finally, over-expression of the hRpn2-derived peptide leads to an increased presence of ubiquitinated proteins in 293T cells. Based on these findings hRpn2-derived peptides capable of specifically targeting hRpn13 function in the proteasome are disclosed herein.

[0007] The disclosure includes a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a peptide, the peptide comprising the amino acid sequence of

SEQ ID NO: 1, wherein 1 to 24 amino acids other than P942, P944, P945, P947, F948, Y950, and I951 are optionally deleted or replaced by another naturally occurring or modified amino acid;

SEQ ID NO: 11 (hRpn2 940-952), wherein 1, 2, 3, 4, or 5 amino acids of SEQ ID NO: 11 other than proline, phenylalanine, tyrosine, or isoleucine, are optionally replaced by another naturally occurring or modified amino acid;

SEQ ID NO: 11, wherein 1 or 2 amino acids other than proline, phenylalanine, tyrosine, or isoleucine, are deleted;

SEQ ID NO: 12, (an hRpn2 derived peptide in which Pro 942, 944, 945, and 947, Phe 948, Tyr 950, Ile 951, and Asp 952) wherein each Xaa is an independently chosen naturally occurring amino acid; or

SEQ ID NO: 13 (an hRpn2 derived peptide in which Pro 942, 944, 945, and 947, Phe 948, Tyr 950, and Ile 951 are conserved and the remaining amino acids are variable), wherein each Xaa is an independently chosen naturally occurring amino acid.

[0008] The disclosure also includes a peptide, and pharmaceutical compositions comprising the peptide and a carrier, in which the peptide has 50%, 60%, 70%, 80, 90% or 95% sequence homology to any one of SEQ ID NO: 1, 10, 11, 12, or 13, and capable of forming van der Waals interactions to hRpn13 methyl groups M31, L33, T36, V38, V85, V93, and V95 and to hRpn13 W108.

[0009] The disclosure includes methods of treating cancer in a patient comprising administering a peptide of the disclosure to the patient. The peptide may be the only chemotherapeutic administered to the patient or may be administered together with an additional chemotherapeutic agent.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] FIGURE 1. The C-terminal region of hRpn2 binds to the hRpn13 Pru domain. Chemical shift perturbation (CSP) values derived from ^1H - ^{15}N spectra of hRpn2 (916-953) alone and with equimolar equivalents of hRpn13 Pru for each hRpn2 amino acid. Prolines, which lack amide signals, as well as unassigned Q940 and E941 are omitted from this analysis and indicated with an asterisk or circle, respectively.

[0011] FIGURE 2. An hRpn2-Derived Peptide Binds to the hRpn13 Pru Domain with 12 nM Affinity. (A) 200 μM hRpn2 (916-953) was injected into a calorimeter cell that contained 20 μM hRpn13 Pru. The binding isotherm (top) was integrated to yield the change in enthalpy as a function of hRpn2 peptide (bottom). (B) The data fit well to a 1-site binding mode with the indicated thermodynamic values.

[0012] FIGURE 3. Order parameters (S^2) for hRpn2 (black) and its Rpn13-bound state (grey). In this analysis, a value below 0.5 is dynamic. This was generated by TALOS+ by using NMR chemical shift assignments for N, HN, C', C α , and C β atoms.

[0013] FIGURE 4. Strictly conserved F948 and Y950/I951 are required for hRpn2 interaction with hRpn13 in 293T cells. (A) 293T cells were transfected with or without 0.5 μg p3XFLAG-CMV7.1-hRpn2 (916-953) plasmid and the cell lysates immunoprecipitated by anti-FLAG antibody and immunoprobed for proteasome subunits hRpn13, hRpt3, and hRpn2, as indicated. (B) Cell lysates (DL), direct load, or (IP), immunoprecipitates, from 293T cells expressing FLAG-hRpn2 (916-953) wild-type (WT) or with the indicated mutations were subjected to immunoprobings with anti-Rpn13 antibody, as indicated. LC, light chain. An untransfected negative control was included. (C) Alignment across species for the C-terminal 38 amino acids of Rpn2. Strictly and moderately conserved amino acids are shaded in black and grey, respectively. This figure was generated by using ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>).

[0014] FIGURE 5. The hRpn2-derived peptide depletes hRpn13 from the proteasome. (A) 293T cells were transfected with p3XFLAG-CMV7.1-hRpn2 (916-953) WT or F948Stop plasmids and the cell lysates immunoprecipitated with anti-hRpt3 antibodies and immunoprobed with antibodies against hRpn13, hRpt3, and hRpn2, as indicated. (B) Cell lysates from 293T cells transfected with HA-ubiquitin alone or together with either p3XFLAG-CMV7.1-hRpn2 (916-953) WT or F948Stop plasmids were immunoblotted with anti-ubiquitin and anti- β -actin antibodies, as indicated.

[0015] FIGURE 6. Highly dynamic hRpn2 (916-953) becomes ordered at the C-terminal region upon binding to hRpn13 Pru. Plots of heteronuclear NOE enhancement values (hetNOE) for hRpn2 (916-953) (solid squares) and hRpn2 (916-953):hRpn13 Pru (open circles). Prolines, unassigned residues (Q940 and E941), and those with too much overlap for reliable integration are indicated by asterisks. Error bars are small and indicated, as calculated by using relax.

[0016] FIGURE 7. hRpn13 is required at the proteasome for robust clearance of ubiquitinated proteins. (A) Dissociation constants (K_d) measured by ITC of hRpn13 Pru for various hRpn2-derived peptides, spanning D916-D953, Q940-D953, P944-D953 and P944-D953 with E946Q, E949Q and D953N substitutions (QQN). (B) Cell lysates or immunoprecipitates derived by anti-FLAG (upper panel) or anti-hRpt3 (lower panel) antibodies from 293T cells expressing FLAG-EGFP (vector), FLAG-EGFP-hRpn2 (916–953), FLAG-EGFP-hRpn2 (940–953) or FLAG-EGFP-hRpn2 (940–947) were subjected to immunoprobings, as indicated. (C) 293T cells expressing FLAG-EGFP (vector) or FLAG-EGFP-hRpn2 (940-953) were treated with cross-linker DSP and the cell lysates or immunoprecipitates (derived by anti-hRpt3 antibodies) were treated with 100 mM DTT, resolved, and immunoprobed for ubiquitin and hRpt3, as indicated.

[0017] FIGURE 8. hRpn2 zippers along an hRpn13 surface with extensive interactions and a proline rich contact surface (A) Backbone heavy atoms for the ten lowest energy structures with best geometry for the hRpn13 Pru:hRpn2 (940-953) complex with hRpn13 displayed in black and hRpn2 is grey. (B) Ribbon diagram for the hRpn13 Pru: hRpn2 (940-953) structure depicting the classic pleckstrin homology fold of hRpn13 Pru (black) and the hRpn2 peptide (grey) extended across a β -strand surface.

[0018] FIGURE 9. The C-terminal 14 amino acids of hRpn2 bind to hRpn13 Pru with 27 nM binding affinity. (A) 200 μ M hRpn2 (940–953) or hRpn2 (944-953) or 1 mM hRpn2 (944-953) QQN mutant was injected into a calorimeter cell containing 20, 18 or 90 μ M hRpn13 Pru, respectively. The binding isotherms (top) were integrated to yield the change in enthalpy as a function of hRpn2 peptide addition. The data fit well to a 1-site binding mode with the indicated thermodynamic values. (B) Graphs of secondary structure (upper panel) and order parameter S^2 (lower panel) for hRpn2-bound hRpn13 (1-150) calculated by TALOS+ (<http://spin.niddk.nih.gov/bax/software/TALOS/>) based on chemical shift assignments.

DETAILED DESCRIPTION

TERMINOLOGY

[0019] The terms “a” and “an” do not denote a limitation of quantity, but rather denote the presence of at least one of the referenced item. The term “or” means “and/or”. The open-ended transitional phrase “comprising” encompasses the intermediate transitional phrase “consisting essentially of” and the close-ended phrase “consisting of.” Claims reciting one of these three transitional phrases, or with an alternate transitional phrase such as “containing” or “including” can be written with any other transitional phrase unless clearly precluded by the context or art. Recitation of ranges of values are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. The endpoints of all

ranges are included within the range and independently combinable. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., “such as”), is intended merely to better illustrate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention as used herein. Unless defined otherwise, technical and scientific terms used herein have the same meaning as is commonly understood by one skilled in the art to which this invention belongs.

[0020] An “active agent” means a compound (including a compound disclosed herein), element, or mixture that when administered to a patient, alone or in combination with another compound, element, or mixture, confers, directly or indirectly, a physiological effect on the patient. The indirect physiological effect may occur via a metabolite or other indirect mechanism.

[0021] A “dosage form” means a unit of administration of an active agent. Examples of dosage forms include tablets, capsules, injections, suspensions, liquids, emulsions, creams, ointments, suppositories, inhalable forms, transdermal forms, and the like.

[0022] “Pharmaceutical compositions” are compositions comprising at least one active agent, such as a compound or salt of Formula (I), and at least one other substance, such as a carrier. Pharmaceutical compositions optionally contain one or more additional active agents. When specified, pharmaceutical compositions meet the U.S. FDA’s GMP (good manufacturing practice) standards for human or non-human drugs.

[0023] The term “carrier” applied to pharmaceutical compositions/combinations of the invention refers to a diluent, excipient, or vehicle with which an active compound is provided. To be pharmaceutically acceptable a carrier must be safe, non-toxic and neither biologically nor otherwise undesirable.

[0024] A “patient” is a human or non-human animal in need of medical treatment. Medical treatment can include treatment of an existing condition, such as a disease or disorder, prophylactic or preventative treatment, or diagnostic treatment. In certain embodiments disclosed herein “medical treatment” means treatment of a diagnosed cancer or known tumor. In certain embodiments the patient is a human patient.

[0025] “Provided together with at least one additional active agent” means the hRpn13 binding peptide of the disclosure and the additional active agent(s) are provided simultaneously in a single dosage form, provided concomitantly in separate dosage forms, or provided in separate dosage forms for administration separated by some amount of time that is within the time in which both the hRpn13 binding peptide of the disclosure and the at least one additional active agent are within the blood stream of a patient. In certain embodiments means the hRpn13 binding peptide of the disclosure and the additional active agent need not be prescribed for a patient by the same medical

care worker. In certain embodiments the additional active agent or agents need not require a prescription. Administration of means the hRpn13 binding peptide of the disclosure or the at least one additional active agent can occur via any appropriate route, for example, oral tablets, oral capsules, oral liquids, inhalation, injection, suppositories or topical contact.

[0026] “Treatment,” as used herein includes providing the hRpn13 binding peptide of the disclosure, either as the only active agent or together with at least one additional active agent sufficient to: (a) slow the progression of a disease, (b) halt the progression of a disease, i.e. arresting its development; and (c) relieving the patient of cancer disease, i.e., the disease is no longer detectable and the patient no longer experiences symptoms. “Treating” and “treatment” also means providing a therapeutically effective amount of a means the hRpn13 binding peptide of the disclosure as the only active agent or together with at least one additional active agent to a patient having a disease. In certain embodiments the disease is cancer.

