

# Introduction

## Session 1 and 2

### (Ubiquitin, proteasome and human disease)

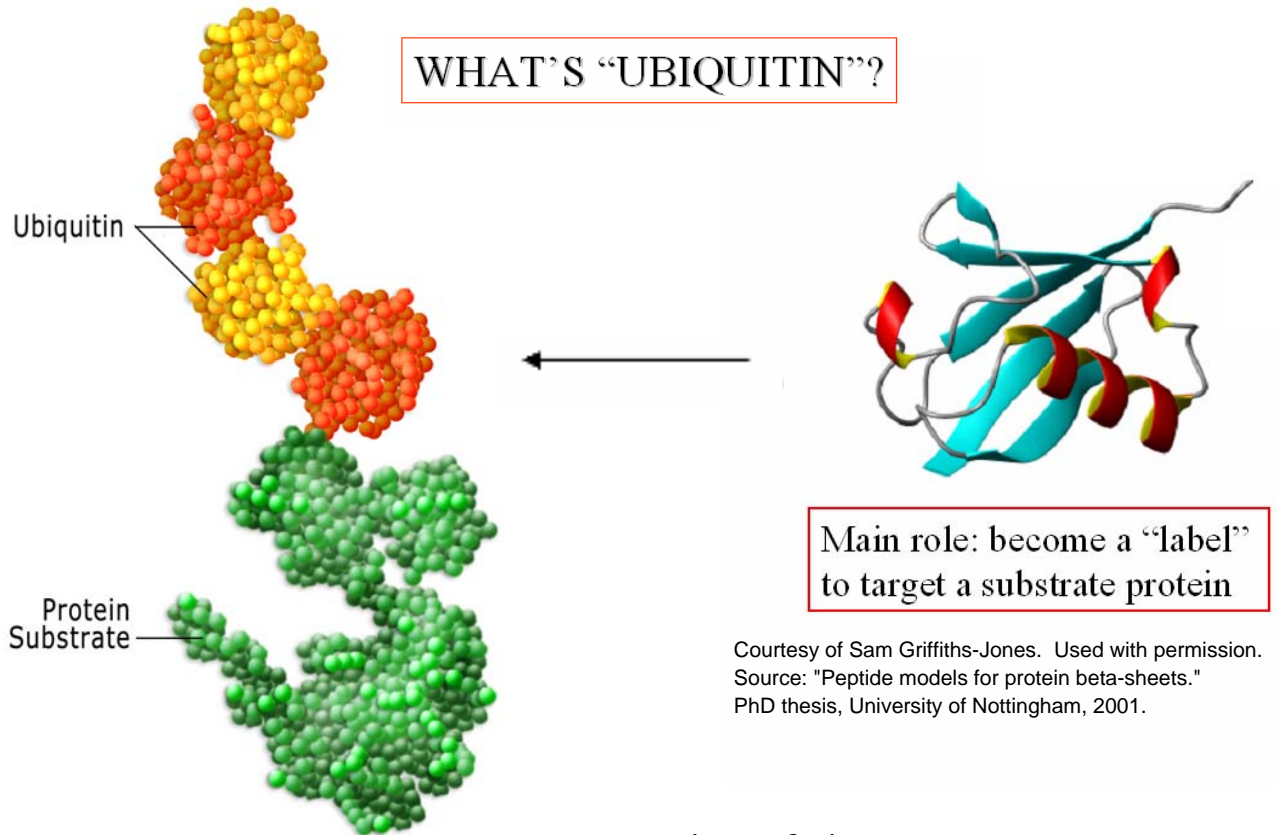
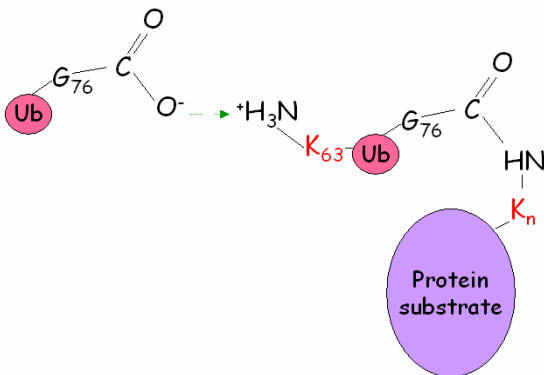


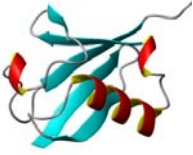
Figure by MIT OCW.  
After Goodsell, D.S. The Oncologist 8, 293-294.

### Appendage of ubiquitin monomers to a protein substrate.

Ubiquitin is a small, 76 aa protein which gets appended to another proteins, as a "label". The protein substrate has amino groups in the side chains of its Lys aa residues. Ubiquitin has a C-terminal Gly. The carboxyl group of this Gly forms an isopeptide bond with the amino group of the Lys in the protein substrate (see figure).

Ubiquitin also has several Lys that can act as internal acceptors for binding to the C-t Gly of new ubiquitin molecules, allowing the formation of a chain.





# DISCOVERY OF THE ROLE OF UBIQUITIN IN PROTEIN DEGRADATION. HISTORICAL FACTS.

Courtesy of Sam Griffiths-Jones. Used with permission.  
Source: "Peptide models for protein beta-sheets."  
PhD thesis, University of Nottingham, 2001.

- ❖ (1975) Ubiquitin was first isolated by **Gideon Goldstein** and colleagues from the thymus (reason why it was originally thought to be a **thymic hormone**).
  
- ❖ But because it was later found in all tissues and eukaryotic organisms it received the name of **UBIQUITIN** (for 'ubiquitous' protein).
  
