**The C-terminus of the PSMA3 proteasome subunit preferentially traps intrinsically disordered proteins for degradation**

**Abstract**

Degradation of intrinsically disordered proteins (IDPs) by a non-26S proteasome process does not require proteasomal targeting by polyubiquitin. However, whether and how IDPs are recognized by the non-26S proteasome, including the 20S complex, remains unknown. Analyses of protein interactome datasets have revealed that the 20S proteasome subunit, PSMA3, preferentially interacts with many IDPs. Employing *in vivo* and cell-free experiments, it was found that a 69-amino-acids-long fragment at the C-terminus of PSMA3 is sufficient to bind the disordered protein p21. A recombinant PSMA3 C-terminus 69 fragment is sufficient to interact with many IDPs, and is therefore designated an IDP trapper. A recombinant IDP trapper blocks the degradation of many IDPs *in vitro* by the 20S proteasome, possibly by competing with the native trapper. In addition, over a third of the PSMA3 trapper-binding proteins have previously been identified as 20S proteasome substrates, and many of the trapper binding proteins are associated with the intracellular proteasomes. The PSMA3-trapped IDPs that are proteasome substrates have the unique features previously recognized as characteristic 20S proteasome substrates *in vitro*. We propose a model whereby the PSMA3 C-terminal region traps a subset of IDPs to facilitate their proteasomal degradation.

Significance Statement

Proteasomal protein degradation is a critical and highly regulated cellular process. The potential substrates often must be polyubiquitinylated if they are to be recognized by the proteasomes. However, another mode of degradation, mostly that of the intrinsically disordered proteins, can occur in the absence of ubiquitination. Here we describe a new mechanism of recognition that is mediated by direct interaction of the potential substrates with a component of the 20S proteasome, the PSMA3 C-terminus. Evidence is provided to show that at least a fraction of the proteins recognized by this new mechanism are rapidly degraded in a cell-free system and in the cells. Given the importance of proteostasis in health and disease, the described mechanism also has therapeutic potential.

**Introduction**

Proteasomal protein degradation plays key roles in diverse cellular processes and cell fate determination (1, 2). The 26S proteasome is a major molecule which catalyzes protein degradation. Its 20S barrel-shaped proteolytic core particle is capped at one or both ends by the 19S regulatory complex (3–6). The 20S proteasome is composed of four stacked rings, two PSMA and two PSMB rings in a barrel shape. Proteolytic activity occurs in the chamber formed by the inner PSMB rings. The outer PSMA rings are identical. and each has seven distinct subunits. The N-termini of the PSMA subunits form a gated opening controlling substrate entry into the proteasome (7, 8).

Prior to undergoing degradation, proteins destined for degradation are first identified as “legitimate” substrates by the proteasomes (9, 10). This is accomplished by protein substrates targeting the proteasome via protein-protein interaction, with the ubiquitin-dependent pathway serving as the major targeting mechanism. Polyubiquitin chains are covalently attached to the substrates, marking them for proteasome recognition and subsequent degradation (11). The polyubiquitin chain binds the 19S regulatory particle of the 26S proteasome directly or through transiently associated 19S proteins (12–15). To date, three of the subunits of the 19S particle, Rpn1, Rpn10 and Rpn13, have been identified as ubiquitin receptors (16, 17).

Intrinsically disordered proteins (IDPs) or proteins with disordered regions (IDRs) are generally more labile proteins (18, 19). Certain proteins, such as IDPs and oxidized proteins, have been shown to undergo ubiquitin-independent proteasomal degradation, including by the 20S proteasomes (20–23). Key questions are whether and how the proteasome recognizes these substrates. This study has found that PSMA3’s C-terminus binds multiple IDPs. Based on this, we proposed a model whereby the 20S catalytic particle has a substrate receptor to trap certain IDPs for proteasomal degradation.