[0027] A “therapeutically effective amount” of a pharmaceutical composition/ combination of this invention means an amount effective, when administered to a patient, to provide a therapeutic benefit such as an amelioration of symptoms, e.g., an amount effective to decrease the symptoms of cancer. For example a patient having cancer may present detectable levels of certain tumor markers, including CA 125, CEA, CA19-9, AFP, PSA, and galactosyl transferase. A therapeutically effect amount is thus an amount sufficient to provide a significant reduction in elevated tumor marker levels or an amount sufficient to provide a return of tumor marker levels to the normal range. A therapeutically effective amount is also an amount sufficient to prevent a significant increase in tumor size relative that usually seen in untreated patients having the same cancer, or significantly reduce tumor size or tumor number, or causes tumors to disappear from the patient’s body altogether.

[0028] A significant increase or reduction in the detectable level of tumor markers, tumor size, or tumor number, is any detectable change that is statistically significant in a standard parametric test of statistical significance such as Student’s T-test, where $p < 0.05$.

HRPN13 BINDING PEPTIDES

[0029] It has been discovered that Rpn13 binds strongly to a peptide derived from the C-terminal region of its neighbor in the proteasome Rpn2. This Rpn2-derived peptide binds endogenous Rpn13 in human cells, and it also displaces Rpn13 from the proteasome. This disclosure maps the amino acids crucial for Rpn13 interaction with the proteasome, and identifies a peptide useful for therapeutic indications, including treating cancerous hematological and solid tumors.

[0030] An *in vitro* GST pull-down assay previously demonstrated direct interaction between hRpn13 and hRpn2, with the hRpn13 Pru domain required and sufficient for this interaction and the C-terminus of hRpn2 required (Hamazaki, J., *EMBO J.* (2006) 25: 4524-4536). This binding site was narrowed down to the extreme C-terminal 20 amino acids in *Saccharomyces cerevisiae* Rpn2 (He, J., *Structure*, (2012) 20: 513-521). This disclosure includes the hRpn13-binding site on hRpn2. This

disclosure also provides peptides derived from the C-terminus of hRpn2 that bind hRpn13 with high affinity, including peptides having a dissociation constant of less than 100 nM, less than 50 nM, and less than 20 nM. In an embodiment the disclosure provides a peptide that binds hRpn13 with a dissociation constant of 12 nM. NMR spectroscopy and cell biology techniques used to identify the amino acids in hRpn2 that are critical for hRpn13 assembly into the proteasome are described herein. The disclosure further includes hRpn2-derived peptides capable of immunoprecipitating endogenous hRpn13 and displacing it from proteasome.

[0031] A 38-amino acid peptide derived from the C-terminal end of hRpn2 (amino acids 916-953, SEQ ID NO: 1) that binds to hRpn13 with high affinity (FIGURE 2) is disclosed herein. The key interacting amino acids are identified via NMR spectroscopy. The region of hRpn2 that interacts with hRpn13 is less dynamic than the remainder of the peptide, and has a propensity toward forming a β -strand. This peptide is capable of binding to full-length hRpn13 in mammalian cells (FIGURE 4). Replacement of a strictly conserved phenylalanine (F948) with arginine abrogates this interaction. This phenylalanine is not sufficient for binding however, as aspartic acid substitution of Y950 and I951 also eliminates the interaction of the hRpn2 peptide with hRpn13.

[0032] The hRpn2-derived peptide has been found to displace hRpn13 from the proteasome (FIGURE 5). This finding highlights the importance of Rpn2 in docking Rpn13 to the proteasome. It also demonstrates the utility of this peptide for therapeutic use.

[0033] The disclosure further provides peptides which bind Rpn13 with high affinity but have sequences considerably shorter than SEQ ID NO: 1. Amino acids F948, Y950, and I951 are conserved in these peptides.

[0034] hRpn2 peptides were generated from the known hRpn13-binding region and tested for binding to hRpn13 by isothermal titration calorimetry (ITC). A dissociation constant (K_d) of 27 ± 10 nM was found for the binding of hRpn2 (940-953) to hRpn13 Pru (FIGURE 7A and FIGURE 9A). Further truncation to hRpn2 (944-953) impaired binding, with an increased K_d value of 1.96 ± 0.22 μ M (FIGURE 7A and FIGURE 9A). hRpn2 (940-953) was next tested to determine whether it interacts with endogenous hRpn13 in 293T cells. FLAG-EGFP-hRpn2 (940-953) or FLAG-EGFP (as a vector control) was expressed in 293T cells, immunoprecipitated from cell lysates by anti-FLAG antibodies, and tested for interaction with hRpn13 by immunoprobining with anti-hRpn13 antibodies (FIGURE 7B, top panel, lane 6). Endogenous hRpn13 immunoprecipitated with hRpn2 (940-953) and a longer construct encompassing 916-953; no such co-immunoprecipitation was observed for FLAG-EGFP (FIGURE 7B, top panel).

[0035] hRpn2 (940-953) was tested to determine whether it can be used to remove hRpn13 from proteasomes. Proteasomes from lysates of cells expressing FLAG-EGFP-hRpn2 (940-953) or FLAG-EGFP (vector control) were immunoprecipitated by anti-hRpn13 antibodies and the presence of endogenous hRpn13 at the proteasome immunoprobed by anti-Rpn13 antibodies. This experiment

revealed loss of hRpn13 at proteasomes in cells expressing hRpn2 (940-953) (FIGURE 7B, third panel, lane 6). Thus, this 14 amino acid region in hRpn2 encompasses the hRpn13-docking site at the proteasome and can be used to test the impact of Rpn13 loss from proteasomes.

[0036] To test whether Rpn13 is required at the proteasome for robust degradation of ubiquitinated proteins, a cross-linking-coupled denaturing immunoprecipitation experiment was performed. 293T cells expressing FLAG-EGFP-hRpn2 (940-953) or FLAG-EGFP (vector control) were treated with dithiobis(succinimidyl) propionate (DSP) for 30 minutes, lysed in radioimmunoprecipitation (RIPA) buffer, and then proteasomes immunoprecipitated with anti-hRpt3 antibodies. Immunoprobings for ubiquitinated proteins revealed an increase of ubiquitinated proteins in cell lysates (FIGURE 7C, left panel) corresponding with a reduction at the proteasome (FIGURE 7C, right panel). Altogether, these data define the hRpn13-binding site at the proteasome to be a 14-amino acid C-terminal region in hRpn2 and indicate that the presence of hRpn13 at the proteasome is essential for robust clearance of ubiquitinated proteins. The hRpn13-binding region in hRpn2 spanning Q940-D953 is referred to as R13IM (Rpn13 Interacting Motif).

[0037] Applicants have determined that hRpn2 zippers along an hRPN13 surface with extensive interactions. The showing above that R13IM can be used to remove hRpn13 from proteasomes and encompasses the full hRpn2 binding site on hRpn13 led applicants to solve the structure of the R13IM/ hRpn13 complex, via NMR. In total, chemical shift values were assigned to 94 and 93 percent of the hRpn13 Pru (spanning N20 to N130) and hRpn2 atoms respectively in this complex. The hRpn13 construct spanned amino acids M1-L150, but the N-terminal 19 and C-terminal 20 amino acids, which are outside of the Pru domain, are randomly coiled, as previously determined for free hRpn13 (Chen et al., *Molecular Cell*, (2010)). A series of NOESY experiments were recorded, including half filtered NOESY experiments to obtain unambiguous intermolecular NOE interactions between hRpn13 Pru and R13IM. In total, 140 unambiguous intermolecular distance constraints were identified and used to solve the structure (TABLE 1).

TABLE 1. Structural statistics for hRpn13 Pru:hRpn2 (940-953)

TABLE 1. Structural statistics for hRpn13 Pru:hRpn2 (940-953)		
NOE restraints		
Total NOEs	1922	
Intramolecular NOEs	1782	
hRpn13 Pru	Intra-residue	550
	Sequential (li-jl=1)	438
	Medium-range (li-jl) ≤ 4	198
	Long-rang (li-jl) > 5	455
hRpn2 (940-953)	Intra-residue	75
	Sequential (li-jl=1)	47
	Medium-range (li-jl) ≤ 4	19
	Long-rang (li-jl) > 5	0
Intermolecular NOEs	140	
Hydrogen bonds		
Intramolecular	45	

TABLE 1. Structural statistics for hRpn13 Pru:hRpn2 (940-953)

Intermolecular	1
Total dihedral angle restraints	
ϕ (°)	179
ψ (°)	179
Ramachandran plot region (%)	
Most-favorable	83.7
Additionally allowed	14.1
Generously allowed	2.3
Disallowed	0
r.s.m.d from average structure (Å) [§]	
Backbone	0.78±0.18
Heavy	1.50±0.24
[§] Statistics for secondary structural elements within hRpn13 Pru (V24-K34, T37-P40, G45-Q51, I57-D63, N68-I75, E81-K83, Y94-K97, R104-W108 and D117-L128) and hRpn2 P942-I951.	

[0038] The ten lowest energy structures with best geometry converged to a backbone root mean square deviation (r.m.s.d.) of 0.78 Å (TABLE 1 and FIGURE 8A). The hRpn13 Pru:R13IM structure exhibits a classic pleckstrin homology fold of hRpn13 Pru, formed by an 8-stranded β -sandwich capped by a C-terminal amphipathic α -helix (FIGURE 8B), as was observed for murine and human Rpn13. R13IM contacts 1190 Å² of hRpn13 Pru, capping its β -strand structure, across from the location of the α -helix by binding between β 2 and a β -sheet composed of β 6 to β 8 (FIGURE 8B).

[0039] Interestingly, in the crystal forms, the R13IM-binding region is occupied by another Rpn13 Pru molecule that similarly buries 1094 Å². Residues located on β 1, β 2 and the β 6- β 7 loop from one Rpn13 Pru molecule interact with F76 from a neighboring Rpn13 molecule in a manner akin to their interaction with hRpn2 F948. Many rearrangements were observed between the free Rpn13 Pru crystal structures and the R13IM-bound hRpn13 Pru and their backbone r.m.s.d. is 2.652 Å. The most striking difference is the reconfiguration of β 1, β 2, and β 6 to bend towards R13IM, like a pincer clamping down on it; the juxtaposed Rpn13 molecule in the crystal requires slightly larger space in this region.

[0040] A model structure of the proteasome from *S. cerevisiae* based on cryoelectron microscopy (cryoEM) reveals the location of Rpn13 relative to Rpn2 (Lander et al., *Nature*, (2012) 482: 186-191, Sakata et al., *PNAS USA*, (2012) 109: 1479-1484); however, this region is poorly defined with the Rpn13-binding C-terminal region of Rpn2 not present in the Rpn2 crystal structure and disordered. Sequence alignment was used here to register the R13IM hRpn2 fragment to that of *S. cerevisiae* and manually docked the hRpn13 Pru:R13IM structure into the cryoEM reconstruction (EMD-2594) with the *S. cerevisiae* Rpn2 structure incorporated (PDB 4CR2) by using UCSF Chimera (Pettersen et al., 2004). The hRpn13-binding region of hRpn2 was fused to the appropriate site in scRpn2 and the resulting modeled structure indicated a favored orientation for Rpn13 in the density map. Extra density was largely confined to the N-terminal end of the Pru domain.