- ❖ (1977) **Harris Goldknopf and Ira Busch** found a DNA-associated protein that had **one C-t but two N-t!** The short arm of this Y-shaped unusual protein was joined through its C-terminal to the  $\epsilon$ -amino group of an internal Lys of the histone H2A.
  
- ❖ **Margaret Dayhoff** soon identified it as **Ubiquitin** (a protein initially described as free by Goldstein).
  
- ❖ (1969-1971) **Avram Hershko** studies regulation of tyrosine aminotransferase by its degradation
  - he found that degradation of the enzyme was **arrested by inhibitors of cellular energy production** (fluoride, azide)  
That was the first indication that an **as-yet-unknown energy-dependent proteolytic system** must exist.
  
- ❖ (1971-1980's) **Hershko** decided to identify this energy-dependent system responsible for the degradation of proteins, by means of classical biochemistry. His aims were:
  - to reproduce the ATP-dependent protein breakdown in a cell-free system.
  - to fractionate such system to find the mode of action of its components.

❖ (1977) **Etlinger and Goldberg** discovered an **ATP-dependent proteolytic system** occurring in **reticulocytes** (red blood cells). Therefore, **Hershko**, helped by his grad student **Aron Ciechanover** and **Irwin Rose** (Fox Chase cancer Center, Philadelphia) decided to isolate the ATP-dependent proteolytic system from these cells.

- Reticulocyte lysates were fractionated on **DEAE-cellulose** (anionic exchange chromatography) into two crude fractions:
  - **Fxn1**(not adsorbed), with most of the hemoglobin .
  - **Fxn2**, proteins adsorbed to the resin and eluted with high salt.
- Fxn2 had lost most of the ATP-dependent proteolytic activity and

only was restored when combining back Fxn1 with 2.

- The active component in Fxn1 was isolated in basis of its **high stability upon heat treatment**: **APF-1**, for **ATP-dependent proteolysis factor 1**.

**...possible activator or regulatory subunit of other component/s of the system??**

- Radiolabeling APF-1 they saw substantial ATP-dependent **binding to high-molecular weight proteins** (gel filtration chromatography).

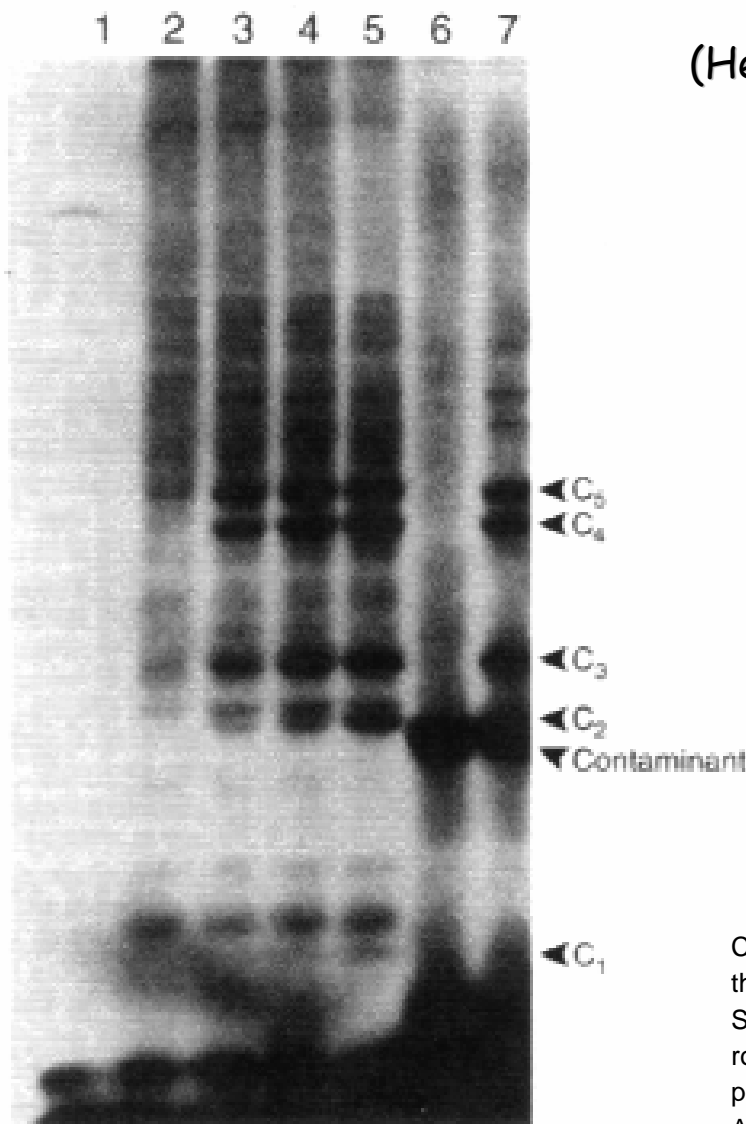
- Interaction extremely stable in the presence of conditions that disrupt non-covalent interactions: interaction should be **covalent** (**peptidic or amidic linkage**).

**...APF-1 interacting not with an active component of the proteolytic system but with protein substrates??**

• By using a good substrate for ATP-dependent proteolysis, **lysozyme**, they found:

1) that similar high-molecular weight derivatives were formed when  $^{125}\text{I}$ -labeled APF-1 was incubated with unlabeled lysozyme than when  $^{125}\text{I}$ -labeled lysozyme was incubated with unlabeled APF-1.

2) analysis of the ratio of radioactivity in APF-1 and lysozyme indicated that the various derivatives consisted of **increasing numbers of APF-1 molecules linked to one molecule of lysozyme**.

