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Regulation of proteasomal degradation by modulating proteasomal initiation regions

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Abstract

Methods for regulating the concentrations of specific cellular proteins are valuable tools for biomedical studies. Artificial regulation of protein degradation by the proteasome is receiving increasing attention. Efficient proteasomal protein degradation requires a degron with two components: a ubiquitin tag that is recognized by the proteasome and a disordered region at which the proteasome engages the substrate and initiates degradation. Here we show that degradation rates can be regulated by modulating the disordered initiation region by the binding of modifier molecules, *in vitro* and *in vivo*. These results suggest that artificial modulation of proteasome initiation is a versatile method for conditionally inhibiting the proteasomal degradation of specific proteins.

Introduction

A powerful and versatile method to regulate the activity of specific proteins in cells is by controlling their abundance in cells. The most common methods developed for this purpose adjust protein synthesis rates. However, this approach is limited by the intrinsic degradation rates of proteins: proteins with high degradation rates may not accumulate substantially even when they are overexpressed, and proteins with low degradation rates may persist for long times in cells even when their synthesis is blocked. Therefore, methods that manipulate degradation rates are now receiving increasing attention.

In eukaryotic cells, most regulated degradation is by the ubiquitin proteasome system (UPS). At the center of the UPS is a large proteolytic complex called the proteasome. One of the simplest ways to regulate proteasomal degradation is through inhibitors of the proteasome's

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Supporting Information

Experimental data of negative control, gel electrophoresis, and additional experiments. This material is available free of charge via the Internet <http://pubs.acs.org>.

Author Contributions

Conceived and designed the experiments: K.T., A.M., and T.I. Performed the experiments: K.T. and T.I. Analyzed the data: K.T. and T.I. Contributed reagents/materials/analysis tools: K.T. and T.I. Wrote the paper: K.T., A.M., and T.I.

proteolytic sites such as bortezomib and carfilzomib, which are promising anti-cancer drugs¹. Proteasome inhibitors do not discriminate between individual proteasome substrate and thus have broad effects on protein turnover and the various cellular processes regulated by the UPS. Thus, a more specific approach that affects individual proteins is desirable².

Proteins are targeted to the proteasome for destruction through the attachment of several copies of the small protein ubiquitin. The modification is catalyzed through the sequential action of a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). Target proteins are recognized by E3 enzymes so that chemical inhibitors of E3 ubiquitin ligases present a more specific method to manipulate proteasomal degradation². However, E3 ligases do not always recognize a single specific protein and the design of E3 inhibitors is challenging³. For this reason, there is demand for simple and versatile methods of degradation regulation with high specificity.

Efficient proteasomal degradation of a protein also requires the presence of a disordered region in the substrate^{4,5}. The proteasome recognizes the substrate at the ubiquitin tag and then engages the substrate and initiates degradation at the disordered region. Once degradation is initiated, the proteasome proceeds along the polypeptide to hydrolyze it sequentially^{4,6}. The proteasome has distinct preferences for the length, location, and amino acid sequence of the initiation region and the initiation step contributes to the specificity of substrate selection^{7,8}.

Here, we propose that it should be possible to tune proteasomal degradation by manipulating the accessibility of the unstructured initiation region in proteasome substrates (Figure 1A). To test this hypothesis, we constructed an experimental system employing model proteins whose unstructured initiation regions can bind modifier molecules, and investigated whether binding alters the efficiency of proteasomal degradation. We show that degradation can be regulated by modifiers for proteasomal initiation regions *in vitro* and in cells. Our results shed light on the regulation mechanism of proteasomal degradation in the cell and provide novel strategies for conditional regulation of the cellular levels of specific proteins.