**Results**

**The 20S proteasome PSMA3 subunit preferentially binds IDPs**

We assumed that an inherent component of the 20S complex could potentially act as an IDP trapper for degradation. To confirm this model, we drew on interactome datasets, assuming that the PSMA-interacting proteins are potential 20S substrates. Analyzing the IMEx data resource, which searches different databases of large-scale protein-protein interaction screens (24), PSMA3 and PSMA1 were found to be the preferred protein-binding constituents (Fig. 1A-B). Next, using the IUPred algorithm (25), we evaluated the percent disorder of the PSMA-interacting proteins, finding that the PSMA3-interacting proteins are uniquely highly enriched for IDPs (Fig. 1C). We also compared PSMA3-interacting proteins found in the IMEx data resource to PSMA3-interacting proteins found in the HI.II.14 dataset from the human interactome project (26). The interacting proteins found in the HI.II.14 dataset are also enriched with IDPs (Fig. 1D). These analyses indicate that PSMA3 is a proteasome subunit that specifically interacts with IDPs.

**PSMA3 interacts with p21 in the cells**

The protein p21 is an IDP (27, 28) that undergoes both ubiquitin-dependent and independent proteasomal degradation (29, 30). It has already been reported that p21 binds to the 20S PSMA3 subunit *in vitro* (31). In order to directly visualize p21’s interaction with PSMA3 in the cells, we used the bimolecular fluorescence complementation (BiFC) assay. In this assay, the reporter fluorescent protein (FP) is divided into two fragments: the C-terminus FPC and the N-terminus FPN, which emit fluorescent signals upon their interaction. PSMA3 and five subunits were fused to the FPC and co-transfected with a chimeric 6xMyc-p21 FPN. The Myc tag minimizes p21 proteasomal degradation (30), but should not affect PSMA3 association. The level of interaction between the 6xmyc p21 FPN and PSMA3-FPC was monitored by quantifying GFP positive cells.

First, we investigated whether the PSMA3-FPC chimera incorporates into the proteasomes using native gel analysis. According to the pattern of migration in the native gel, a fraction of the PSMA3-PFC successfully incorporated into 20S and 26S proteasome complexes (Fig. 2A). To quantify the fraction of the incorporated chimeric PSMA3, we conducted successive proteasome depletion experiments (Fig. 2B-C). The proteasomes were depleted from the cellular extract through immunoprecipitation of the endogenous 20S proteasome PSMA1 subunit and monitored for the presence of the chimeric PSMA3-FPC. We found that the PSMA3-FPC chimera was depleted as efficiently as the endogenous proteasome subunit PSMA1 (Fig. 2D). As expected, under the same conditions, the 19S proteasome subunit PSMD1 was also depleted, although with lower efficiency. These results suggest that the vast majority of the PSMA3-FPC chimera protein is incorporated into the proteasomes. In the test, p21 FPN gave a stronger signal when co-transfected with PSMA3 FPC than did PSMA5 FPC (Fig. 2E-F). Both constructs were expressed to the same level (Fig. 2G). These data suggest that PSMA3 preferentially interacts with p21 in the cells.

**The PSMA3 C-terminus is sufficient to interact with p21**

The PSMA subunits differ primarily at their C-termini (32). We generated and examined PSMA3 truncated C-terminus mutants and found that a sharp reduction in PSMA3 and p21 interaction was obtained by the truncation of the 187-255 C-terminus region (Fig. S1). Because the PSMA3 C-terminus (Ct 187-255) is exposed to its surroundings in the context of the 20S and 26S proteasomes, it is accessible to the putative IDP substrates (Fig. S2). Consequently, we assumed that the PSMA3-Ct is the most likely p21-interacting region. To examine this possibility and to show that the PSMA3 C-terminus is sufficient to interact with p21, we constructed PSMA5-PSMA3 chimeric constructs in which the C-terminus of PSMA5 was replaced with the homologous region of PSMA3. Two chimeric PSMA5-PSMA3 were constructed: one with a long PSMA3 Ct fragment (Ct 187-255) and the other with a shorter Ct187-229 fragment (Fig. S3A). The chimeric constructs had higher levels of GFP than the PSMA5 (Fig. 3A), suggesting that the PSMA3 Ct is more efficient in interacting with p21. To validate the GFP data, we conducted co-immunoprecipitation experiments, for which we used a 6xmyc-tagged p21 construct lacking the FPN moiety. We found it co-immunoprecipitated with the chimeric PSMA5-PSMA3 constructs but not with the naïve PSMA5 (Figure 3B). The reciprocal constructs in which the C-terminus of the PSMA3 was replaced by that of PSMA5 were constructed, but these expressed very poorly (Figure S3B and C). These data suggest that the PSMA3-Ct is sufficient to interact with p21 also in the PSMA5 context.