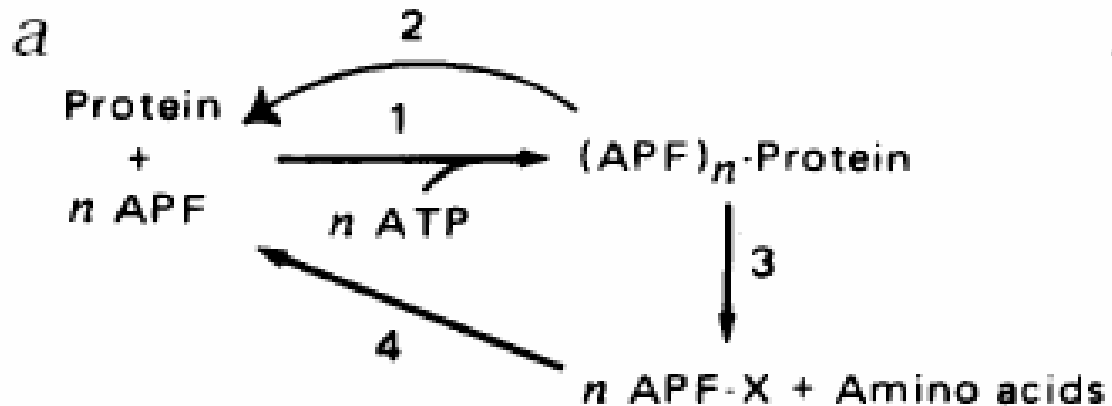


(Hershko *et al.*, 1980)

Courtesy of A. Hershko. Used with permission of the author.

Source: Figure 1 in Hershko et al. "Proposed role of ATP in protein breakdown: conjugation of protein with multiple chains of the polypeptide of ATP-dependent proteolysis." PNAS 1980 April; 77(4): 1783–1786.

## (Model of action proposed by Hershko *et al.*, 1980)



Courtesy of A. Hershko. Used with permission of the author.

Source: Figure 6 in Hershko *et al.* "Proposed role of ATP in protein breakdown:

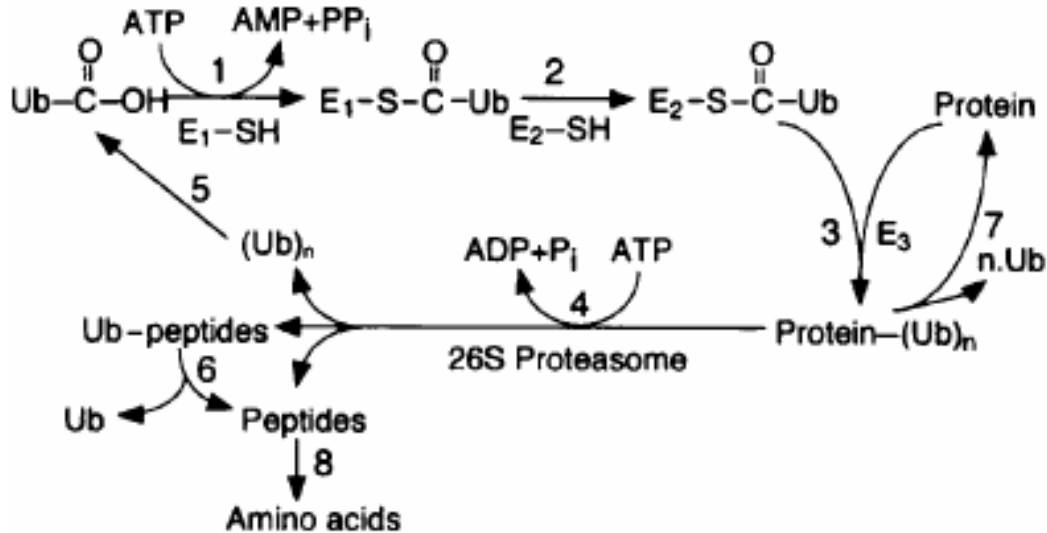
conjugation of protein with multiple chains of the polypeptide of ATP-dependent proteolysis."

PNAS 1980 April; 77(4): 1783–1786.

Several molecules of APF-1 linked to  $\epsilon$ -amino groups of the protein substrate by an **APF-1-protein amide synthetase (step 1)**. Proteins ligated to several APF-1 are broken down by a **specific protease** that recognizes such conjugates (**step 3**). The protein is broken down to free amino acids and to APF-1 still linked by isopeptide linkage to a lysine or a small peptide, APF-1-X. Finally, free APF-1 would be released for re-use by specific **amidase-isopeptidase (step 4)**. A hypothetical **'correcting' isopeptidase** would release free APF-1 and substrate protein from erroneous ligations (editing activity; **step 2**).

❖ Short after Hershko's model proposal, **Keith Wilkinson and Arthur Haas** (post-doctoral fellows in Irwin Rose's lab) indicated that **APF-1 was indeed UBIQUITIN**.

# Ubiquitin proteolytic pathway



Courtesy of Annual Reviews. Used with permission.

Source: Figure 1A in Hershko A, Ciechanover A. The ubiquitin system. Annu Rev Biochem. 1998;67:425-79.

**A:** Conjugation of ubiquitin to the target substrate

E1: Ub-activating enzyme

E2: Ub-conjugating enzyme; Ubc's

E3: Ub-ligase

Image removed due to copyright considerations.

See Figure 1 in Ciechanover A, Orian A, Schwartz AL.

"Ubiquitin-mediated proteolysis: biological regulation via destruction." Bioessays. 2000 May; 22 (5): 442-51.