Results and discussion

Modification of an unstructured region with a small molecule regulates proteasomal degradation

To test whether the modification of an unstructured initiation region by a small molecule can alter the efficiency by which it is degraded, we relied on well-characterized model proteasome substrates whose proteolysis is easily followed *in vitro* and *in vivo* (Figure 1B). These model proteins consist of a central folded domain derived from *E. coli* dihydrofolate reductase (DHFR) and contain an ubiquitin-like domain (UbL) domain derived from yeast Rad23 fused to DHFR's N-terminus to allow the proteasome to recognize the substrate. A small titin immunoglobulin domain (I27) followed by a 27 amino acid-long unstructured region derived from yeast cytochrome *b*₂ at which the proteasome initiates degradation was attached to DHFR's C terminus (to create UbL-DHFR-I27-tail)⁷. Thus, degradation proceeds along the polypeptide chain from its C terminal end toward the N terminus. To manipulate the accessibility of the C-terminal tail of the model substrate, we directly

inserted the sequence FLNCCPGCCMEP between the folded domain and the disordered tail. This sequence contains a tetracysteine motif, which is recognized with high affinity and specificity by biarsenic compounds⁹. Biarsenics have been developed to label proteins specifically and membrane permeable molecules such as the resorufin derivative ReAsH are commercially available (Figure 1B)^{10, 11}. To test whether ReAsH binds to the tetracysteine substrate, we synthesized the protein by coupled transcription and translation in *E. coli* extract, incubated it with ReAsH for 15min and analyzed the end product by SDS-PAGE and fluorescence imaging. After the electrophoresis, green light illumination revealed a single major red fluorescent band at the molecular weight expected for the ReAsH-tetracysteine model substrate complex, indicating ReAsH modification of tetracysteine model substrate (Figure 1C).

We predict ReAsH-modification of the disordered region will change its physico-chemical properties such as structure, bulk, flexibility, and hydrophobicity. These changes in turn may affect the proteasome's ability to initiate degradation thus stabilizing the entire protein against proteolysis. To test this prediction, we synthesized radiolabeled substrate by *in vitro* transcription and translation and presented it to purified yeast proteasome in the presence of ATP. We took samples at different times after the reaction was initiated and analyzed the amount of protein remaining by SDS-PAGE and autoradiography. The protein was degraded efficiently but addition of 20 μ M ReAsH stabilized it by decreasing the degradation rate at least 10-fold and also reducing the amount of degradation (see below) (Figure 2A). Degradation was by the proteasome because it was inhibited in the presence of proteasome inhibitor MG132 (Supporting Figure S1). ReAsH inhibited degradation directly through its interaction with the initiation region because deletion of the ReAsH binding sequence from the proteasome substrate also abolished any effect of ReAsH on degradation (Figure 2B). Substrate lacking the tetracysteine motif was degraded in the presence and absence of ReAsH with similar efficiency (Figure 2B). Inhibition of degradation depended on the amount of ReAsH added, indicating that an increasing fraction of substrate was modified with ReAsH and became non-degradable (Figure 2A). Thus, the extent of degradation could be tuned by the ReAsH concentration. Finally, ReAsH inhibition was rapid compared to the rate of degradation *in vitro* so that degradation was blocked immediately after the addition of ReAsH (Figure 2C).

It would be possible that ReAsH binding blocks proteasome translocation because it cannot pass through degradation channel even though the receptor can recognize the ReAsH-modified unstructured region. To test this possibility, we inserted the ReAsH-bound tetracysteine motif to the center of model substrate, between DHFR and titin I27 domain (Supporting Figure S2A), and monitored degradation. If ReAsH created a roadblock, the proteasome would produce a fragment of approximately 30 kDa, consisting of the N-terminal part of the substrate until just past the tetracysteine motif. However, the purified yeast proteasome degraded the tetracysteine-inserted substrate completely without the formation of any detectable fragments even in the presence of ReAsH (Supporting Figure S2B), indicating that the ReAsH-bound sequence was able to pass through the degradation channel of the proteasome. This result is supported by the previous observation that the proteasome's degradation channel permits the concurrent passage of at least three polypeptide chains¹². We conclude that the ReAsH-modification of the disordered region

inhibits the initiation of proteasomal degradation by changing the physico-chemical properties of the initiation site in a manner that prevents recognition by the proteasome. Future investigation is required to determine the precise nature of the unfavorable interactions.