Next, we utilized the split luciferase reporter to monitor p21’s interaction with PSMA3 and with the PSMA3 Ct 187-255 fragment. The utilized constructs were efficiently expressed (Fig. 3C). Luciferase activity was restored not only in the presence of full length PSMA3, but also with the Ct 187-255 fragment (Fig. 3D). These data suggest that the PSMA Ct 187-255 fragment binds p21. Therefore, we termed it the p21 trapper region.

**Recombinant PSMA3 p21-trapper fragment interacts with many IDPs**

To explore whether the p21 trapper is active in binding other IDPs, we generated chimeric GST-PSMA3 187-255 recombinant proteins. The chimera GST-PSMA5-188-241 and recombinant GST were used as controls (Fig. 4A). We examined the recombinant trapper’s ability to pull down specific endogenous IDPs, such as c-Fos and p53, and ectopically expressed 6xmyc p21. Remarkably, PSMA3 187-255 specifically pulled down all these proteins (Fig. 4B). The interaction was highly specific compared to the two controls. These data suggest that the PSMA3 fragment active in binding p21 also binds a subset of IDPs.

To obtain a more systemic view of the PSMA3 trapper domain, we used cellular extracts of over 1400 proteins in pull-down experiments. We identified 152 proteins that were retained in the GST-PSMA3 187-255 column and none in the control PSMA5-188-241. We termed the PSMA3 trapper binding proteins PSMA3-TBP. Interestingly, PSMA3-TBPs are significantly intrinsically disordered (Fig. 4C).

Previously, we had identified a group of proteins that is readily degraded by the 20S proteasome *in vitro* and designated it as 20S-IDPome (20). Interestingly, out of 152 PSMA3-TBPs, 57 overlaps were obtained with the 20S-IDPome (Fig. 4D). Random intersection between the groups was estimated based on a Z-test with the null distribution calculated by 10,000 simulations. Based on this calculation, the number of proteins shared by these two groups is highly significant over the random distribution (Fig. 4E). The finding that some of the PSMA3-TBPs are 20S substrates may suggest that the trapper domain of the PSMA3 mediates degradation by the 20S proteasome *in vitro*. To substantiate this possibility, we conducted 20S degradation *in vitro* of cellular extracts in the presence and absence of the recombinant trapper domain (Figure 4F). We found that the recombinant GST-PSMA3 trapper markedly compromised 20S proteasomal degradation of the substrates. We ruled out the possibility of the recombinant polypeptides inhibiting the 20S catalytic activity (Fig. S4). The decoy effect was specific and was not repeated by the control recombinant GST. These results suggest that *in vitro*, the 20S proteasomal degradation of a large number of IDPs is regulated by the PSMA3 trapper (Fig. 4G).

**Many of the PSMA3-TBPs share the 20S proteasome substrate hallmarks**

We previously reported that proteins of the 20S-IDPome are not only highly disordered, but also display a unique signature (20) in that they are significantly highly enriched for: RNA binding proteins (RBPs); proteins with low complexity region (LCR); proteins with prion-like domain (PrLD); and GR/PR di-peptide repeats interactor proteins (33). Interestingly, the PSMA3-TBPs also have the 20S-IDPome signature (Fig. 5A-E). To determine whether the signature is important for trapper recognition or for degradation, we separately analyzed the PSMA3-TBPs that were identified as 20S substrates (57 proteins) and compared them to those that were not (95 proteins) (Fig. 4D). Interestingly, the former group was significantly more disordered and displayed the 20S-IDPome signature in comparison to the latter group (Fig. 5 F-J).