**B:** Degradation of the polyubiquitinated substrate by the 26S proteasome complex and Ub recycling by deubiquitinating enzymes (Ub C-terminal hydrolases, DUBs or UCH's)



❖ (1980's) **Hershko and Ciechanover** purified the components of the first part of the reaction (**APF-1-protein amide synthetase**): **E1, E2 and E3**

enzymes, by 'covalent' affinity chromatography over immobilized Ub.

❖ The '**protease**' step was further clarified with the characterization and purification of the **26 proteasome complex**, by **Martin Rechsteiner** and colleagues (1986).

❖ It was first thought that multiple ubiquitin attached to different Lys sites in the substrate protein but **Hershko** (1985) and then **Alexander Varshavsky** and colleagues (1989) demonstrated that **a single poly-ubiquitin chain could be form through attachment to a single internal Lys residue.**

Attachment of subsequent Ub to the first one occurs through linkages of the Gly C-t of the next Ub to **specific lysines** present in the precedent Ub. Poly-ubiquitin chains might have **different roles** depending on the Lys utilized:

**K48** → recognition by proteasome

**K63** → signal for traffic and degradation

in the lysosome, vacuole; subunit selectivity

**K29, K6, etc..**

❖ First evidence showing that ubiquitin constitutes **a signal for protein**

**degradation *in vivo*** (**Hershko lab; 1982**): **immunochemical analysis of Ub adducts in cells** → by using anti-Ub Abs they saw that, after incubation of cells in the presence of **aa analogs** the resulting **abnormal proteins were short-lived** and their rapid degradation was accompanied by a **transient increase in Ub adducts.**

But the strongest confirmation came with **Alexander Varshavsky's** work.

**UBIQUITIN CONJUGATION REQUIRED FOR  
PROTEIN DEGRADATION *IN VIVO***  
(first evidence through the studies carried out by  
**Alexander Varshavsky, Dan Finley and Aaron  
 Ciechanover)**

❖ **Varshavsky** studied **chromosome structure and regulation of gene expression** and came to Boston in the fall of 1977 where, a month later, became a **faculty member in the Biology Department here at MIT**.

❖ He became interested in the **role of protein degradation in gene regulation and expression** at the light of Hershko's and Wilkinson's experiments (showing that the **ATP-dependent proteolytic factor 1**, was ubiquitin).

❖ Considering that he already knew that Ub had been found attached to histones, he became very excited when in 1980 he read a paper, by **Yamada** and coll. that described a **conditionally lethal, temperature sensitive mouse cell line called ts85** (they had seen that in this cell line at restrictive temperature a nuclear protein **disappeared** and Varshavsky confirmed it was **UbH2A**).

→ **Dan Finley**, from his lab, and **A. Ciechanover** helped him to study **ts85** and saw that cells were arrested in **S/G2** phase of cell division cycle and had induced synthesis of heat shock proteins at non permissive temperature.

They found that **ts85** mouse cells had **a temperature-sensitive Ub-activating enzyme (E1)** and that these cells stopped degrading the bulk of their normally short-lived proteins at the nonpermissive temperature.

❖ Later studies (by **Hunt** and coll.), with rapidly dividing fertilized clam eggs, led to the discovery of **CYCLINS** and in 1984 **Varshavsky** proposed that **cyclins were degraded at the exit from mitosis by the ubiquitin system**.



Ubiquitination is an essential regulatory process that occurs at multiple levels and in all mammalian cellular pathways

- ❖ selective protein degradation
- ❖ modulation of transcription, translation and protein localization

For example..

..in the regulation of apoptosis

..in the regulation of transcription

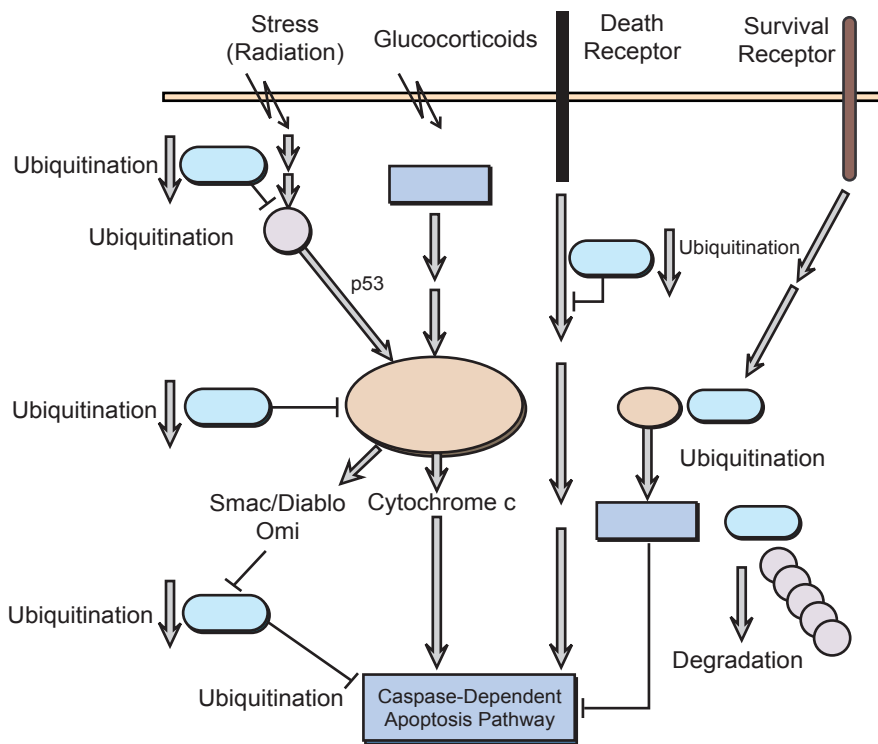


Image removed for copyright considerations.  
 Source: Figure 5 in Muratani M, W. P. Tansey.  
 "How the Ubiquitin-proteasome System Controls  
 Transcription." Nat Rev Mol Cell Biol. 4,  
 no. 3 (Mar 2003):192-201.