Ligand-induced folding of disordered initiation region inhibits proteasomal degradation

Next we tested how general the effect may be or whether our observations so far depend on some unknown indirect effect of ReAsH. Some of the mutants of staphylococcus nuclease (SNase), such as SNase (140–149) in which the C-terminal 10 residues of SNase are truncated, are intrinsically disordered but fold in the presence of the nucleotide inhibitor adenosine-3',5'-diphosphate (prAp)¹³. We constructed a model proteasome substrate in which SNase (140–149) served as the initiation region. The protein again consisted of a central DHFR domain with the Rad23 UbL domain fused to its N terminus and SNase (140–149) fused to its C terminus (Figure 3A). The protein was efficiently degraded by purified yeast proteasome, indicating that the C-terminal SNase (140–149) domain served as an unstructured initiation site. Addition of prAp suppressed degradation at least 10-fold (Figure 3B and Supporting Figure S3A). We conclude that transformation of the unstructured initiation region into a compact folded structure inhibits the initiation of degradation by the proteasome. This result demonstrates that noncovalent binding to the unstructured region is sufficient for inhibiting proteasomal degradation.

Antibody binding to an unstructured region can modulate proteasomal degradation

The above results suggest that the initiation region of a proteasome degradable protein can be targeted to stabilize specific proteins by ligand binding. The easiest way to develop tightly binding ligands to a given disordered region may be by deriving antibodies to the target sequence. Recent advances in antibody production allow the preparation of antibodies against almost any target conveniently¹⁴. Thus, we tested whether the degradation of a model substrate can be modulated by an antibody that binds to the unstructured initiation region in a proteasome degradable protein. We again designed the substrate around a central DHFR domain but now targeted it to the proteasome by fusing four ubiquitin domains to its N-terminus¹⁵. The DHFR domain was followed by the same 27 amino acid long disordered region derived from cytochrome *b*₂ described above but now followed by a C-terminal hexahistidine (His₆)-tag (Figure 4A). The substrate was efficiently degraded by purified yeast proteasome but adding an antibody directed against the His₆-tag inhibited degradation approximately nine-fold (Figure 4B and Supporting Figure S3B). The antibody did not inhibit degradation of a model substrate in which its His₆ recognition site was disrupted by mutation of the His₆ sequence to AHAAHA (Figure 4C and Supporting Figure S3B). This result suggests that degradation of a protein can be inhibited by blocking proteasomal initiation at the relevant unstructured region by antibody binding. The milder inhibition observed here compared to that observed in the ReAsH-mediated inhibition experiments may be caused by the reversible and dynamic nature of non-covalent binding. Nevertheless, this strategy can be a convenient, effective and versatile method to inhibit proteasomal degradation of specific proteins.

ReAsH-modification of the unstructured region can modulate proteasomal degradation in cells

Next we tested whether it is possible to tune the degradation of a protein in cells by modulating its proteasomal initiation region. To this end, we fused FLAG tags to the N termini of model substrates described above and transiently expressed them in HEK293T cells (Figure 5). To characterize the degradation kinetics, we stopped protein synthesis with the translation inhibitor cycloheximide (CHX) and monitored protein levels by western blotting with antibodies against the FLAG tag. As expected, the cellular abundance of the substrate decreased over time after treatment with CHX (Figure 5A). Degradation was blocked by treatment with the proteasome inhibitor MG132 suggesting that protein degradation was by the proteasome (Supporting Figure S4A). Degradation was inhibited approximately three-fold by treatment of the cells with ReAsH (Figure 5A). ReAsH bound to the tetracysteine motif in the model substrate because we could detect a characteristic red fluorescent band at the expected molecular weight after SDS-PAGE analysis and fluorescence imaging of cell extracts (Supporting Figure S5A). Inhibition depended on the presence of the tetracysteine motif in the disordered region of the model substrate, suggesting that ReAsH acted directly on the proteasome initiation step (Figure 5B). ReAsH inhibited degradation less effectively in cells than *in vitro*, most likely because the labeling efficiency of the tetracysteine motif by ReAsH was lower in the cellular environment. Nonetheless, our observation suggests that the method to regulate proteasomal proteolysis by modifying the unstructured region is effective in cells.

