Substrate selection by the proteasome during degradation of protein complexes

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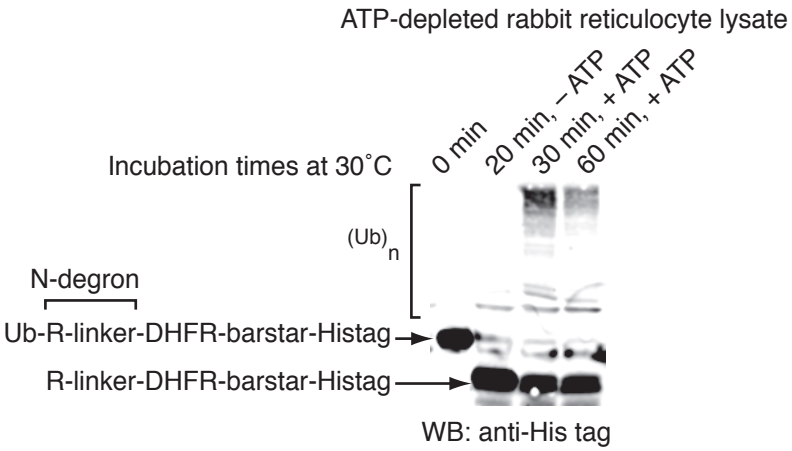

Supplementary Figure 1
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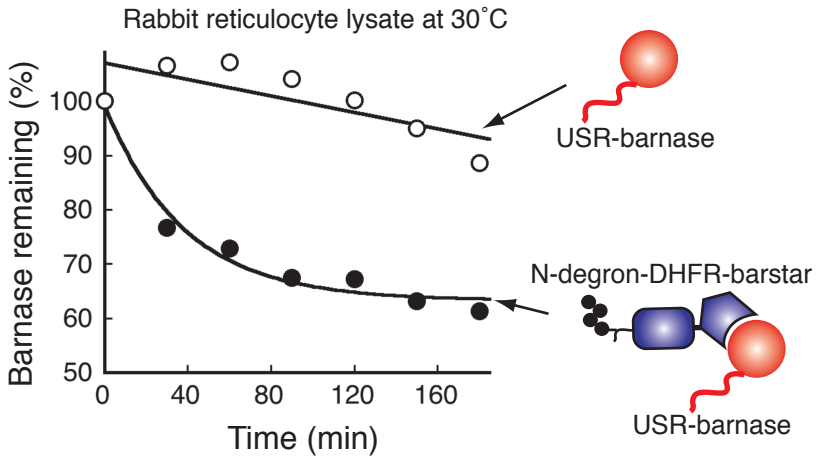
Supplementary Figure 1

Ubiquitination and degradation of an N-degron-containing fusion protein. N-degron-DHFR-barstar was incubated in reticulocyte lysate at 30 ˚C first in the absence of ATP and then after the addition of ATP and an ATP-regenerating system. Samples were removed at the indicated times and analyzed by SDS PAGE and Western blotting. The N-terminal ubiquitin is cleaved to reveal an Arg residue at the newly created N-terminus, which leads to the ubiquitination and subsequent degradation of the protein.



Supplementary Figure 2
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Supplementary Figure 2

Degradation of a protein lacking a ubiquitin modification bound to a ubiquitinated adaptor. Barnase containing a N-terminal proteasome initiation site was incubated with the proteasome in reticulocyte lysate at 30 ˚C by itself or bound to N-degron-DHFR-barstar. The graph plots the amount of protein remaining at different times as a percentage of total protein at the beginning of the reaction.

SUPPLEMENTARY METHODS

Protein expression and purification. For experiments in reticulocyte lysate, barnase substrates were cloned into pGEM-3Zf(+) vector and expressed as radioactive proteins by *in vitro* transcription and translation in reticulocyte lysate (Promega Corp.) supplemented with [^{35}S]methionine and partially purified by high speed centrifugation and ammonium sulfate precipitation. For experiments with purified components, barstar and barnase were overexpressed and purified from *E. coli* using standard methodology. The barstar constructs N-degron-DHFR-barstar, Ub_4-barstar, Ub_4-USR2-barstar, Ub_4-USR3(Ser-rich)-barstar, Ub_4-USR4-barstar, and Ub_4-DHFR-barstar were cloned into a pET3a vector and expressed from the T7 promoter in *E. coli* strains BL21(DE3)pLysS or Rosetta(DE3)pLysS (Novagen). Bacterial strains were grown at 37 °C to an optical density of 0.4, protein expression was induced with 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG), and incubation continued for an additional 2 hours. Expressed proteins were purified by a talon metal affinity column (BD Biosciences) in buffer containing 50 mM sodium phosphate buffer [pH 7.0] and 300 mM NaCl, and eluted from the resin with buffer containing 50 mM sodium phosphate [pH 7.0] and 150 mM imidazole. The eluted fractions were bound to a 1 ml MonoQ or MonoS ion exchange column (Pharmacia). Proteins were eluted with a linear salt gradient of 0 - 0.6 M NaCl.

Barnase lacking an unstructured region was purified as described[1]. Briefly, the barnase gene has previously been cloned in plasmid pMT416 and expressed in *E. coli* strain JM105 by inducing the lac promoter with 1 mM IPTG for 2 hours after the cells reached

log phase[1]. Barnase is secreted into the periplasmic space and released into the medium by an acid. The released barnase was purified by SP-Trisacryl and MonoS cation exchange columns as described[2]. The eluted barnase were further purified by size exclusion chromatography on a Shepacryl S200 column (Pharmacia) in buffer containing 50 mM Tris/Cl buffer [pH 7.4] and 200 mM NaCl.

Barnase containing an initiation site was purified as described[3]. Briefly, it was subcloned into a pQE60 vector (Qiagen), where the protein is under the control of a tac promoter. Lac repressor was co-expressed from the plasmid pREP4 (Qiagen). *E. coli* M15 cells harboring both plasmids were induced in late log phase with 1 mM IPTG for 4 hours before collection. Cells were broken open by sonication, inclusion bodies were collected and washed, and protein was extracted from the inclusion bodies with 8 M guanidine hydrochloride. Protein was then dialyzed against 1.5 M guanidine hydrochloride, diluted 100 times into 50 mM sodium acetate [pH 5.0], and concentrated in a stirred cell.

Barstar was expressed in *E. coli* strain JM109 from the vector pMT643 (ref. 4) by inducing the tac promoter controlling the barstar gene with 1 mM IPTG for 4 hours after the cells reached late log phase. Cells were broken open by sonication, and the cell debris was removed by centrifugation. Barstar was purified by precipitation from the supernatant with 80% ammonium sulfate, followed by size exclusion (Sephadex G-75, Pharmacia) and anion exchange (Mono Q, Pharmacia) column chromatography.

The identities of the gene expression constructs were confirmed by DNA sequencing, and proteins were characterized by SDS-PAGE and Western blotting.

SUPPLEMENTSRY REFERENCES

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3. Huang, S., Murphy, S. & Matouschek, A. Effect of the protein import machinery at the mitochondrial surface on precursor stability. *Proc. Natl. Acad. Sci. USA* **97**, 12991-12996 (2000).
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