Figure by MIT OCW. After Yang and Xu, "Regulation of apoptosis: the ubiquitous way." FASEB J 17 (2003) 790-799.

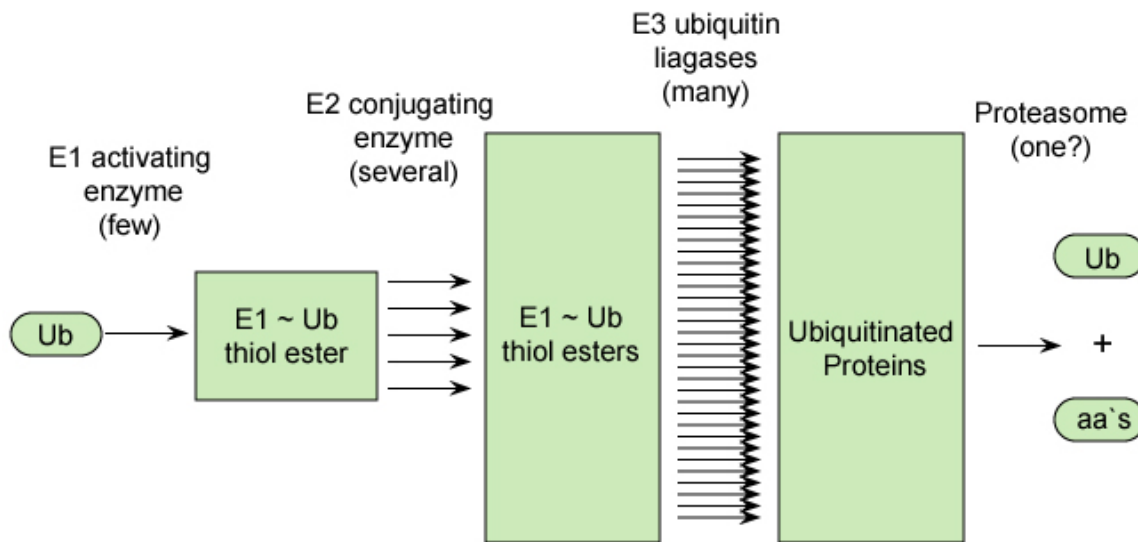


Figure by MIT OCW.

How are the substrates recognized by the E3s?

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See Figure 2 in Ciechanover A. et al. "Ubiquitin-mediated proteolysis: biological regulation via destruction." *Bioessays*. 2000 May; 22(5): 442-51.

## "N-End Rule" Discovery

❖ **Hershko and Ciechanover** saw a discrepancy while attempting to purify APF-1 (Ub): the preparation had a high dry-weight to low protein-content ratio.

Was APF-1 a **ribonucleoprotein**? When they treated the preparation with RNase A this abrogated the degradation of BSA mediated by APF-1, although not that of lysozyme.

They later found the weight to protein content discrepancy was in fact caused by the protein detection method (Lowry assay) which is based in the detection of **aromatic aa<sub>s</sub>** (Ub had a low content on those).

Then what caused the RNase effect on BSA Ub-dependent degradation?

❖ Some proteins in order to be degraded must be **recognized by specific destabilizing aa residues located in their N-t ('N-End rule')**. In some cases

these destabilizing residues are added by **post-translational modification**,

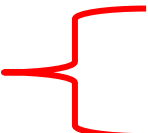
carried out by specific amidases and transferases.

In the specific case of BSA, the RNase was destroying the tRNA<sup>Arg</sup> that was necessary along with arginyl-tRNA protein transferase to convert the N-t acidic (Asp) residue of BSA to Arg. Only this modified form of BSA can bind to the E3 $\alpha$ .

(The lysozyme has a Lys in the N-t so doesn't need to undergo this post-translational modification to be recognized by the E3).

❖ **Varshavsky, Finley and others** discovered further aspects of the N-End

rule and established a family of N-t degradation signals called **N-Degrans**,

 N-t destabilizing residue  
internal Lys (site of Ub attachment)

# The ubiquitin system of *S. cerevisiae*

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See Figure 5 in Hershko, A. et al. "Basic Medical Research Award. The ubiquitin system." *Nature Medicine* 2000 Oct; 6 (10): 1073-81.

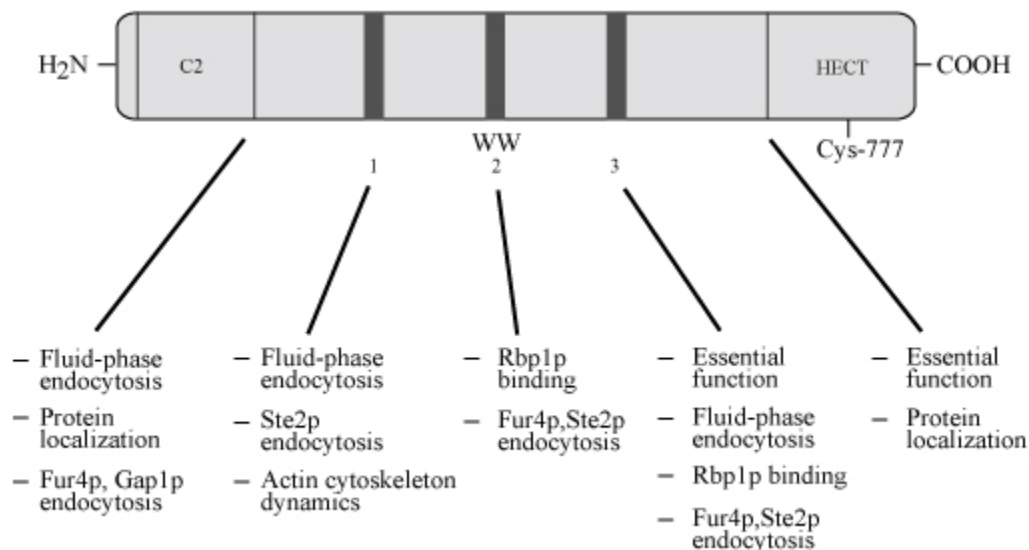
## Ubiquitin is not the only protein used as a tag in cells:

UBL	Variants	Function	Known Substrates
Ubiquitin	Mono  Poly (Lys29) Poly (Lys48)  Poly (Lys63)	Endocytosis/lysosomal degradation, meiosis, chromatin remodeling  26S proteasomal degradation? 26S proteasomal degradation  Post-replicative DNA repair, translation, endocytosis	Histones, ion channels, receptors  UFD substrates Many short lived proteins, mis-folded proteins  L28 ribosomal protein, TRAF6, PCNA, plasma membrane proteins
ISG15 (UCRP)		Immune response	Serpin 2a, Stat1, ERK1, others
AUT7 (APG8)	GATE16 (Golgi-associated ATPase Enhancer of 16 kDa), LC3 (microtubule-associated protein 1 light chain 3), GABARAP	Autophagy, cytoplasm-to-vacuole targeting, vesicular transport	Phosphatidylethanolamine
APG12		Autophagy, cytoplasm-to-vacuole targeting	APG5
NEDD8 (RUB 1)		Auxin response, meiosis-to-mitosis transition	Cullins
SUM01 (SMT3)	SUMO2, 3	Nuclear transport, chromosome segregation, transcriptional regulation	Many
HUB1		Cell polarity	Sph1, Hbt1
FAT10		Apoptosis	Unknown
URM1 (Ubiquitin Related Modifier 1)		Growth at high temperature	Unknown
MNSF		T-cell activation	TCR $\alpha$ -like protein

Figure by MIT OCW.  
After Table 1 in Schwartz and Hochstrasser, TIBS 28 (2003) 321-328.

## Two main kinds of E3 ubiquitin ligases

1) **HECT-domain proteins** (homologous to E6-AP carboxyl terminus): coordinate the transfer of Ub to the substrate by forming a **thiol-ester bond with Ub** prior to transferring it to a substrate.



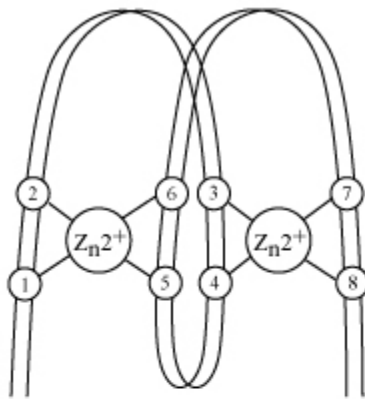
Example: Rsp5p

Figure by MIT OCW.

2) **RING finger proteins**: coordinate the transfer of Ub to the substrate by **bringing the E2-ubiquitin complex** to the substrate.

- Consensus sequence: 8 conserved Cys and His that coordinate 2 Zn ions in a “cross-braced” fashion.
- Some act singly, such as Ubr1/E3 $\alpha$ ; others exist in complex, such as the SCF complexes (Skp1, yeast Cdc53, or mammalian cullin, and E-box protein)





F-box protein: substrate binding. Fbws bind substrate through WD-40 domains; Fbls, through Leu-rich repeats; Fbxs, use different motifs.

Skp1: scaffold that keeps together the cullin and the F-box.

Cullin: mediates attachment of Nedd8, Skp1, and Roc1.

Roc1/Rbx1 (Hrt1 in yeast): C-t RING finger domain; stabilizing interaction between the E2 and the rest of the complex.

Nedd8 (Rub1 in yeast): Ubi-like protein that enhances the ubiquitin-ligating activity of the complex.

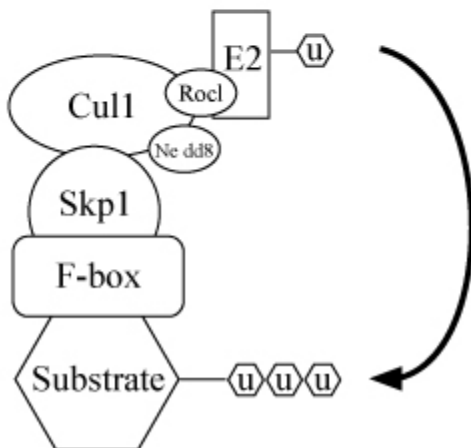
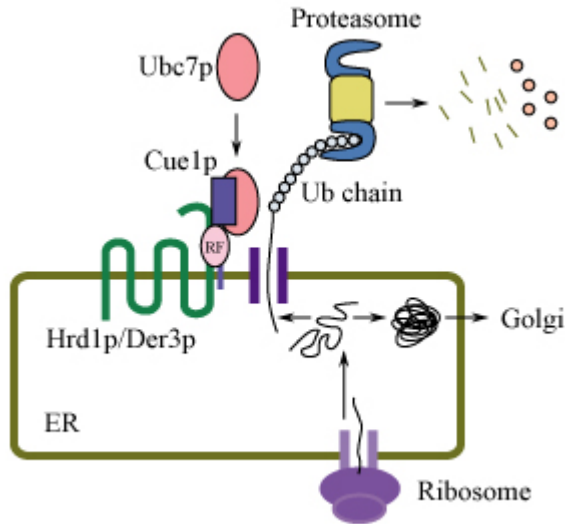


Figure by MIT OCW.