[0041] It was previously found that amino acid substitution of hRpn2 F948 or Y950/I951 results in loss of interaction with hRpn13 (Lu et al., *PLoS One* (2015) 10: e0140518). This finding is

consistent with the structure of the hRpn13:R13IM, as hRpn2 F948 and Y950 are buried by hRpn13 V38 and V85, as well as hRpn13 V95 and R104 for hRpn2 Y950 and hRpn13 V93 for hRpn2 F948. The aromatic group of hRpn2 F948 is involved in hydrophobic interactions with the methyl groups of hRpn13 M31, V38, V85 and V93. hRpn2 F948 also forms hydrogen bonds with hRpn13 V38 through backbone atoms. The aromatic group of hRpn2 F950 is involved in hydrophobic interactions with the methyl groups of hRpn13 L33, T36, V38, V85, V95 and side chain atoms of R104. Hydrophobic interactions are also formed between hRpn2 I951 and hRpn13 T36. These interactions are supported by intermolecular NOE data.

[0042] To test further the significance of F948-D953, applicants expressed hRpn2 (940-947) in 293T cells, as was done previously for R13IM (FIGURE 7B). We found that hRpn2 (940-947) was unable to immunoprecipitate endogenous hRpn13 (FIGURE 7B, top panel, lane 7), nor was it able to expulse hRpn13 from the proteasome (FIGURE 7B, third panel, lane 7).

[0043] R13IM is rich in prolines, which make important contributions at the contact surface; all R13IM prolines adopt a trans configuration in their Rpn13-bound state. Strictly conserved P942, P944, and P945 bury hRpn13 W108, as defined by NOE interactions. hRpn13 also contributes prolines to the interaction surface, with P112 at the edge against hRpn2 P942, and hRpn13 P40 at a central location, joining hRpn13 W108 in burying hRpn2 P945. The many interactions involving P942 provide an explanation for the measured reduction in hRpn2 affinity towards hRpn13 upon deletion of Q940 to E943 (FIGURE 7A and FIGURE 9A). hRpn2 P947 also forms many contacts with hRpn13 amino acids, including T37, T39 and P40. These three amino acids combine with M31, L33, T36 and V38 from hRpn13 to form extensive hydrophobic interactions with E946-I951 of hRpn2.

[0044] Charge complementarity exists between R13IM, which is rich in acidic amino acids, and the hRpn13 Pru domain, which presents basic residues such as K97, K103 and R104 at the hRpn2-binding surface. This complementarity may contribute to long-range recruitment of hRpn13 to this region on hRpn2. Indeed, substitutions E946Q, E949Q and D953N in R13IM lead to a 20-fold reduction in affinity (FIGURE 7A and FIGURE 9A).

[0045] The disclosure includes peptides 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24 the N-terminal amino acids of SEQ ID NO: 1 (hRpn2 916-953) are deleted. Amino acid 953 may also be deleted. These peptides bind hRpn13 with high affinity. The disclosure includes hRpn2 940-953 (SEQ ID NO: 10) hRpn2 940-952 (SEQ ID NO: 11). The disclosure also includes peptides derived from hRpn2 940-953 or hRpn2 940-952 in which one or more amino acids other than F948, Y950, or I951 is deleted or replaced with another naturally occurring or non-naturally amino acid. It is preferred that the prolines in hRpn2 940-953 are conserved, or that only 1, 2, 3, or 4 of the prolines are substituted.

[0046] The disclosure includes peptides of SEQ ID NO: 1, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, or SEQ ID NO: 13, wherein one, two, or several amino acids (other than F948, Y950, or I951) are substituted, deleted, inserted, and/or added, as long as the ability to bind hRpn13 with high affinity, e.g. hRpn13 binding affinity of less than 100 nM, less than 50 nM, less than 20 nM, or less than 12 nM. The number of residues substituted, deleted, inserted, and/or added is may be five amino acids or less, four amino acids or less, three amino acids or less, two amino acids or less, or one amino acid.

[0047] The amino acid modification preferably retains the properties of the original amino acid side chains. Examples of the properties of amino acid side chains are shown below: hydrophobic amino acid side chains (A, I, L, M, F, P, W, Y, V); hydrophilic amino acid side chains (R, D, N, C, E, Q, G, H, K, S, T); and side chains having the following functional groups or characteristics in common: aliphatic side chains (G, A, V, L, I, P); hydroxy group-containing side chains (S, T, Y); sulfur atom-containing side chains (C, M); carboxylic acid- and amide-containing side chains (D, N, E, Q); base-containing side chains (R, K, H); and aromatic ring-containing side chains (H, F, Y, W). The substitution of one amino acid by another in the same group, e.g. an aliphatic side chain amino acid with another aliphatic side chain amino acid, is a “conservative substitution.” In certain embodiments any substitution is a conservative substitution. The disclosure includes peptides of SEQ ID NO: 1, 10, or 11 having unmodified sequences, or having modified sequences in which one or more amino acids other than F948, Y950, I951, and preferably other than P942, P944, P945, P947 are substituted with a conservative substitution. The disclosure also includes peptides of SEQ ID NO: 1, 10, or 11 having modified sequences in which one or more amino acids other than F948, Y950, I951, and preferably other than P942, P944, P945, P947 are substituted with a conservative substitution, substituted with an alanine, or deleted. In certain embodiments no more the 20 percent or no more than 10 percent of the amino acids of SEQ ID NO: 1, 10, or 11 are substituted, modified, or deleted. The peptides of the disclosure may include modifications such as glycosylation, side chain oxidization, and phosphorylation, unless the peptides lose their hRpn13 binding affinity. Other modifications include, for example, D-amino acids and other amino acid analogues that can be used to increase the serum half-life of the peptides.

[0048] In an embodiment the disclosure includes a peptide of SEQ ID NO: 13 in which 1, 2, 3, 4, or 5 amino acids designated Xaa are replaced by an amino acid does not occur in the corresponding wild type hRpn2 sequence. In certain embodiments the amino acid Xaa is a conservative replacement for the amino acid that occurs in the wild type sequence. For example in SEQ ID NO: 13:

Xaa₁ Xaa₂ Pro Xaa₃ Pro Pro Xaa₄ Pro Phe Xaa₅ Tyr Ile Asp

one or more of the following conditions is met:

Xaa₁ is not glutamine, but is preferably D, E, or Q;

Xaa₂ is not glutamic acid, but is preferably D, N, or Q;

Xaa₃ is not glutamic acid, but is preferably D, N, or Q;

Xaa₄ is not glutamic acid, but is preferably D, N, or Q; or

Xaa₅ is not glutamic acid, but is preferably D, N, or Q.

The following peptides have been identified as hRpn13 inhibitors:

SEQ ID NO	Description	Required Amino Acids
SEQ ID NO: 1	Amino acids 916 to 953 of hRpn2	F948, Y950, I951 are required P942, P944, P945, P947 are preferred Amino acids 916 to 939 may be deleted Amino acids 916 to 939 may be modified or substituted with another amino acid, in some embodiments the 1 to 24 of amino acids 916 to 939 are conservatively substituted
SEQ ID NO: 10	Amino acids 940 to 953 of hRpn2	F948, Y950, I951 are required P942, P944, P945, P947 are preferred
SEQ ID NO: 11	Amino acids 940 to 952 of hRpn2	F948, Y950, I951 are required P942, P944, P945, P947 are preferred

[0049] Peptides of the disclosure may be produced by any of the methods known in the art for obtaining and producing the peptides. They may be chemically synthesized peptides or recombinant peptides produced by gene recombination techniques.

[0050] Chemically synthesized peptides of the disclosure can be synthesized according to chemical synthesis methods such as the Fmoc method (fluorenylmethyloxycarbonyl method) and the t-Boc method (t-butyloxycarbonyl method). The peptides of the disclosure can also be synthesized utilizing various commercially-available peptide synthesizers.

[0051] The peptides of the disclosure can be produced as recombinant proteins by obtaining DNAs having the nucleotide sequences encoding the peptides, or variants or homologs thereof, and introducing them into a suitable expression system.

PHARMACEUTICAL COMPOSITIONS

[0052] Peptides disclosed herein can be administered as the neat chemical, but are preferably administered as a pharmaceutical composition. Accordingly, the disclosure provides pharmaceutical compositions comprising a compound or pharmaceutically acceptable salt of a hRpn13 binding peptide of the disclosure, such as a peptide of SEQ ID NO: 1, 10, 11, 12, or 13, or any of the modifications of these peptides discussed herein, together with at least one pharmaceutically acceptable carrier. The pharmaceutical composition/ combination may contain hRpn13 binding peptide of the disclosure as the only active agent, or may contain at least one additional active agent.

In certain embodiments it is preferred that the additional active agent is a 26S proteasome inhibitor. In certain embodiments the pharmaceutical composition is in a dosage form that contains from about 0.1 mg to about 2000 mg, from about 10 mg to about 1000 mg, from about 100 mg to about 800 mg, or from about 200 mg to about 600 mg of a hRpn13 binding peptide of the disclosure and optionally from about 0.1 mg to about 2000 mg, from about 10 mg to about 1000 mg, from about 100 mg to about 800 mg, or from about 200 mg to about 600 mg of an additional active agent in a unit dosage form. The pharmaceutical composition may also include a molar ratio of a compound of a hRpn13 binding peptide of the disclosure, and an additional active agent. For example the pharmaceutical composition may contain a molar ratio of about 0.5:1, about 1:1, about 2:1, about 3:1 or from about 1.5:1 to about 4:1 of the hRpn13 binding peptide of the disclosure and the additional active agent.

[0053] hRpn13 binding peptides of the disclosure may be administered orally, topically, parenterally, by inhalation or spray, sublingually, transdermally, via buccal administration, rectally, as an ophthalmic solution, or by other means, in dosage unit formulations containing conventional pharmaceutically acceptable carriers. The pharmaceutical composition may be formulated as any pharmaceutically useful form, e.g., injectable dosage form, an infusion, as an aerosol, a cream, a gel, a pill, a capsule, a tablet, or a syrup. Some dosage forms, such as tablets and capsules, are subdivided into suitably sized unit doses containing appropriate quantities of the active components, e.g., an effective amount to achieve the desired purpose.

[0054] Carriers include excipients and diluents and must be of sufficiently high purity and sufficiently low toxicity to render them suitable for administration to the patient being treated. The carrier can be inert or it can possess pharmaceutical benefits of its own. The amount of carrier employed in conjunction with the compound is sufficient to provide a practical quantity of material for administration per unit dose of the compound.

[0055] Classes of carriers include, but are not limited to binders, buffering agents, coloring agents, diluents, disintegrants, emulsifiers, flavorants, glidants, lubricants, preservatives, stabilizers, surfactants, tableting agents, and wetting agents. Some carriers may be listed in more than one class, for example vegetable oil may be used as a lubricant in some formulations and a diluent in others. Exemplary pharmaceutically acceptable carriers include sugars, starches, celluloses, powdered tragacanth, malt, gelatin; talc, and vegetable oils. Optional active agents may be included in a pharmaceutical composition, which do not substantially interfere with the activity of the compound of the present invention.