ReAsH modification of an unstructured proteasomal initiation region can also regulate ubiquitin-independent proteasomal degradation

Some proteins are targeted to the proteasome without ubiquitin modification and the most established example is degradation of ornithine decarboxylase (ODC) ¹⁶. We asked whether ubiquitin-independent degradation could also be inhibited by modulating proteasomal initiation by small molecule binding. The C-terminal tail of ODC can serve as a transferable degradation signal and lead to the destruction of eGFP when fused to its C terminus ^{17, 18}. We targeted eGFP to the proteasome by fusing the 42 amino terminal amino acids of ODC to its C terminus (to make eGFP-tail^{ODC}) and then inserted the tetracysteine motif between eGFP and the ODC tail (to create eGFP-Cys₄-tail^{ODC}). We expressed the eGFP-ODC tail fusion proteins transiently in HEK293T cells and followed their turnover by shutting off translation with CHX and estimating protein abundance by SDS-PAGE and western blotting against eGFP (Figure 6). The eGFP-ODC tail fusions turned over with similar rates (Figure 6B, C). The proteins were degraded by the proteasome because degradation was inhibited when we added the proteasome inhibitor MG132 (Supporting Figure S4B). Addition of the ReAsH ligand inhibited turnover of the eGFP-ODC tail fusion with the tetracysteine insert (eGFP-Cys₄-tail^{ODC}) approximately five-fold but had no measurable effect on turnover of the protein without the tetracysteine insert (eGFP-tail^{ODC}) (Figure 6B, C). The ReAsH modification of eGFP-Cys₄-tail^{ODC} in the cell was again confirmed by observation of a red fluorescent band at the expected molecular weight after SDS-PAGE analysis and fluorescence imaging of cell extracts (Supporting Figure S5B). We conclude that degradation of ubiquitin-independent substrates was also suppressed by ReAsH modification of the unstructured initiation region. These observations suggest that molecules that bind to

proteasomal initiation regions can suppress the degradation of any specific substrate proteins *in vitro* and *in vivo*.

Degradation of natural proteasome substrates may be modulated by binding partners

The degradation of natural proteins may be regulated in an analogous manner. For example, the transcription factor Sox4 is degraded by the proteasome and overexpressed abnormally in some tumors¹⁹. Sox4 degradation is inhibited by its binding partner syntenin and syntenin's binding site is predicted to be unstructured in the absence of syntenin²⁰. The stabilization of Sox4 by syntenin may be involved in tumorigenesis and may be effected by prevention of the proteasome from initiating degradation of Sox4. Another example is the largely unstructured protein NAD(P)H: quinone oxidoreductase 1 (NQO1). NQO1 can be degraded *in vitro* by the 20S proteasome in a ubiquitin-independent manner. Binding of NQO1's cofactor FAD to NQO1 stabilizes the structure of NQO1 and inhibits its degradation by the proteasome²¹.

Roughly 40% of human proteins are either entirely disordered (intrinsically disordered proteins or IDPs) or contain intrinsically disordered regions (IDRs), but they form rigid tertiary structure in the presence of their ligands (such as cofactors)^{22, 23}. The IDRs often serve as ligand binding sites in cellular interaction networks and they can also serve as proteasome initiation sites²³⁻²⁵. Therefore, above observations suggest that modulation of the structure of IDPs or IDRs by ligand binding may modulate their degradation rates.

Comparison with conventional proteasome inhibitors

Proteasome inhibitors are emerging as a new class of anticancer drugs²⁶. However, their use is limited by their toxicity. Proteasome inhibitors lack specificity and lead to the accumulation of a wide variety of cellular proteins, including toxic proteins. Although the development of upstream inhibitors of the UPS, such as E2 and E3 inhibitors, improves specificity², it remains difficult to target individual proteins. The method proposed here interferes in degradation specifically and can therefore target key proteins for stabilization without affecting other processes.