Since the 20S-IDPome group was identified based on *in vitro* degradation studies using heat-treated cellular extracts, the physiological relevance of the findings were unclear. Therefore, we performed a similar study using a published dataset of cellular nascent substrates that were identified by virtue of their proteasomal association and degradation in cells (34). Remarkably, many of the PSMA3-TBPs are cellular nascent proteasome substrates (Fig. 6A), far more than would be expected by random distribution (Fig. 6B). Furthermore, cellular nascent proteasome substrates display the 20S-IDPome signature (Fig. 5C-G), particularly, the group of proteins that were both PSMA3-TBPs and nascent substrates. However, the possibility that this group of proteins undergo ubiquitin-dependent degradation *in vivo* remains open. These data suggest that PSMA3-TBPs bearing the 20S-IDPome signature are more prone to proteasomal degradation *in vitro* and *in vivo*.

**Discussion**

Understanding the mechanisms of substrate recognition by the proteasome is critical for appreciating the importance of proteostasis. The mechanism controlled by substrate ubiquitination that mediates recognition by the 19S regulatory particle of the 26S proteasome is well known (11). However, our knowledge of ubiquitin-independent mechanisms is very limited. Ornithine decarboxylase proteasomal degradation is ubiquitin independent and mediated by antizyme. However, how the substrate is targeted for recognition by the proteasome remains an open question (35). This study investigated a mechanism that is based on the inherent structures of the proteasome and the substrates, and has provided evidence that the PSMA3 C-terminus region plays the role of trapper of a group of IDPs. We demonstrated that the trapper interacts *in vivo* with the IDP p21 and pulls down c-Fos and p53. We further showed that the recombinant trapper inhibits degradation of proteins by the 20S proteasome, possibly by competing with the substrates to bind the trapper in its natural context. Interestingly, the trapper binds a large number of proteins sharing the hallmarks of the previously identified 20S substrates (20). These findings are consistent with a model whereby the PSMA3 trapper recognizes the substrate for degradation.

How the PSMA3 trapper recognizes a large number of IDPs is not known. The trapper C-terminus tail is highly acidic; however, the PSMA5 also has an acidic tail but is inactive in IDP binding. This is not because the PSMA5 is less accessible to the surface, as even the PSMA5 C-terminus in isolation did not bind IDPs. Although a charged tail might be important, it seems not to be sufficient. Additional structural analysis is required to resolve this interesting question.

Not all the trapper binding proteins are optimal proteasome substrates in the process of ubiquitin-independent degradation. Interestingly, the trapper binding proteins that are either degraded by the 20S proteasome *in vitro* or identified as proteasome nascent substrates in the cells, share the hallmarks of the 20S-IDPome; namely, they are IDP, RBP, GR/PR di-peptide repeats interactor proteins (33), containing a low complexity region (LCR) and a prion-like domain (PrLD). Trapper binding proteins that were not well degraded by the proteasomes only poorly display this signature. Remarkably, components of liquid droplets share the same signature (20). An interesting possibility is that the trapper preferentially recognizes liquid droplets for degradation.

A key question is how the IDP trapper/degradation is regulated to permit substrate discrimination. The trapper region is post-translationally modified, particularly at the 250 residue (phosphosite dataset). Also, it has been clearly demonstrated that IDPs undergo rather extensive post-translational modifications (36–38). These modifications, largely S/T phosphorylation, might regulate trapper binding and degradation. Additionally, previously, we reported on nanny proteins that interact with IDPs to escape their proteasomal degradation (39). Nanny proteins therefore might function to help the substrate in escaping the trapper. According to these models, trapper interaction takes place by default unless inhibited. This model is substantially different from the ubiquitination model where the substrate is stable unless marked by ubiquitin.

**Materials and methods:**

**Tissue culture**

The human embryonic kidney cell line HEK293 and the human osteosarcoma cell line U2OS were grown in DMEM supplemented with 8% fetal bovine serum, 100 units/ml penicillin, and 100 g/ml streptomycin and cultured at 37°C in a humidified incubator with 5.6% CO2. HEK293 cells were transfected using the calcium phosphate method. U2OS cells stably expressing the chimeric PSMA3 subunit were created using the Gateway cloning system (Invitrogen).