[0056] The pharmaceutical compositions/ combinations can be formulated for oral administration. These compositions contain between 0.1 and 99 weight % (wt.%) of a hRpn13 binding peptide of the disclosure and usually at least about 5 wt.% of a hRpn13 binding peptide of the disclosure. Some embodiments contain from about 25 wt. % to about 50 wt. % or from about 5 wt. % to about 75 wt. % of an hRpn13 binding peptide of the disclosure.

METHODS OF TREATMENT

[0057] The pharmaceutical compositions/ combinations disclosed herein are useful for treating patients. The compositions disclosed herein are useful for treating diseases and disorders which are modulated by proteolysis via the ubiquitin-proteasome pathway. In certain embodiments the patient is afflicted with cancer. In other embodiments the patient is afflicted with a viral infection, a neurodegenerative disorder, or an inflammatory disorder. In certain embodiments the disease is hematological cancer, HPV associated cancer, ovarian cancer, prostate cancer, gastric cancer, breast cancer, or colorectal cancer.

[0058] This disclosure provides methods of treatment, by providing a therapeutically effective amount of an hRpn13 binding peptide of the disclosure to a patient in need of treatment. A hRpn13 binding peptide of the disclosure may be provided as the only active agent or may be provided together with one or more additional active agents. In certain embodiments the hRpn13 binding peptide of the disclosure is administered together with a 26S proteasome inhibitor.

[0059] An effective amount of a pharmaceutical composition of the disclosure includes an amount sufficient to (a) slow the progression of disease (b) halt the progression of the disease; or (c) relieve the patient of the disease, i.e. the disease is no longer detectable and the patient is no longer experiencing symptoms. In certain embodiments the disease is cancer. An effective amount of a pharmaceutical composition of the disclosure includes an amount sufficient to significantly reduce the level of cancer markers in a patient's blood, serum, or tissues. An effective amount of a pharmaceutical composition described herein will also provide a sufficient concentration of the active agents in the concentration when administered to a patient. For cancer treatment a sufficient concentration of an active agent includes the concentration of the agent in the patient's body necessary to reduce cancer symptoms or slow cancer progression. Such an amount may be ascertained experimentally, for example by assaying blood concentration of the agent, or theoretically, by calculating bioavailability.

[0060] Pharmaceutical compositions and methods of treatment in which hRpn13 binding peptide of the disclosure is provided together with one or more additional active agents are included herein. In preferred embodiments the hRpn13 binding peptide of the disclosure is provided a 26S proteasome inhibitor, either in a single pharmaceutical composition or in a separate dosage forms with instructions to the patient to use the hRpn13 binding peptide of the disclosure and additional active agent together.

[0061] According to the methods of the disclosure, the hRpn13 binding peptide of the disclosure and at least one additional active agent may be: (1) co-formulated and administered or delivered simultaneously in a combined formulation; (2) delivered by alternation or in parallel as separate formulations; or (3) by any other combination therapy regimen known in the art. When delivered in alternation therapy, the methods of the disclosure may include administering or

delivering the hRpn13 binding peptide of the disclosure and an additional active agent sequentially, e.g., in separate solution, emulsion, suspension, tablets, pills or capsules, or by different injections in separate syringes. In general, during alternation therapy, an effective dosage of each active ingredient is administered sequentially, i.e., serially, whereas in simultaneous therapy, effective dosages of two or more active ingredients are administered together. Various sequences of intermittent combination therapy may also be used.

[0062] Methods of treatment include inhibiting hRpn13 *in vivo* by providing an amount of a hRpn13 binding peptide of the disclosure to a patient, sufficient to produce a concentration of the peptide in the patient's blood or plasma that would inhibit hRpn13 *in vitro*. In this instance the concentration includes an *in vivo* concentration, such as a blood or plasma concentration. The concentration of compound sufficient to inhibit hRpn13 *in vitro* may be determined from an assay of hRpn13 inhibition such as the assay provided in Example 2, herein.

[0063] Methods of treatment include providing certain dosage amounts of a hRpn13 inhibitor of the disclosure to a patient. Dosage levels of each active agent of from about 0.1 mg to about 140 mg per kilogram of body weight per day are useful in the treatment of the above-indicated conditions (about 0.5 mg to about 7 g per patient per day). The amount of active ingredient that may be combined with the carrier materials to produce a single unit dosage form will vary depending upon the patient treated and the particular mode of administration. In certain embodiments about 0.1 mg to about 2000 mg, from about 10 mg to about 1500 mg, from about 100 mg to about 1000 mg, from about 200 mg to about 800 mg, or from about 300 to about 600 mg of an hRpn13 binding peptide of the disclosure and optionally from about 0.1 mg to about 2000 mg, from about 10 mg to about 1500 mg, from about 100 mg to about 1000 mg, from about 200 mg to about 800 mg, or from about 300 to about 600 mg of a compound of an additional active agent, for example 26S proteasome. It is preferred that each unit dosage form contains less than 1200 mg of active agent in total. Frequency of dosage may also vary depending on the compound used and the particular disease treated.

[0064] It will be understood, however, that the specific dose level for any particular patient will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex, diet, time of administration, route of administration, and rate of excretion, drug combination and the severity of the particular disease in the patient undergoing therapy.

SPECIFIC EMBODIMENTS

[0065] The disclosure includes the following specific embodiments.

[0066] 1. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a peptide, the peptide comprising the amino acid sequence of

SEQ ID NO: 1, wherein 1 to 24 amino acids other than P942, P944, P945, P947, F948, Y950, and I951 are optionally replaced by another naturally occurring or modified amino acid;

SEQ ID NO: 11, wherein 1, 2, 3, 4, or 5 amino acids of SEQ ID NO: 11 other than proline, phenylalanine, tyrosine, or isoleucine, are optionally replaced by another naturally occurring or modified amino acid;

SEQ ID NO: 11, wherein 1 or 2 amino acids other than proline, phenylalanine, tyrosine, or isoleucine, are deleted; or

SEQ ID NO: 13, wherein each Xaa is an independently chosen naturally occurring amino acid.

[0067] 2. The pharmaceutical composition of Embodiment 1, the peptide comprising SEQ ID NO: 12.

[0068] 3. The pharmaceutical composition of Embodiment 1, the peptide comprising SEQ ID NO: 11.

[0069] 4. The pharmaceutical composition of Embodiment 1, the peptide comprising SEQ ID NO: 11, wherein 1, 2, 3, 4, or 5 amino acids of SEQ ID NO: 11 other than proline, phenylalanine, tyrosine, or isoleucine are replaced by another naturally occurring or modified amino acid or wherein 1 or 2 amino acids other than proline, phenylalanine, tyrosine, or isoleucine, are deleted.

[0070] 5. The pharmaceutical composition of Embodiment 1, the peptide comprising SEQ ID NO: 10.

[0071] 6. The pharmaceutical composition of Embodiment 1, the peptide comprising SEQ ID NO: 1.

[0072] 7. The pharmaceutical composition of Embodiment 1, the peptide comprising SEQ ID NO: 1, in which 1 - 24 amino acids have been deleted from the N-terminal and in which 1 amino acid has optionally been deleted from the C-terminal.

[0073] 8. The pharmaceutical composition of any one of Embodiments 1 to 7, wherein the peptide binds to hRpn13 with an affinity of 20 nM or less.

[0074] 9. A method of treating cancer in a patient, comprising administering the pharmaceutical composition of any one of Embodiments 1 to 8 to the patient.

[0075] 10. A method of treating cancer in a patient comprising administering a peptide comprising the amino acid sequence of

SEQ ID NO: 11, wherein 1, 2, 3, 4, or 5 amino acids of SEQ ID NO: 11 other than proline, phenylalanine, tyrosine, or isoleucine, are optionally replaced by another naturally occurring or modified amino acid;

SEQ ID NO: 11, wherein 1 or 2 amino acids other than proline, phenylalanine, tyrosine, or isoleucine, are deleted; or

SEQ ID NO: 13, wherein each Xaa is an independently chosen naturally occurring amino acid.

[0076] 11. The method of Embodiment 9 or 10, wherein the cancer is a hematological cancer, HPV associated cancer, ovarian cancer, prostate cancer, gastric cancer, breast cancer, or colorectal cancer.

[0077] 12. The method of Embodiment 9 or 10, wherein the peptide is administered orally.

[0078] 13. The method of any one of Embodiment 9 to 12, wherein peptide is a first chemotherapeutic agent and is administered together with at least one additional chemotherapeutic agent.

[0079] 14. The method of Embodiment 13, wherein the additional chemotherapeutic is a 26S proteasome inhibitor.

[0080] 15. A peptide comprising SEQ ID NO: 1, wherein 1 to 24 amino acids other than P942, P944, P945, P947, F948, Y950, and I951 is deleted or replaced by another naturally occurring or modified amino acid;

SEQ ID NO: 11, wherein 1, 2, 3, 4, or 5 amino acids of SEQ ID NO: 11 other than proline, phenylalanine, tyrosine, or isoleucine, are replaced by another naturally occurring or modified amino acid;

SEQ ID NO: 11, wherein 1 or 2 amino acids other than proline, phenylalanine, tyrosine, or isoleucine, are deleted; or

SEQ ID NO: 13, wherein each Xaa is an independently chosen naturally occurring amino acid and is not identical to hRpn2 940-952.

[0081] 16. The peptide of Embodiment 15, the peptide comprising SEQ ID NO: 11, wherein 1, 2, 3, 4, or 5 amino acids of SEQ ID NO: 11 other than proline, phenylalanine, tyrosine, or isoleucine are replaced by another naturally occurring or modified amino acid or wherein 1 or 2 amino acids other than proline, phenylalanine, tyrosine, or isoleucine, are deleted.

[0082] 17. The peptide of Embodiment 15 or 16, wherein the peptide binds to hRpn13 with an affinity of 20 nM or less.

EXAMPLES

ABBREVIATIONS

Pru Pleckstrin-like receptor for ubiquitin domain

RP Regulatory Particle

GENERAL METHODS

PLASMIDS AND ANTIBODIES

[0083] The DNA fragment encoding amino acids 916 to 953 (SEQ ID NO. 1) of hRpn2 was amplified by PCR and cloned into the bacterial expression vector pRSET and mammalian expression vector p3XFLAG-CMV7.1. Antibodies used in this study include anti-FLAG (Sigma), anti-hRpn2 (cell signaling), anti-hRpt3 (Abcam), and anti-hRpn13 (Abcam).

IMMUNOPRECIPITATION AND IMMUNOBLOTTING

[0084] 293T cells (purchased from ATCC) were cultured in DMEM supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere and 5% CO₂. Plasmid DNA was transfected into 293T cells using Lipofectamine2000 (Invitrogen) according to the manufacturer's instruction. Cells were harvested by gentle centrifugation at 500g for 5 minutes at 4°C after washing with PBS, and cell pellets were resuspended in 1% Triton-TBS buffer (50 mM Tris, pH7.5, 150 mM NaCl, 1 mM EDTA and protease inhibitor cocktail) followed by rocking at 4°C for 30 minutes. The supernatant was collected following centrifugation at 16,000g for 10 minutes and incubated with antibodies at 4°C overnight. Protein G sepharose beads (Sigma) were used to pull down interacting proteins by 3-hour incubation at 4°C. After extensive washing with 1% Triton TBS buffer, SDS-PAGE loading buffer was used to elute proteins from beads for immunoblotting.