Our findings suggest a strategy to inhibit degradation of specific proteins by modifier molecules that bind to the unstructured proteasome initiation regions in the target proteins. Antibodies against unstructured initiation regions can serve as seed molecules to modulate proteasome degradation. Antibodies are prepared easily and inhibit degradation of specific proteins *in vitro*, but their application in cells is limited by their biochemical properties (size, stability, affinity). Recently developed affinity reagents, such as antibody fragments and antibody mimics such as monobodies may be more effective at inhibiting proteasomal degradation *in vivo*²⁷⁻²⁹. We propose that modulation of proteasomal initiation may be used as the basis of specific protein targeted therapeutics in cancer and other diseases associated with the aberrant proteasomal degradation. Some tumor suppressors, such as cyclin-dependent kinase inhibitor protein, are inactivated by the aberrant proteasomal degradation in several cancers³⁰. In other tumor cells the activity of the NF- κ B transcription factor is dysregulated³¹. NF- κ B is kept inactive in normal cells by its natural inhibitor I κ B, which is in turn degraded by the proteasome when NF- κ B is activated³¹. Thus, cyclin-dependent

kinase inhibitor and I κ B would be good candidates for artificial stabilization by unstructured region binders to combat cancer.

Conclusion

We have proposed a novel concept for the regulation of proteasomal degradation. We have shown that the proteolysis of specific proteins can be suppressed when a feature in the protein, the disordered proteasome initiation region, was modulated by a ligand. We present three examples, binding of the small molecule ReAsH to tetracysteine motifs in artificial proteasome substrates, the binding of a ligand to an IDP, and a degron-specific antibody. This strategy of regulation of proteasomal degradation may be applied to cellular proteins and used for artificial control of proteasomal degradation of specific proteins.

Methods

Chemicals

ReAsH and BAL were purchased from Life Technologies. Adenosine-3', 5'-diphosphate (prAp) was purchased from Sigma-Aldrich. Cycloheximide (CHX) was purchased from Wako Chemical. The following antibodies were purchased; anti-His-tag polyclonal antibody, anti-GFP antibody, and anti- α -Tubulin antibody from Medical & Biological Laboratories; anti-DYKDDDDK tag antibody (1E6) from Wako Chemical.

Proteasome Purification

Yeast proteasomes were purified from *S. cerevisiae* strain YYS40 (MATa *rpn11::RPN11^{3 $\times$ FLAG}-HIS3 leu2 his3 ura3 trp1 ade2 can1 ssd1*) following the previous protocols⁷. Briefly, harvested yeast cells were resuspended in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 10% (v/v) glycerol, 1 mM DTT, and ATP regeneration mix (4 mM ATP, 0.2 mg ml⁻¹ creatine kinase and 20 mM creatine phosphate) and lysed using a BeadBeater (Biospec) with 0.5-mm glass beads. After clarification by centrifugation and filtration, the cell lysate was mixed with anti-FLAG M2 resin (Sigma-Aldrich) and incubated 2 h at 4°C with rocking. The resin was washed with wash buffer (50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 10% (v/v) glycerol, 1 mM DTT, and 2 mM ATP). The proteasome was eluted with wash buffer supplemented with 0.15 mg ml⁻¹ 3 $\times$ FLAG peptide (Sigma-Aldrich). The eluted purified proteasome was concentrated by ultrafiltration (Amicon) and stored at -80°C in 15% (v/v) glycerol.

Protein constructs

DNA constructs encoding substrate proteins for an in vitro experiment were cloned into pGEM-3Zf+ (Promega). Substrate proteins were constructed on the basis of proteins previously used: UbL tagged DHFR-titin I27 with a 27-residue unstructured region at the C terminus⁷. A tetracysteine-motif-inserted protein was constructed by directly inserting the sequence FLNCCPGCCMEP between titin I27 domain and the 27-residue unstructured region. For the in-cell experiment, this model substrate was cloned into pcDNA3 (Life Technologies). pd1EGFP-N1 encoding eGFP-ODC tail fusion protein was purchased from Clontech (CA, USA). A modified tetracysteine tag (FLNCCPGCCMEP) was inserted between eGFP and the ODC tail. pMT7SN encoding wild type SNase was kindly gifted by

Dr. Kuwajima (The University of Tokyo). SNase (140–149) was connected to the C terminus of UbL tagged DHFR through a short linker. For the antibody-mediated degradation inhibition, a linearly fused tetraubiquitin and a 34-residue unstructured region containing a His₆ tag at the C terminus were attached to the N terminus and the C terminus of DHFR, respectively, as described previously⁷.