**Plasmids, transfection and infection**

PSMA subunits (kindly provided by Prof. K. Tanaka, Tokyo Metropolitan Institute of Medical Science, Japan) were cloned into pBiFC-VN173 (Addgene plasmid no. 22010), a gift from Prof. Chang-Deng Hu (Purdue University). The 6xmyc p21 (kindly provided by Prof. Chaim Kahana, Weizmann Institute of Science, Israel), was cloned into pBiFC-CC155 (Addgene plasmid no. 22015), a gift from Prof. Chang-Deng Hu. pCI 6xmyc p21, pcDNA3 CFP and pcDNA3 CFP PSMA3 187-255aa. Luc1 and Luc2, the N' terminal and C' terminal fragments of split Gaussia-luciferase, were kindly provided by Prof. Adi Kimchi (Weizmann Institute of Science, Israel.).

**Immunoblot analysis**

Cells were lysed with NP40 buffer (20 mM Tris-HCl pH7.5, 320 mM sucrose, 5 mM MgCl2, 1% NP40), supplemented with 1 mM dithiothreitol (DTT) and protease and phosphatase inhibitors (Sigma). A Laemmli sample buffer (final concentration 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue, 0.0625 M Tris-HCl pH6.8) was added to the samples, heated at 950C for three minutes and loaded on a polyacrylamide-SDS gel. Proteins were transferred to 0.45 m cellulose nitrate membranes. Antibodies: Mouse anti-HA, mouse anti-tubulin and mouse anti-human p53 Pab1801 were purchased from Sigma. Mouse anti-myc was produced by the Weizmann Institute Antibody Unit. Rabbit anti-PSMD1, a subunit of the 19S proteasome, was purchased from Acris. Rabbit anti-PSMA4, a subunit of the 20S proteasome (40), was kindly provided by Prof. C. Kahana, Weizmann Institute of Science, Israel. Secondary antibodies used were horseradish peroxidase-linked goat, anti-rabbit, and anti-mouse (Jackson ImmunoResearch). Signals were detected using the EZ-ECL kit (Biological Industries).

**Co-immunoprecipitation**

Cell lysates were incubated with a primary antibody for 16 hours. Samples were washed six times with NP40 buffer. Bound and associated proteins were eluted with Laemmli sample buffer or HA peptide (Sigma) according to the standard protocol (ref?).

**Nondenaturing PAGE**

Samples were prepared and run as described (41).

**Protein-fragment complementation assays (PCAs)**

We employed the protein-fragment complementation approach (42). To directly visualize p21 interaction with PSMA3 in the cells, we adopted the bimolecular fluorescence complementation (BiFC) assay (43). In this assay, the reporter fluorescent protein (FP) is split into two fragments; the C-terminus FPC and the N-terminus FPN, which emit a fluorescent signal upon their interaction. Cells were co-transfected with PSMA3 subunit-FPC, potential substrate-FPN, and H2B-red fluorescent protein (RFP), the latter used to identify the transfected cells. Cells with successful BiFC are green (VFP), while H2B-RFP makes the cell nuclei red. For flow cytometry analysis, cells were harvested 48 hours post-transfection, washed and resuspended in PBS. Samples were analyzed with a BD LSR II flow cytometer using FACSDiva software (BD Biosciences). The fluorescent intensities of VFP and RFP in RFP positive cells were recorded and extracted using FlowJo software (

We also used split Gaussia luciferase. In this system, the receptor protein was fused to the C'-terminal fragment of Gaussia luciferase (denoted Luc2) and p21 fused to the N'- terminal fragment of the luciferase (denoted Luc1). Upon interaction, the luciferase enzyme reconstructs and its activity, which generates bioluminescence in the presence of its substrate, can be detected. Cell lysates were plated in a 96-well white plate, 30 L per well. Using automated injection in a Veritas microplate luminometer (Turner BioSystems) the lysates were mixed with luciferase substrate. The solution was prepared by diluting coelenterazine (Nanolight) to a final concentration of 20 M in an assay buffer (25 mM Tris pH 7.75, 1 mM EDTA, 0.6 mM reduced glutathione, 0.4 mM oxidized glutathione and 75 mM urea). A bioluminescent signal was read after injection of 100 L of substrate solution and integrated over 10 s.