SAMPLE PREPARATION

[0085] hRpn2 (916–953) was expressed in *Escherichia coli* as a fusion protein with GST (SEQ ID NO. 2) and a PreScission protease cleavage site. Cells were lysed by sonication and cellular debris removed by centrifugation at 31,000g for 30 minutes. The cell lysate was incubated with glutathione S-sepharose resin and washed extensively with Buffer 1 (20 mM sodium phosphate, 300 mM NaCl, 2 mM DTT, pH 6.5). hRpn2 (916-953) was eluted from the resin and separated from GST by overnight incubation with 50 unit/mL PreScission protease in Buffer 2 (20 mM sodium phosphate, 50 mM NaCl, 2 mM DTT, pH 6.5). Further purification was achieved by size exclusion chromatography with a Superdex 75 column on an FPLC system. ¹⁵N ammonium chloride, ¹³C glucose, and D₂O were used for isotope labeling. hRpn13 (1–150) was prepared as described (Schreiner, P., et al., *Nature*, (2008) 453 (7194): 548-552) with a method similar to that described above for hRpn2, but with a histidine tag in place of GST and Talon resin used for affinity purification. PreScission protease was used for elution and removal of the tag and all buffers were identical. All NMR experiments were performed in Buffer 2, but with addition of 10% D₂O and 0.1% sodium azide.

ISOTHERMAL TITRATION CALORIMETRY (ITC) EXPERIMENTS

[0086] ITC was performed at 25°C on a MicroCal iTC200 system. hRpn13 (1-150) (SEQ. ID NO. 3) and hRpn2 (916-953) samples were dialyzed extensively against ITC buffer (20 mM sodium phosphate, 50 mM NaCl and 10mM βME [pH 6.5]). One aliquot of 0.5 μL followed by eighteen aliquots of 2.1 μL 200 μM hRpn2 (916-953) were injected at 1000 rpm into the calorimeter cell (volume 200.7 μL), which contained 20 μM hRpn13. Blank experiments were performed by replacing

protein samples with buffer and this blank data was subtracted from the experiment data during analyses. The integrated interaction heat values were normalized as a function of protein concentration, and the data were fit with MicroCal Origin 7.0 software. Binding was assumed to be at one site to yield the binding affinity K_a ($1/K_d$), stoichiometry and other thermodynamic parameters.

NMR EXPERIMENTS

[0087] All NMR experiments were conducted at 25°C on Bruker Avance 600, 700, 800, or 850 MHz spectrometers equipped with cryogenically cooled probes. ^1H , ^{15}N , ^{13}C HNCACO, HNCO, HNCACB, CBCACONH and 3D-dispersed NOESY (200 ms mixing) spectra were acquired on 0.6 mM ^{15}N -, ^{13}C -, and 70% ^2H -labeled hRpn2 (916-953) or on a mixture of 0.7 mM ^{15}N -, ^{13}C -, and 70% ^2H -labeled hRpn2 (916-953) and equimolar unlabeled hRpn13 Pru. ^1H , ^{13}C HSQC and 3D ^{13}C -edited NOESY spectra were recorded on a mixture of 0.5 mM ^{13}C -labeled hRpn2 (916-953) and equimolar unlabeled hRpn13 Pru with a NOESY mixing time of 150 ms or on 0.5 mM ^{13}C -labeled hRpn2 (916-953) with a NOESY mixing time of 300 ms.

[0088] Distance constraints for structure calculations were obtained by using an ^{15}N -dispersed NOESY spectrum recorded on a mixture of 0.6 mM ^{15}N -, ^{13}C -labeled hRpn13 Pru and equimolar ^{15}N -, ^{13}C -labeled hRpn2 (940-953) with a 150 ms mixing time as well as ^{13}C -edited NOESY spectra on mixtures of 0.7 mM ^{15}N -, ^{13}C -labeled hRpn13 Pru and equimolar unlabeled hRpn2 (940-953) (120 ms mixing time) or 0.7 mM ^{15}N -, ^{13}C -labeled hRpn2 (940-953) and equimolar unlabeled hRpn13 Pru (100 ms mixing time). Intermolecular NOE distance constraints were determined by using a ^{13}C -half-filtered NOESY spectrum (100 ms mixing time) recorded on a mixture of 0.7 mM ^{15}N -, ^{13}C -labeled hRpn13 Pru and equimolar unlabeled hRpn2 (940-953). The ^{13}C -edited NOESY spectra were acquired on samples dissolved in D_2O . NMR Pipe was used to process data and XEASY was used to visualize and analyze spectra.

[0089] Chemical shift perturbation (CSP) analysis was done by using ^1H , ^{15}N HSQC experiments recorded on ^{15}N labeled hRpn2 (916-953) and equimolar unlabeled hRpn13 Pru. CSP values were calculated according to equation (1), as described in by Chen and Walters (Chen, X. and Walters, K.J., *Methods Mol. Biol.*, (2012) 832: 279-303).

$$\text{CSP} = \sqrt{0.2\Delta\delta_{\text{N}}^2 + \Delta\delta_{\text{H}}^2} \quad (1)$$

[0090] $\Delta\delta_{\text{H}}$, change in proton value (in parts per million); $\Delta\delta_{\text{N}}$, change in nitrogen value (in parts per million).

EXAMPLE 1. A 38-AMINO ACID HRPN2-DERIVED PEPTIDE THAT BINDS TO THE HRPN13 PRU DOMAIN

[0091] Previously an hRpn2 peptide that spans amino acids 797-953 (SEQ. ID NO. 4) was found to bind to ^{15}N -labeled hRpn13 (Husnjak, K., et al., (2008) 453: 481-488). To further define the hRpn13-binding region on hRpn2, we generated a truncated peptide that spans amino acids 916-953, as described in General Methods. We acquired 2D ^1H , ^{15}N NMR experiments on 0.6 mM free ^{15}N

labeled hRpn2 916-953 and after the addition of equimolar hRpn13 Pru domain (not shown). Sample concentrations were determined by using calculated extinction coefficients for each protein and absorbance at $\lambda = 280\text{nm}$ as well as LC-MS; the complex was purified from any excess component by size exclusion chromatography. ^1H - ^{15}N HSQC spectra of hRpn2 (916-953) were obtained alone and with equimolar equivalents of the Rpn13 Pru domain. The NMR experiment revealed a subset of hRpn2 signals to shift significantly while others were unaffected by the presence of hRpn13 Pru.

[0092] The chemical shift perturbation (CSP) values derived from the ^1H - ^{15}N HSQC spectra of hRpn2 (916-953) alone and with the Rpn13 Pru domain are presented in FIGURE 1 for each hRpn2 amino acid. Prolines, which lack amide signals, are omitted from this analysis and indicated with an asterisk. Comparison of the hRpn2 NMR spectra with and without hRpn13 Pru domain illustrated large chemical shift perturbations for F948, E949, Y950, and I951. Adding hRpn13 to hRpn2 (916-953) produced only minor chemical shift perturbations at the N-terminal end of hRpn2 (916-953). By contrast, significant shifting was calculated for E943-I951. Chemical shift perturbation analysis was performed as described in the General Methods section.

EXAMPLE 2. AN HRPN2-DERIVED PEPTIDE BINDS TO HRPN13 PRU WITH 12 NM AFFINITY

[0093] Isothermal titration calorimetry (ITC) was used to determine the affinity of hRpn13 Pru and hRpn2 (916-953). 200 μM hRpn2 (916-953) was injected into a calorimeter cell that contained 20 μM Rpn13 Pru domain. This initial injection was 0.5 μL , after which 2.1 μL was used. The thermogram indicated a high affinity binding profile with favorable enthalpy (FIGURE 2A). The data fit to a 1-site binding mode with a K_d value of 12 nM (FIGURE 2B). The following additional thermodynamic parameters were determined: n 0.811 $\pm$ 0.00339, ΔH -1.569 $\times 10^4$, and ΔS -16.4 cal $\text{mol}^{-1}\text{K}^{-1}$. The mean concentration of cytosolic proteasome in intact neurons is estimated to be 190 nM, a concentration 15-fold greater than the K_d value for hRpn2 interaction with hRpn13.

EXAMPLE 3. STRUCTURAL PROPERTIES OF THE HRPN13-BINDING REGION OF HRPN2

[0094] TALOS+ (<http://spin.niddk.nih.gov/bax/software/TALOS>) was used to predict the secondary structure and dynamic properties of hRpn2 in its free and Rpn13-bound state from our assigned N, NH, C', $\text{C}\alpha$, and $\text{C}\beta$ values. In both analyses, the peptide was predominately found to be unstructured. However, amino acids involved in binding to hRpn13 Pru demonstrated a propensity to form a β -strand, especially when bound to hRpn13. These include E949, Y950 and I951.

[0095] The region involved in binding to Rpn13 was also found to be less dynamic compared to the remainder of hRpn2. Most of the hRpn2-derived peptide was found to be dynamic, as indicated by S^2 values of ~ 0.5 (FIGURE 3). The Rpn13-binding region however showed higher S^2 values, which increased in the presence of Rpn13.

[0096] From the dynamic region, two signals of unequal population were observed in the hRpn2 free state for A929 and I935, and in the hRpn13-bound state, V925, E926, and V928 also exhibited two sets of signals in the NMR spectra (not shown). This phenomenon suggests that these five amino acids exchange between two distinct states. It is likely that this exchange is triggered by proline isomerization, as nearby P927 exhibited two sets of C α , C β and C' signals (not shown). This proline was the only one for which two sets of signals were observed.

EXAMPLE 4. HRPN2 (916-953) PEPTIDE INTERACTS WITH ENDOGENOUS HRPN13 IN 293T CELLS

[0097] To test whether hRpn2 (916-953) is competent for interaction with endogenous hRpn13 in a human cell line, we sub-cloned this fragment in frame with an N-terminal FLAG tag (SEQ. ID NO. 5) into the mammalian expression vector p3XFLAG-CMV7.1. The resulting plasmid was transfected into 293T cells and the cell lysate immunoprecipitated with anti-FLAG antibody. The immunoprecipitate was immunoprobed for hRpn13 by using anti-Rpn13 antibody, which confirmed the interaction of the hRpn2-derived peptide with endogenous hRpn13 in 293T cells (FIGURE 4A, top panel).