Substrates for *in vitro* experiments

Radioactive substrates were *in vitro* translated using a RYTS Kit (Protein Express), supplemented with ³⁵S-methionine. The substrates were partially purified by ammonium sulfate precipitation. To label the model substrate with ReAsH, substrate proteins are incubated for 15 min under final concentrations of ReAsH. To induce folding of SNase (140–149), the protein was incubated in the presence of 1 mM CaCl₂ and 1 mM prAp. To prepare complex of substrate and antibody, substrate proteins are incubated in the presence of 25 μg ml⁻¹ anti-His₆ tag antibody for 15 min.

SDS-PAGE of substrate-ReAsH complexes

Substrate-ReAsH complexes were mixed with SDS-PAGE sample buffer containing 0.5 mM TCEP instead of β-mercaptoethanol or DTT, and then loaded onto the SDS-PAGE gel. The protein-ReAsH complexes were visualized on the unstained and unfixed gel by the 600 nm channel (excitation at 520 nm, emission at 600 nm) of Odyssey FC imager (LI-COR Biosciences, Lincoln, NE, USA).

Proteasomal Degradation Assay

Degradation assays were performed under first-order single-turnover conditions with 50 nM purified yeast proteasome and picomolar-range radioactive substrates at 30°C in 5% (v/v) glycerol, 5 mM MgCl₂, 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 1 mg ml⁻¹ BSA, 1 mM ATP, 10 mM creatine phosphate, and 0.1 mg ml⁻¹ creatine phosphokinase. Unstructured-region modifiers (ReAsH, prAp, or antibody) were added to the reaction mixture at the indicated concentrations. A fraction of the protein at each time point was subjected to SDS-PAGE. The gel was immediately dried, exposed on an imaging plate, and visualized with BAS-1000 (Fuji-film). The remaining substrate proteins were quantified by imaging analysis using Multi Gauge (Fuji-film). The degradation data thus obtained were fitted with a single exponential curve using Kaleida Graph (Synergy).

ReAsH labeling and Cycloheximide chase

HEK293T cells were kindly gifted by Prof. Isobe (University of Toyama). HEK293T cells were cultured in DMEM supplemented with 10% (v/v) FBS and penicillin–streptomycin. Semi-confluent cells were transfected with plasmids carrying the gene of the substrate using X-treme GENE HP DNA transfection reagent (Roche Lifescience). Cells transiently expressing the model substrate were stained with 1 μM ReAsH in Opti-MEM medium (reduced serum medium) for 90 min, treated with BAL Wash Buffer for 30 min at 37°C to remove nonspecific labeling, and subjected to CHX chase assay. Cells collected at each time point (0, 6, 12, and 18 h) were washed with PBS and suspended in TCA for 30 min. The precipitate was resolved in resolving buffer (50 mM Tris-HCl, 2% (w/v) SDS, 1% (v/v) β-

mercaptoethanol, 12.5 mM EDTA). Total protein concentrations were determined using the Bradford assay and equal amount of total protein were subjected to SDS-PAGE. Remaining substrate proteins were quantified by Western blotting, performed using an Odyssey Fc infrared imaging system (LI-COR Biosciences) following the manufacturer's protocol.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Modification of an unstructured region regulates proteasomal degradation

(A) Schematic representation of a method of regulation of proteasomal degradation via an unstructured initiation region. The unstructured region acts as an initiation site for unfolding and degradation by the proteasome (left). The modification of the unstructured region by another molecule inhibits the unfolding and degradation (right). (B) Schematics of UbL-tagged model substrates. A model substrate with a tetracysteine motif was constructed by insertion of an optimized sequence containing the tetracysteine motif between the folded domain and the unstructured region. (C) ReAsH modification of the model substrate with

tetracysteine motif. Radio-labeled UbL-tagged model substrates with or without the tetracysteine motif were incubated in the absence or presence of 20 μ M ReAsH and loaded on SDS-PAGE gel. The model substrates were visualized by the fluorescence of ReAsH (left) or autoradiography (right).