# Ubiquitination involving degradation of proteins targeted to the secretory pathway:



## 'ERAD' or Endoplasmic Reticulum Associated Degradation

Figure by MIT OCW.  
 After Fang et al. "RING finger ubiquitin protein ligases: implications for tumorigenesis, metastasis and for molecular targets in cancer." *Semin Cancer Biol.* 2003 Feb; 13 (1): 5-14.

## Vacuolar/ Lysosomal Degradation

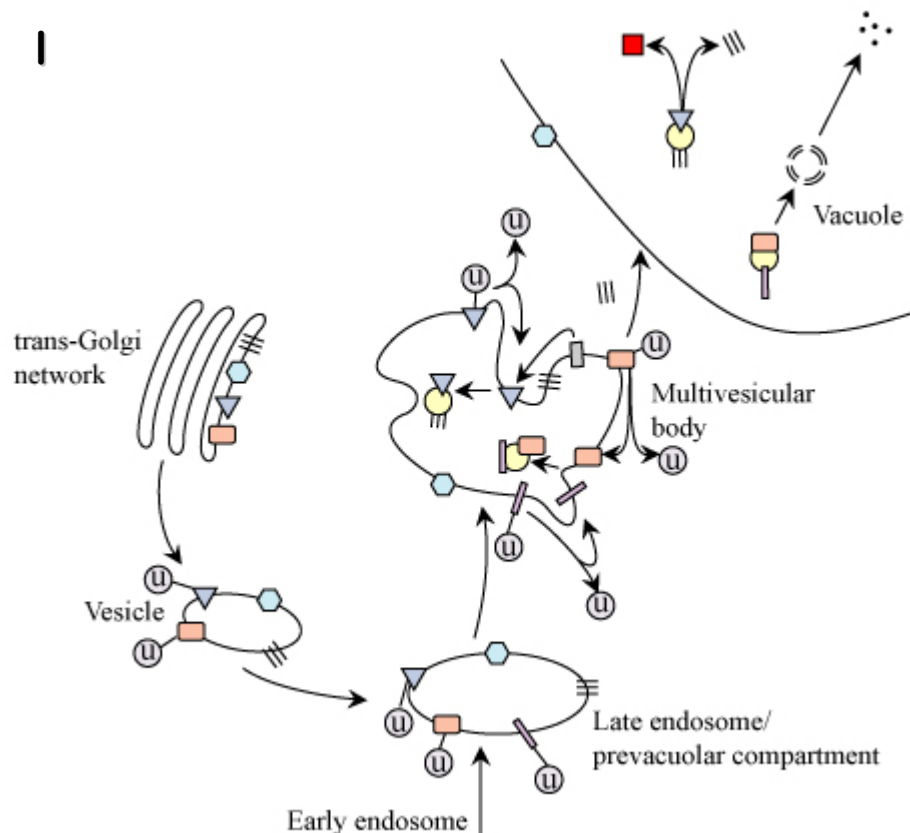


Figure by MIT OCW.  
 After Fig. 5 in Horák, J. (2003). The role of ubiquitin in down-regulation and intracellular sorting of membrane proteins: insights from yeast. *Biochim. Biophys. Acta* 1614, 139-155.