EXAMPLE 5. STRICTLY CONSERVED F948 AND Y950/I951 ARE REQUIRED FOR HRPN2 INTERACTION WITH HRPN13 IN 293T CELLS

[0098] This experiment assessed the importance of hRpn2 amino acids F948, Y950, and I951 for interaction with hRpn13. F948 was replaced with arginine (SEQ. ID NO. 6), a stop codon (SEQ. ID NO. 7), both Y950 and I951 were replaced with aspartic acid (SEQ. ID NO. 8), and F948 was simultaneously replaced with arginine and Y950 and I951 with aspartic acid (SEQ. ID NO. 9). All hRpn2 peptides included an N-terminal FLAG sequence. These FLAG-hRpn2 variants were transfected into 293T cells along with FLAG-hRpn2 (916-953) and subjected to immunoprecipitation with anti-FLAG antibody followed by immunoprobings with anti-Rpn13 antibody, as described for FIGURE 4A. All three of these substitutions resulted in a peptide that was unable to immunoprecipitate endogenous hRpn13 from 293T cells (FIGURE 4B). The importance of F948 and Y950 is further supported by their conservation among eukaryotic Rpn2 species; F948 is strictly conserved whereas Y950 is highly conserved (FIGURE 4C).

EXAMPLE 6. HRPN2 (916-953) BINDS TO PROTEASOME FREE HRPN13 IN 293T CELLS

[0099] If the 916-953 region in hRpn2 is used to dock hRpn13 into the proteasome, then hRpn13 will not be able to interact with the peptide while seated in the proteasome. We thus tested whether the hRpn2-derived peptide can immunoprecipitate endogenous proteasome subunits hRpn2 and hRpt3 after immunoprecipitation with anti-FLAG antibody. This experiment demonstrated neither of these RP components was immunoprecipitated by the peptide (FIGURE 4A, bottom two panels).

DL stands for direct load and illustrates that peptide expression does not affect levels of hRpn2 or hRpt3. Thus, hRpn13 immunoprecipitated by the hRpn2-derived peptide is not bound to the proteasome.

EXAMPLE 7. OVER-EXPRESSION OF THE HRPN2-DERIVED PEPTIDE DEPLETES HRPN13 FROM THE PROTEASOME

[0100] This experiment assessed whether over-expression of the hRpn2-derived peptide depletes hRpn13 from the proteasome. For this purpose, proteasome was immunoprecipitated with anti-hRpt3 antibody and the presence of hRpn13 was detected by immunoprobining with anti-hRpn13 antibody. Whereas hRpn13 co-immunoprecipitated with anti-hRpt3 antibody in untransfected 293T cells, expression of hRpn2 (916-953) peptide significantly reduced the amount of hRpn13 at the proteasome (FIGURE 5A, top panel, compare lane 4 with lane 5). Expression of the F948Stop mutant of this peptide, which does not bind hRpn13 (FIGURE 4B), does not deplete hRpn13 from proteasome (Fig. 5A, lane 6). As expected, the assembly of hRpn2 into the proteasome was not affected by expression of the hRpn2-derived peptide (FIGURE 5A, second panel).

EXAMPLE 8. OVER-EXPRESSION OF THE HRPN2-DERIVED PEPTIDE IN 293T CELLS LEADS TO THE ACCUMULATION OF UBIQUITINATED PROTEINS

[0101] This experiment tested whether over-expression of the hRpn2-derived peptide in 293T cells leads to the accumulation of ubiquitinated proteins. Lysates from cells transfected with HA-ubiquitin alone or together with either FLAG-hRpn2 (916-953) WT or F948Stop were subjected to immunoblotting with anti-ubiquitin antibody. Cells expressing F948Stop were used as a negative control, and immunoblotting with anti-actin antibody to confirm equivalent loading. This experiment revealed an increase in the presence of ubiquitinated proteins in the cell lysate for 293T cells over-expressing the hRpn2 WT-derived peptide (FIGURE 5B). This increase was not observed for the negative control peptide (F948Stop) that does not bind to Rpn13.

EXAMPLE 9. NMR RELAXATION EXPERIMENT DEMONSTRATES BINDING TO THE C-TERMINAL REGION OF HRPN2 (916-953)

[0102] This experiment examines the dynamic state of the hRpn2 peptide in the presence and absence of hRpn13 Pru domain. The peptide by itself is highly dynamic but becomes ordered in the region spanning E939-D952 in the presence of hRpn13 Pru domain (FIGURE 6). This result offers further validation for the very C-terminal region being important in binding and becoming ordered as it binds.

CLAIMS

1. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a peptide and the peptide having the amino acid sequence of

SEQ ID NO: 1, wherein 1 to 24 amino acids other than P942, P944, P945, P947, F948, Y950, and I951 are optionally deleted or replaced by another naturally occurring or modified amino acid;

SEQ ID NO: 11, wherein 1, 2, 3, 4, or 5 amino acids of SEQ ID NO: 11 other than proline, phenylalanine, tyrosine, or isoleucine, are optionally replaced by another naturally occurring or modified amino acid;

SEQ ID NO: 11, wherein 1 or 2 amino acids other than proline, phenylalanine, tyrosine, or isoleucine, are deleted; or

SEQ ID NO: 13, wherein each Xaa is an independently chosen naturally occurring amino acid.

2. The pharmaceutical composition of Claim 1, the peptide comprising SEQ ID NO: 12.

3. The pharmaceutical composition of Claim 1, the peptide comprising SEQ ID NO: 11.

4. The pharmaceutical composition of Claim 1, the peptide comprising SEQ ID NO: 11, wherein 1, 2, 3, 4, or 5 amino acids of SEQ ID NO: 11 other than proline, phenylalanine, tyrosine, or isoleucine are replaced by another naturally occurring or modified amino acid or wherein 1 or 2 amino acids other than proline, phenylalanine, tyrosine, or isoleucine, are deleted.

5. The pharmaceutical composition of Claim 1, the peptide comprising SEQ ID NO: 10.

6. The pharmaceutical composition of Claim 1, the peptide comprising SEQ ID NO: 1.

7. The pharmaceutical composition of Claim 1, the peptide comprising SEQ ID NO: 1, in which 1 - 24 amino acids have been deleted from the N-terminal and in which 1 amino acid has optionally been deleted from the C-terminal.

8. The pharmaceutical composition of any one of Claims 1 to 7, wherein the peptide binds to hRpn13 with an affinity of 20 nM or less.

9. A method of treating cancer in a patient, comprising administering the pharmaceutical composition of any one of Claims 1 to 8 to the patient.

10. A method of treating cancer in a patient comprising administering a peptide comprising the amino acid sequence of

SEQ ID NO: 11, wherein 1, 2, 3, 4, or 5 amino acids of SEQ ID NO: 11 other than proline, phenylalanine, tyrosine, or isoleucine, are optionally replaced by another naturally occurring or modified amino acid;

SEQ ID NO: 11, wherein 1 or 2 amino acids other than proline, phenylalanine, tyrosine, or isoleucine, are deleted; or

SEQ ID NO: 13, wherein each Xaa is an independently chosen naturally occurring amino acid.

11. The method of Claim 9 or 10, wherein the cancer is a hematological cancer, HPV associated cancer, ovarian cancer, prostate cancer, gastric cancer, breast cancer, or colorectal cancer.

12. The method of Claim 9 or 10, wherein the peptide is administered orally.

13. The method of any one of Claims 9 to 12, wherein peptide is a first chemotherapeutic agent and is administered together with at least one additional chemotherapeutic agent.

14. The method of Claim 13, wherein the additional chemotherapeutic is a 26S proteasome inhibitor.

15. A peptide comprising SEQ ID NO: 11, wherein 1, 2, 3, 4, or 5 amino acids of SEQ ID NO: 11 other than proline, phenylalanine, tyrosine, or isoleucine, are replaced by another naturally occurring or modified amino acid;

SEQ ID NO: 11, wherein 1 or 2 amino acids other than proline, phenylalanine, tyrosine, or isoleucine, are deleted; or

SEQ ID NO: 13, wherein each Xaa is an independently chosen naturally occurring amino acid and is not identical to hRpn2 940-952.

16. The peptide of Claim 15, the peptide comprising SEQ ID NO: 11, wherein 1, 2, 3, 4, or 5 amino acids of SEQ ID NO: 11 other than proline, phenylalanine, tyrosine, or isoleucine are replaced by another naturally occurring or modified amino acid or wherein 1 or 2 amino acids other than proline, phenylalanine, tyrosine, or isoleucine, are deleted.

17. The peptide of Claim 15 or 16, wherein the peptide binds to hRpn13 with an affinity of 20 nM or less.

FIGURE 1. hRpn2 Chemical Shift Perturbation with Addition of hRpn13 Pru Domain

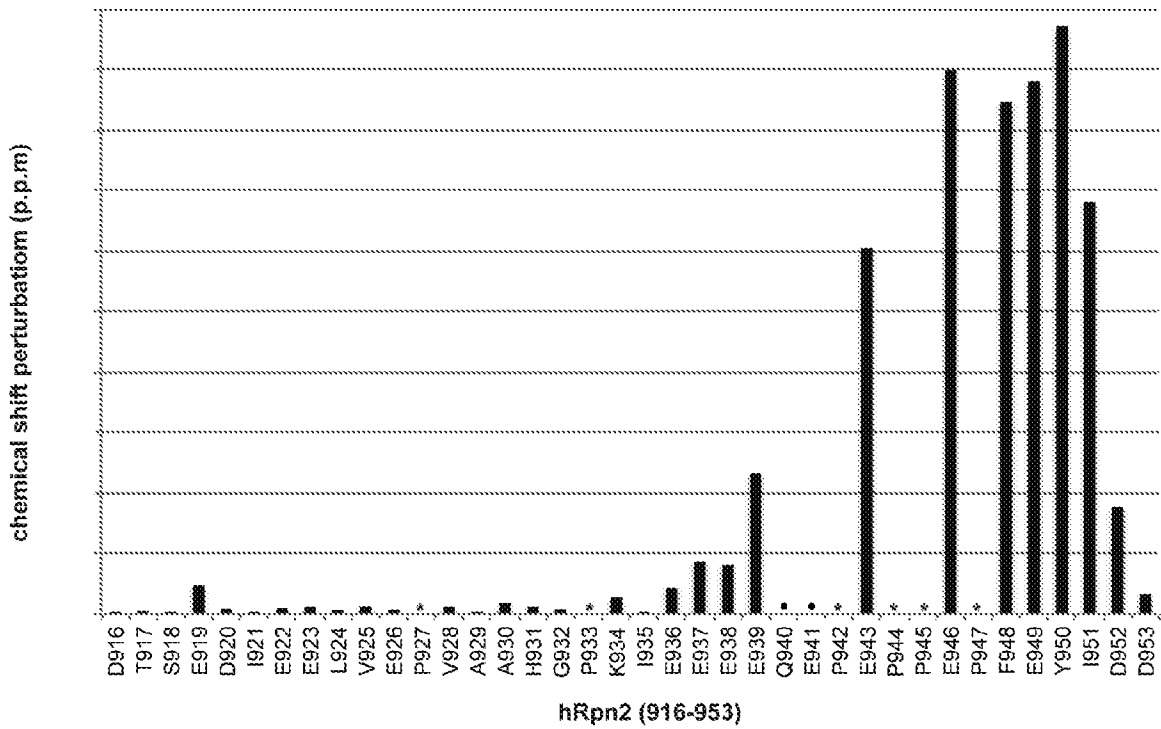
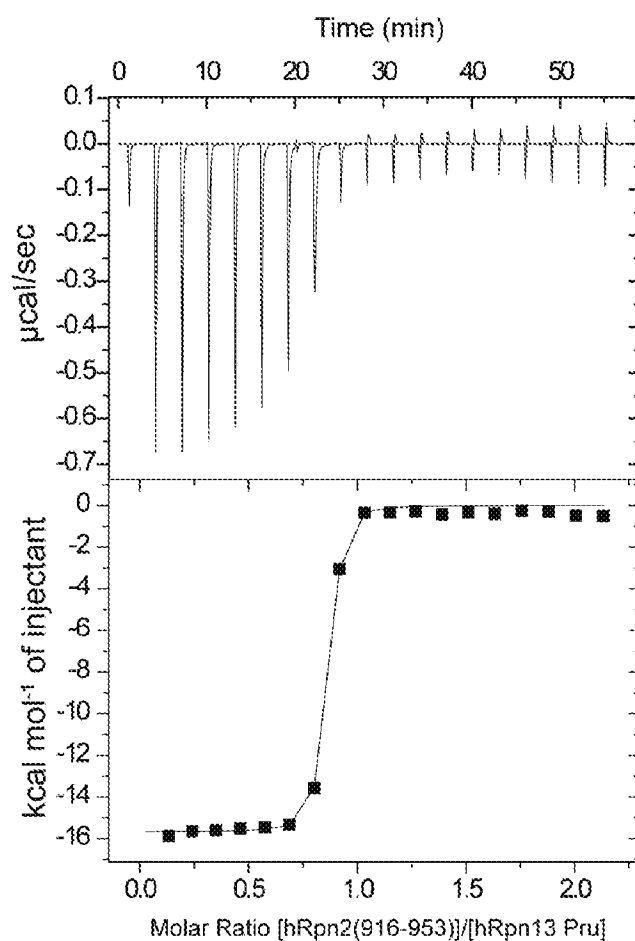