Figure 2. Modification of unstructured region with ReAsH regulates degradation

(A) Degradation kinetics for a model substrate with tetracysteine motif by yeast purified proteasome in the presence of different concentrations of ReAsH (blue circles, green triangles, black diamonds, and red squares represent 0, 5, 10, and 20 μ M, respectively). (B) Degradation kinetics for a model substrate without tetracysteine motif by yeast purified proteasome in the absence (blue circles) or presence (red squares) of 20 μ M ReAsH. (C) Degradation kinetics for a model substrate with tetracysteine motif by yeast purified proteasome with or without interruption by adding 20 μ M ReAsH at 7 min after starting

reaction. The plot shows the amount of protein estimated by autoradiography in SDS-PAGE gel bands (shown on the right) over time as a percentage of the initial protein amount. Data points represent mean values determined from three repeat experiments; error bars indicate s.e.m.

Figure 3. Ligand-induced folding of disordered initiation region inhibits proteasomal degradation

(A) Schematic representation of a substrate consisting of an N-terminal UbL domain, followed by DHFR and finally SNase (140–149). prAp binds to SNase (140–149) and induces its folding. (B) Degradation kinetics for UbL-DHFR-SNase (140–149) by yeast-purified proteasomes in the absence (blue squares) or presence (red squares) of 1 mM prAp. Plots show the amount of protein estimated by autoradiography in SDS-PAGE gel bands (shown in Supporting Figure S3A) over time as a percentage of the initial protein amount.

Data points represent mean values determined from three repeat experiments; error bars indicate s.e.m.

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Figure 4. Binding of an antibody to an unstructured region regulates proteasomal degradation (A) Schematic representation of a substrate consisting of an N-terminal tetraubiquitin fused in frame, followed by DHFR, a 27-residue unstructured region, and finally a C-terminal His₆ tag. The anti-His₆-tag antibody binds specifically to the C-terminal His₆ tag. (B)(C) Degradation kinetics for the model substrates with (B) or without (C) His₆ tag in the unstructured initiation region by yeast purified proteasome in the absence (blue squares) or presence (red squares) of 25 $\mu\text{g ml}^{-1}$ anti-His₆-tag antibody. Plots show the amount of protein estimated by autoradiography in SDS-PAGE gel bands (shown in Supporting Figure

S3B) over time as a percentage of the initial protein amount. Data points represent mean values determined from three repeat experiments; error bars indicate s.e.m.

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Figure 5. ReAsH-modification of an unstructured region regulates degradation in the cell
 (A) Degradation kinetics for a model substrate with ReAsH binding motif in HEK293T cells treated with 1 μ M ReAsH (red squares) or 0.05% (v/v) DMSO (blue diamonds). (B) Degradation kinetics for a model substrate without ReAsH binding motif in HEK293T cells treated with 1 μ M ReAsH (red squares) or 0.05% (v/v) DMSO (blue diamonds). Plots show the amount of protein estimated by western blot with anti-FLAG antibody for the detection of substrate proteins (lower) and anti-tubulin antibody as a loading control (upper) (shown

on the right) over time as a percentage of the initial protein amount. Data points represent mean values determined from three repeat experiments; error bars indicate s.e.m.

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Figure 6. ReAsH-modification of an unstructured degron regulates ubiquitin-independent degradation of a substrate in the cell

(A) Construction of eGFP–ODC tail fusion proteins with tetracysteine motif. An optimized ReAsH binding motif was inserted between eGFP and C-terminal tail of ODC. (B)(C) Degradation kinetics of eGFP–Cys₄–tail^{ODC} (B) and eGFP–tail^{ODC} (C) in HEK293T cells treated with 1 μ M ReAsH (red squares) or 0.05 % (v/v) DMSO (blue diamonds). Plots show the amount of protein estimated by western blot with anti-GFP antibody for the detection of substrate proteins (lower) and anti-tubulin antibody as a loading control (upper) (shown on

the right) over time as a percentage of the initial protein amount. Data points represent mean values determined from three repeat experiments; error bars indicate s.e.m.

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