**GST pulldown**

Recombinant GST proteins bound to glutathione agarose were incubated in a rotator with treated or naïve cell lysate for 16 hours at 4°C. Beads were washed six times with 300 l NP40 buffer and recombinant GST and associated proteins were eluted from glutathione agarose beads with 70 l of 10 mM glutathione in 50 mM Tris-HCl pH 9.5.

***In vitro* degradation assay**

Degradation of IDP-enriched lysate by purified 20S proteasomes was carried out in a degradation buffer (100 mM Tris-HCL pH 7.5, 150 mM NaCl, 5 mM MgCl2, 2 mM DTT) at 37ºC for three hours. The degradation reaction was halted with the addition of Laemmli sample buffer to the samples. The samples were then heated at 95ºC for five minutes and fractionated by SDS-PAGE. Following electrophoresis, proteins were visualized by gel staining or transferred to cellulose nitrate membranes. Purified 20S proteasome was detected by immunoblotting with rabbit anti-PSMA4 antibody.

**Data analysis**

Data analysis was performed with a web-tool for plotting box plots (<http://boxplot.tyerslab.com>) and Microsoft Excel. The FunRich software (<http://www.funrich.org>) was used to plot proportional Venn diagrams. Gene ontology, prediction of structural disorder, PrLD, LCR and GR/PR di-peptide repeats interactor proteins were performed as previously reported (20). The expected intersection between two groups was evaluated by a Z-test with the null distribution calculated by a 10,000 simulation randomly choosing x from set A, y from set B, and noting the intersection between the randomly chosen element. Protein sequences were obtained from UniProt (http://www.uniprot.org/) using the annotated Swiss-Prot bank. Statistical tests and plotting were performed using MATLAB 2016b, The MathWorks, Natick, 2014.

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**Figure Legends:**

Figure 1: **PSMA3 preferentially interacts with IDPs.** (A) Crystal structure of PSMA ring adapted from (45). PSMA subunits are identified by numbers. The N termini of the PSMA subunits protrude into the center of the ring, forming a gate restricting access into the 20S proteasome. (B) Pie chart presenting identified protein interactions of each PSMA subunit as a percentage of all identified protein interactions with PSMA subunits. We used the IMEx data resource to assemble an interaction list for the subunits. (C-D) Boxplot presenting the fraction of disordered residues found in the interacting proteins' sequences. Non-overlapping notches provide a 95% confidence that medians differ. Disordered residues were predicated with the IUPred algorithm. (C) Distribution of PSMA subunits interacting proteins from IMEx data resource. (D) Distribution of PSMA3 interacting proteins from HI.II.14 dataset and IMEx data resource. The PSMA subunits are color coded.

Figure 2: **PSMA3 interacts with p21 in the cells.** (A) U2OS cells stably expressing PSMA3-FPC. Cell lysates were enriched with proteasomes by ultracentrifugation and loaded on native gel. The membrane was probed with antibody against either HA-tag or the endogenous subunit PSMA4. (B) Schematic description of the antibodies used against the different 26S proteasome subunits for the described co-immunoprecipitation experiments to demonstrate the possible incorporation of a chimeric PSMA3 subunit into proteasomes. The endogenous PSMA1 subunit was first immunoprecipitated and the level of the co-immunoprecipitated subunits was monitored using antibodies to detect the endogenous PSMD1, a subunit of the 19S proteasome, and anti-HA to detect the chimeric PSMA3. (C) The schematic description of the experimental strategy of serial consecutive immunoprecipitation steps. (D) The results obtained from each of the steps described in Panel C. HEK293 cells expressing HA PSMA3 FPC were harvested 24 h post-transfection. Cells’ lysate was subjected to four subsequent immunoprecipitations of proteasomes via the endogenous PSMA1 subunit. Ten percent of cell lysate was kept for analysis after each immunoprecipitation. (E) Cells were transfected with either PSMA3 FPC or PSMA5 FPC together with p21 FPN (see scheme). We also transfected the cells with H2B RFP, which provides RFP labeling of the transfected cells’ nuclei. Successful BiFC using fluorescent microscopy, 20x objective 48 hours post-transfection. (F) Intensities of at least 10,000 cells for each PSMA-p21 combination were recorded by flow cytometry. Standard deviation bars represent two independent experiments. (G) Expression level of the proteins in the cells presented in Panel D was examined.