FIGURE 2. Affinity of hRpn2 (916-953) Binding to hRpn13 Pru Domain

A.



B.

Parameter	Value
n	0.811 ± 0.00339
ΔH	$-1.569 \times 10^4 \pm 125.8 \text{ cal mol}^{-1}$
ΔS	$-16.4 \text{ cal mol}^{-1} \text{ K}^{-1}$
K_d	$12.32 \pm 3.11 \text{ nM}$

FIGURE 3. Order Parameters for Free hRpn2 and hRpn2 Bound to hRpn13

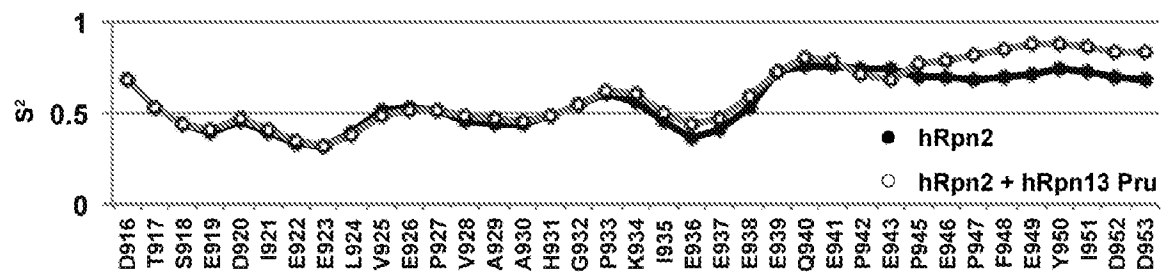


FIGURE 4. F948 and Y950/I951 are required for hRpn2 interaction with hRpn13 in 293T cells

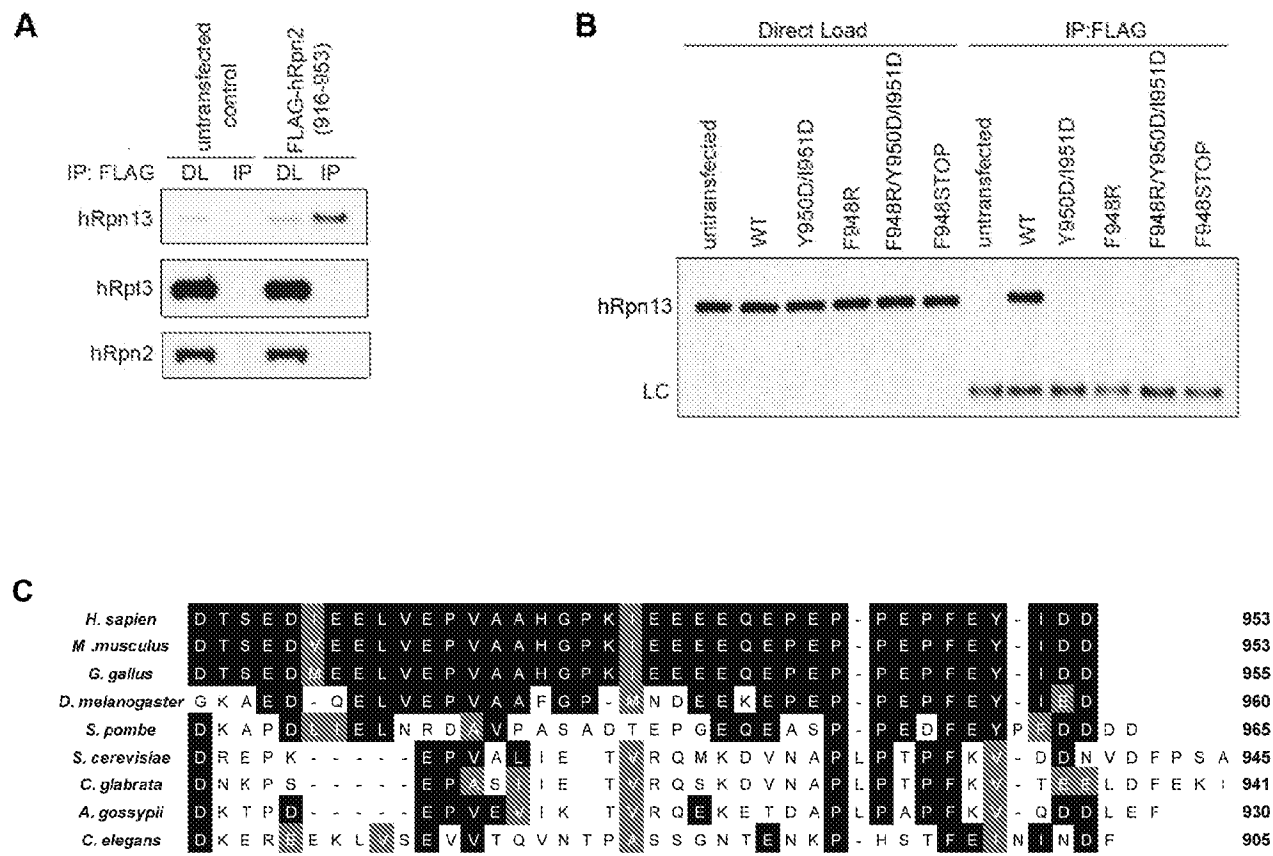


FIGURE 5. The hRpn2-Derived Peptide Depletes hRpn13 From the Proteasome

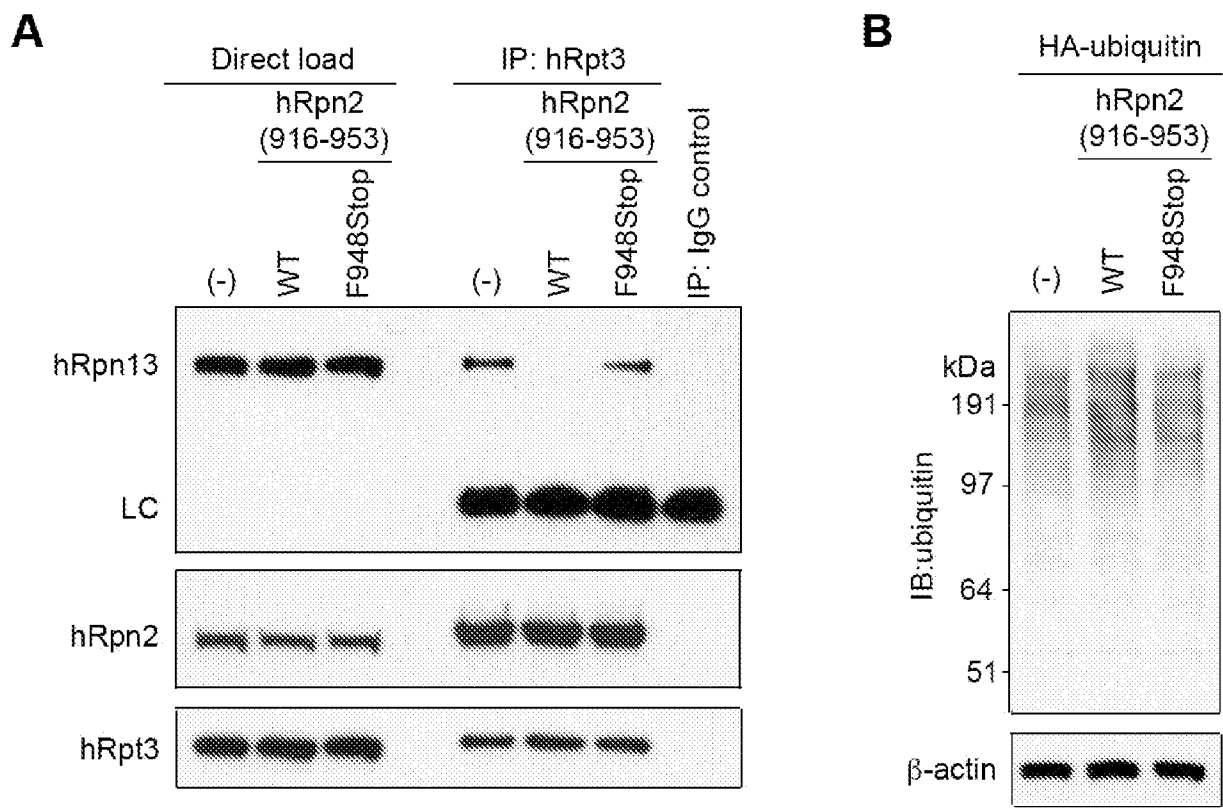


FIGURE 6. Heteronuclear NOE experiment demonstrating the C-terminal region of hRpn2 to become ordered as it binds to hRpn13 Pru domain.