# Infectious agents hijack cell machinery involved in ubiquitination and protein degradation

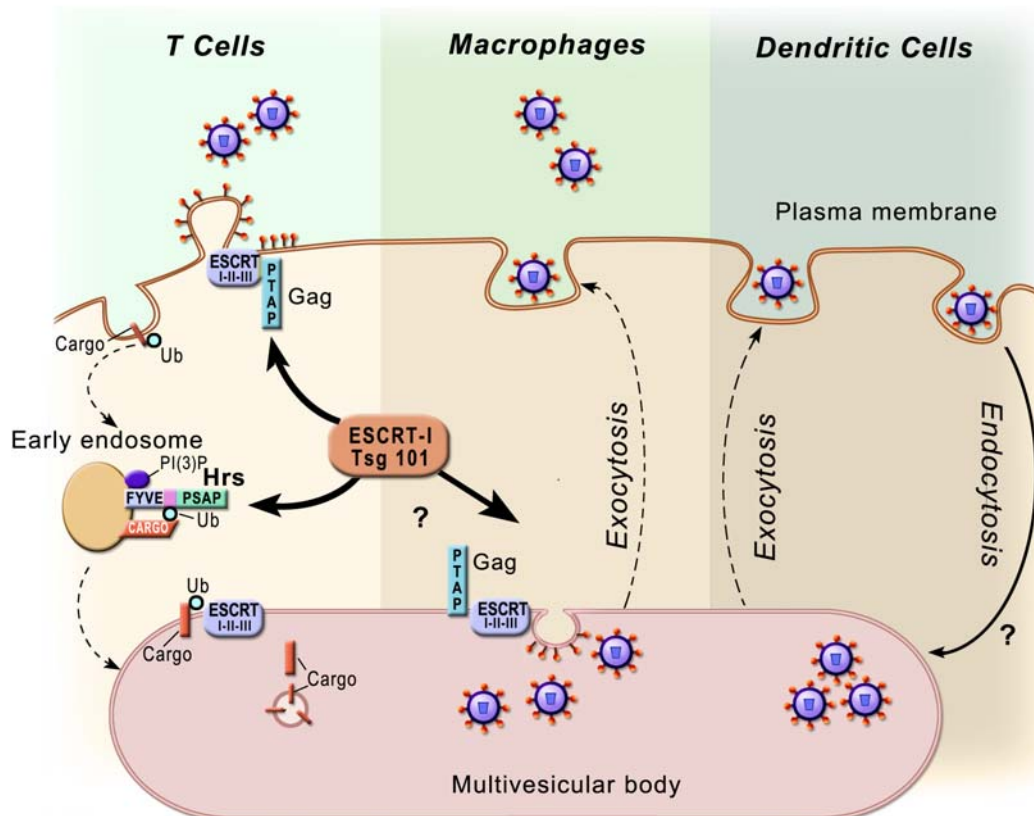


Figure by MIT OCW.

After Fig. 2 in Amara, A and Littman, DR. 2003. After Hrs with HIV. *J. Cell Biol.*, 162: 371-375.

Example: HIV virus budding off cells by using host machinery involved in traffic of membranes

# What can go wrong in the UPS (Ubiquitin Proteasome System) to cause disorder and/or disease?

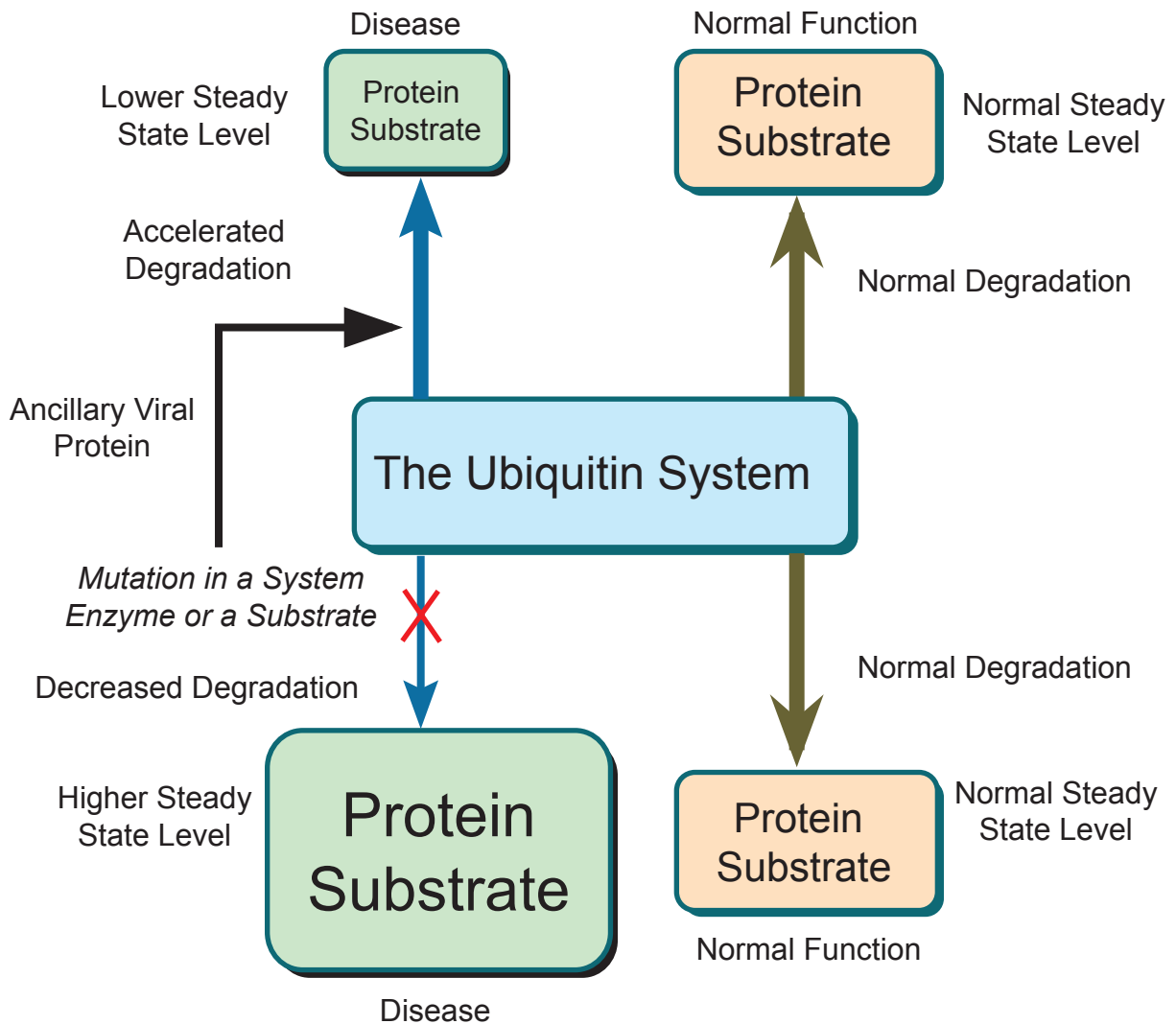


Figure by MIT OCW.

After Fig 3 in Hershko, A. et al. "Basic Medical Research Award. The ubiquitin system." Nature Medicine 2000 Oct; 6 (10): 1073-81.

## Example: The UPS and the pathogenesis of neurodegeneration

Image removed due to copyright considerations.

See Figure 2 in Ciechanover, A. and Brundin, P. 2003. The ubiquitin proteasome system in neurodegenerative diseases: sometimes the chicken, sometimes the egg. *Neuron* 40: 427-446.