Figure 3: **The PSMA3 C-terminus is sufficient to interact with p21.** (A) Cells were transfected with the PSMA5 and three chimera (see Fig. 3S) together with p21-FPV and fluorescence intensities of at least 10,000 cells for each case were monitored and recorded by flow cytometry. Standard deviation bars represent three independent experiments. \*p = 0.03, \*\*p = 0.001 using a two-tailed Student t test. (B) Schematic illustration of the experimental strategy with the antibodies used for immunoprecipitation (IP) or immunoblotting (IB), the latter to detect myc-tagged p21. HEK293 cells were transiently transfected as indicated with 6xmyc p21 and chimeric PSMA5 subunits. Cells were harvested 48 hours post-transfection, lysed and subjected to IP with HA beads to immunoprecipitate chimeric PSMA5 subunits. Total lysate and IP samples were analyzed by SDS-PAGE and immunoblotting. (C) Representative SDS-PAGE and immunoblot analysis of the overexpressed proteins that were tested for interaction. Ponceau staining was used as a loading control. (D) The scheme of divided luciferase experiment is shown above the obtained data analyzed by Boxplot representing the overall bioluminescent signals corresponding to interaction between p21 and either PSMA3 or PSM3 187-255. The samples are numbered based on Panel C. n=14-18. \*\*\* P ≤ 0.007-0.002.

Figure 4: **Isolated PSMA3 C-terminus interacts with many intrinsically disordered proteins.** (A) Illustration of constructs used and experimental strategy. (B) Purified GST, GST PSMA3 trapper and GST PSMA5 C terminus bound to glutathione agarose beads were incubated with HEK293 cell lysate overexpressing 6xmyc p21 or naïve HEK293 cell lysate. GST constructs and the interacting proteins were eluted with 10 mM reduced glutathione. GST constructs were visualized with Ponceau and interacting proteins; myc-p21 and endogenous c-Fos and p53 were detected by immunoblot (IB). (C) The different GST-chimeric proteins described in A were incubated with cellular extract. The bound proteins were identified by MS. One hundred fifty-seven proteins were retained on the GST PSMA3 trapper fragment, whereas only nine were retained on the GST PSMA5 C terminus fragment. The former group was analyzed for IDP/IDR content. The Boxplot shows the IDP/IDR content of the proteome compared to the trapped proteins by the PSMA3 C-terminus fragment and to the 20S IDPome group. \*\*\*\* P ≤ 0.001 (D) Venn diagram of the proteins retained on the column containing the GST-PSMA3 trapper fragment and the 20S IDPome. (E) The expected average number of shared proteins between the two groups in Panel D was evaluated by a Z-test with the null distribution calculated by 10,000 simulations. The expected intersection number is approximately 25 proteins, whereas the observed number is 57 (red dot, P-Value < 0.00001). (F) Hek293 IDP-enriched lysate was incubated for three hours at 37oC with purified 20S proteasome, GST-PSMA3 trapper and GST as indicated. Protocol for 20S purification was previously described (20). Proteins were visualized with InstantBlue stain. (G) A model describing the steps of IDP recognition by the trapper and degradation by the 20S.