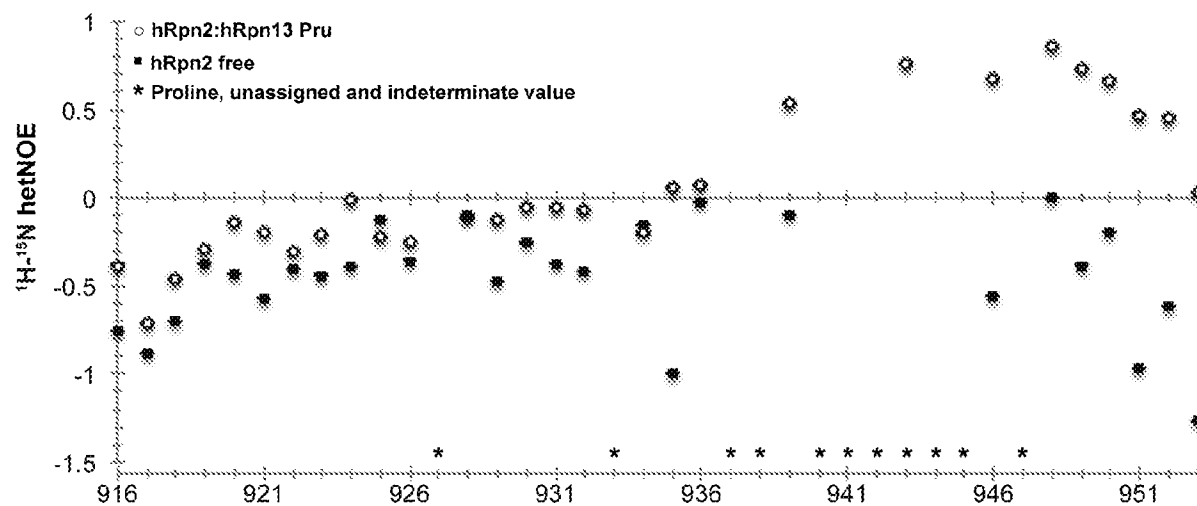
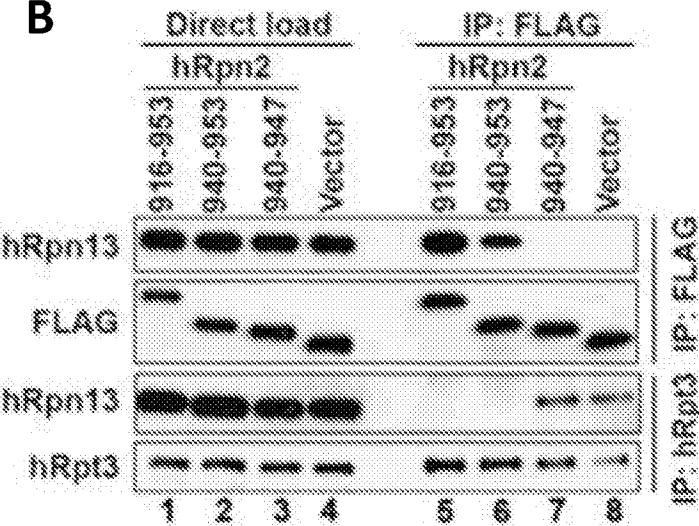


FIGURE 7. hRpn13 is required at the proteasome for robust clearance of ubiquitinated proteins

A

hRpn2	K_d (μ M)
940-953	0.027 ± 0.010
944-953	1.96 ± 0.22
944-953 (QQN)	40.32 ± 3.05

B



C

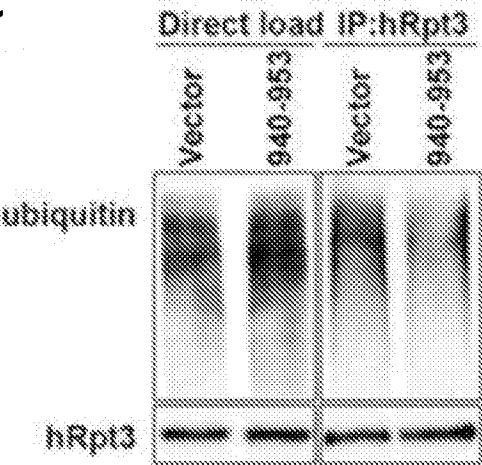


FIGURE 8. NMR Structure of a complex between R13IM (grey) and hRpn13 (black).

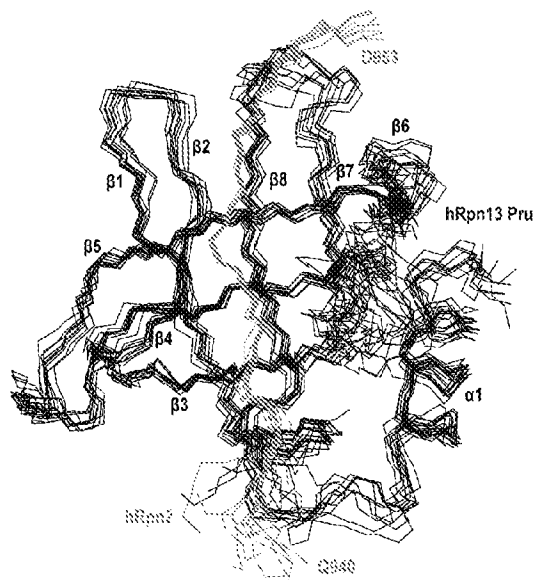
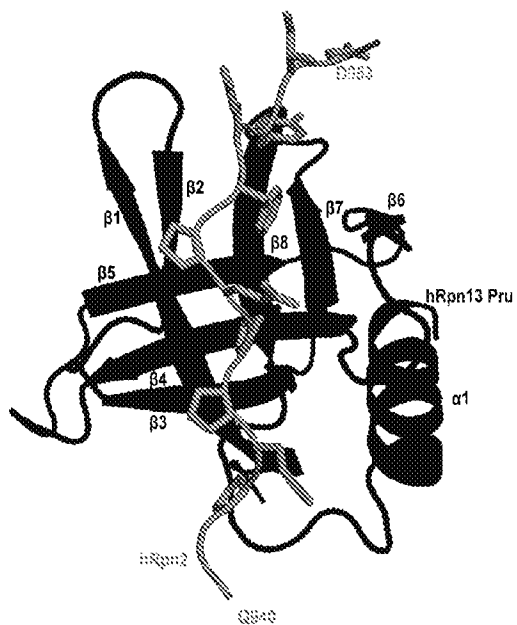
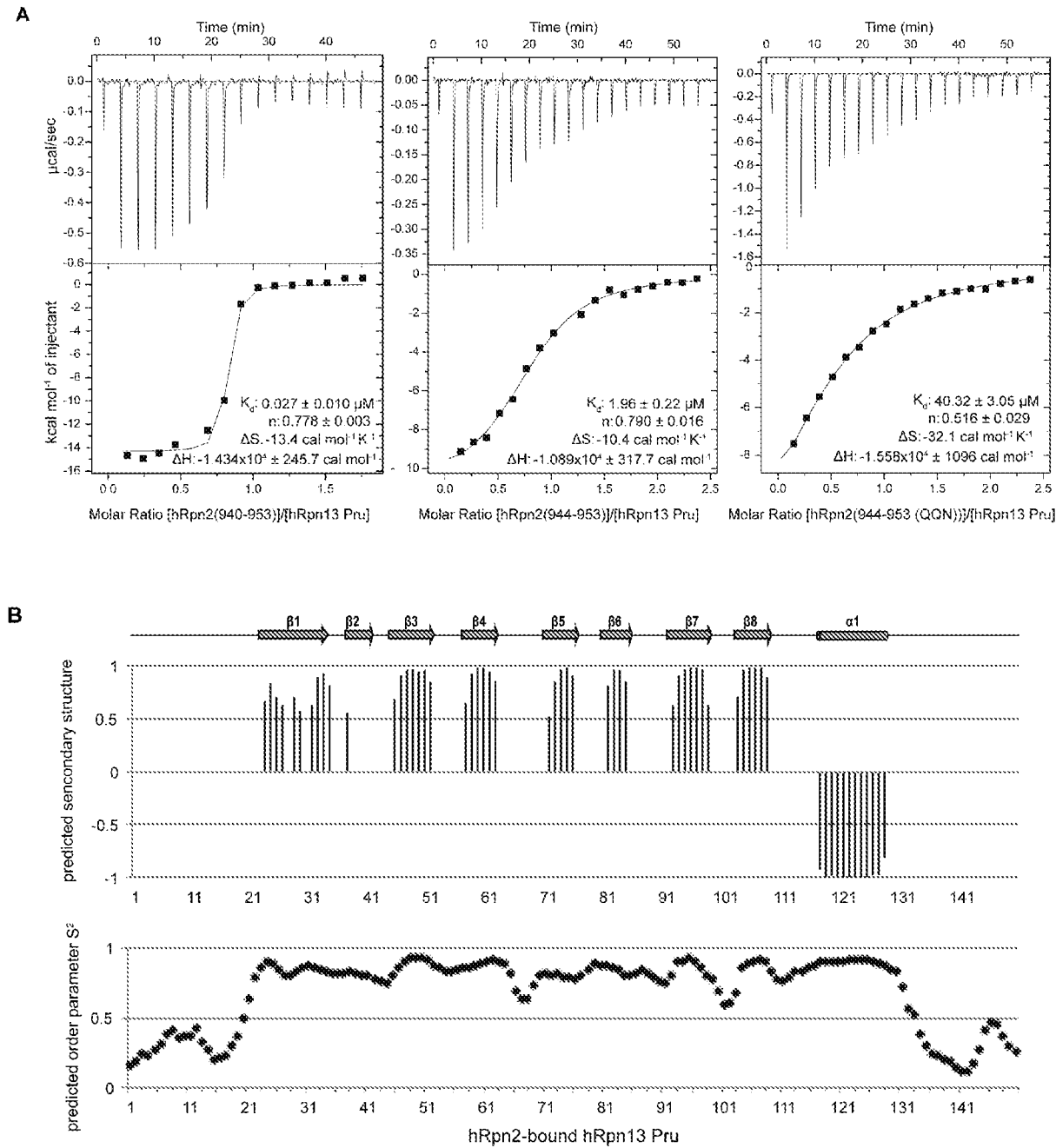
A**B**

FIGURE 9. Calorimetry and Secondary Structure of C-terminal 14 Amino Acids of hRpn2 Binding to hRpn13



INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/053352

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/00 C07K14/435 A61K38/00 A61P35/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Anonymous: "Uniprot: AC 088771", 24 June 2015 (2015-06-24), XP055329150, Retrieved from the Internet: URL: http://www.uniprot.org/uniprot/088771.txt?version=48 [retrieved on 2016-12-14] abstract	1-8, 15-17
X	Anonymous: "Uniparc-UPI0000209778", 13 September 2006 (2006-09-13), XP055329156, Retrieved from the Internet: URL: http://www.uniprot.org/uniparc/UPI0000209778 [retrieved on 2016-12-14] abstract	1-8, 15-17
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Further documents are listed in the continuation of Box C.



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Date of the actual completion of the international search

20 December 2016

Date of mailing of the international search report

05/01/2017

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Authorized officer

Zellner, Eveline

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/053352

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	page 514, column 1, paragraph 1; figures 2, 5 page 517, column 2, paragraph 1 -----	1-17
X	US 2008/207497 A1 (RAMAKRISHNA VENKY [US] ET AL) 28 August 2008 (2008-08-28)	1-12, 15-17
Y	paragraph [0020]; sequence 1261 -----	1-17
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X,P	XIUXIU LU ET AL: "A High Affinity hRpn2-Derived Peptide That Displaces Human Rpn13 from Proteasome in 293T Cells", PLOS ONE, vol. 10, no. 10, 14 October 2015 (2015-10-14), page e0140518, XP055328539, DOI: 10.1371/journal.pone.0140518 abstract -----	1-17

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Information on patent family members

International application No

PCT/US2016/053352

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