Figure 5: **Many of the PSMA3-TBPs share the 20S proteasome substrate hallmarks** (A-E) Three groups of proteins are compared and labeled -1 for overall proteome, 2 for PSMA3-TBPs and 3 for 20S-IDPome. These group of proteins were compared for: (A) the average disorder degree (Boxplot); (B) percent of RBPs in the group; (C) percent of proteins positive for low complexity region (LCR); (D) percent of proteins positive for prion-like domain (PrLD); and (E) percent of proteins positive for GR/PR di-peptide repeats interactor proteins. (F-J) Under this set of panels, the 20S IDPome group of proteins (Group 1, 505 proteins) is compared with a fraction of the PSMA3-BPs that is shared by Group 1 (group 2, 57 proteins) and that is not shared by Group 1 (Group 3, 95 proteins). The comparison was done for: (F) the average disorder degree (Boxplot); (G) percent of RBPs in the group; (H) percent of proteins positive for low complexity region (LCR); (I) percent of proteins positive for prion-like domain (PrLD); and (J) percent of proteins positive for GR/PR di-peptide repeats interactor proteins. \*p = 0.004 \*\*p = 0.00003 \*\*\*p = 0.0007 using a two-tailed Student t test. NS is non-significant. NS, P > 0.05, \* P ≤ 0.05, \*\* P ≤ 0.01, \*\*\* P ≤ 0.001.

Figure 6: **Many of the PSMA3-TBPs are proteasomal associated in the cells** (A) Venn diagram of the nascent proteasome substrates (nascent peptides, 1192 proteins) and the PSMA3-BPs (trapper). (B) The expected overlap between the two groups in Panel A was evaluated by a Z-test with the null distribution calculated by 10,000 simulations. The expected average overlapped group is 20 proteins, whereas the observed number is 58 (red dot) (P-value <0.00001). (C-G) Under this set of panels, the proteome (Group 1) is compared to the following groups: PSMA3-TPs (Group 2 n=152); proteins that are found in Group 2 and also in the nascent substrate proteins (Group 3 n=58); and to proteins that are of PSMA3-TPs group but not found in the nascent substrate proteins (Group 4 n=94). The comparison was done for: (C) the average disorder degree (Boxplot); (D) percent of RBPs in the group; (E) percent of proteins positive for low complexity region (LCR); (F) percent of proteins positive for prion like domain (PrLD); and (G) percent of proteins positive for GR/PR di-peptide repeats interactor proteins. NS, P > 0.05, \* P ≤ 0.05, \*\* P ≤ 0.01, \*\*\* P ≤ 0.001.

Figure S1: The PSMA3 C-terminus binds p21.

(A) Shows the predicted structure of PSMA3 26 C-terminus mutant and the structure of the deleted region. Crystal structure adapted from (45). (B) As in A, but the PSMA3 69 C-terminus is described. (C) HEK293 cells were transiently transfected as indicated with 6xmyc p21 FPN, chimeric PSMA3 WT, deletion mutants and H2B RFP constructs. The latter monitors for the transfected cells. The percent of the GFP positive cells is compared between the different constructs.

Figure S2: PSMA3 C-terminus is exposed in the context of the 20S and 26S proteasome complexes.

(A)Cryo-EM structure of the 26S proteasome marking PSMA3 Ct adapted from Huang et al., 2016. (B) Structure of the 20S proteasome marking PSMA3 Ct and. The indicated C-terminal portions are labeled in magenta, and remaining PSMA3 and PSMA5 subunits are labeled in cyan and green, respectively.

Figure S3: PSMA5 and three chimeric constructs.

(A) Illustration of chimeric constructs of PSMA5 and PSMA3 C-terminus region used in our experiments. Crystal structures adapted from (45). (B) Illustration of the chimeric constructs of PSMA3 and PSMA5 C-terminus region is shown. (C) The expression level of the constructs shown in B.

Figure S4: The PSMA3 trapper does not inhibit proteasome catalytic activity.

Purified 20S proteasome was incubated for 30 min at 37°C as indicated with purified GST, GST PSMA3 trapper, GST PSMA5 C-terminus and proteasome inhibitor MG132 in the presence of the chymotrypsin-like fluorogenic substrate Suc-LLVY-AMC. Standard deviation bars represent three independent experiments.