

# CPB 310 Tutorial on “Chemical biology: Probing Cell Biology with Small Molecules”

for a lecture to be given by  
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- **Background Biology:**
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- **Inhibition of protein degradation**
  - Use of epoxomicin as a proteasome inhibitor
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- **Induction of protein degradation**
  - Use of PROTACS for targeting proteins for proteolytic degradation  
(PROteolysis Targeting Chimeric moleculeS)



# Protein Degradation

- Cells are in dynamic state of protein production and protein degradation
- Many key enzymes & regulatory proteins are controlled via selective protein degradation.
- Examples:
  - cyclins – regulate progression through the cell cycle; degradation of cyclin B results in a cell exiting M-phase of the cell cycle.
  - initiation of innate immune response; NF- $\kappa$ B regulation by I $\kappa$ B.



# Protein Degradation

- Protein degradation is compartmentalized in:
  - Proteasomes – macromolecular structures that break down proteins
  - Organells (i.e. lysosomes)
- Ubiquitin is a 76-residue protein that is used to mark other proteins for destruction
- Ubiquitination – marking of a protein for proteasome degradation
- Proteases are enzymes that break peptide bonds btw residues
- Proteolysis is the directed degradation of proteins by proteases



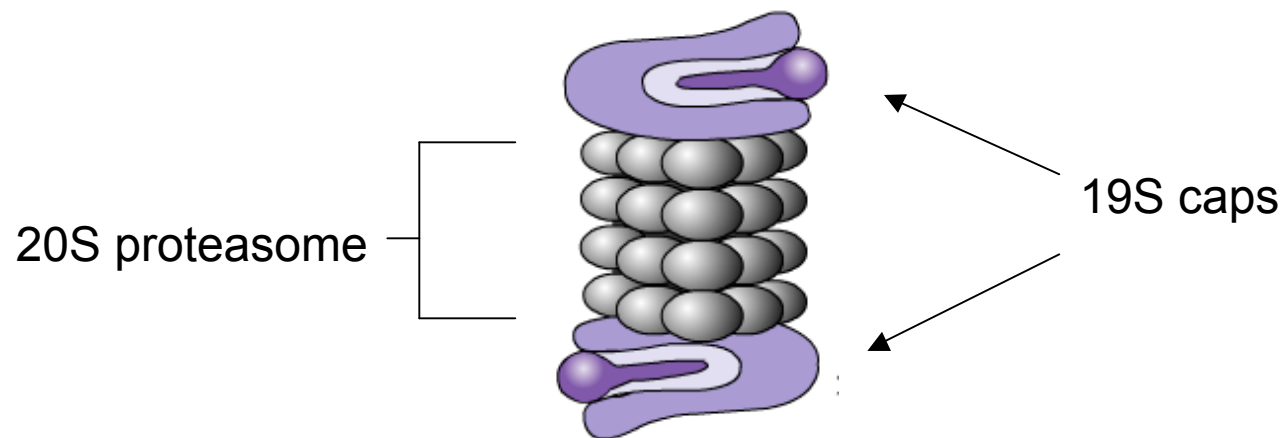
# Protein Degradation – Ubiquitin pathway

- Ubiquitination occurs through ligation of ubiquitin to the target protein (typically binds to Lys residues)
- 3 additional proteins are required for this to occur.  
They are:
  1. E1 – ubiquitin-activating enzyme
  2. E2 – ubiquitin-carrier protein
  3. E3 – ubiquitin-protein ligase
- After ligation of ubiquitin, the target protein is marked and is consumed by the proteasome and destroyed
- The proteasome contains a cavity where proteolysis occurs

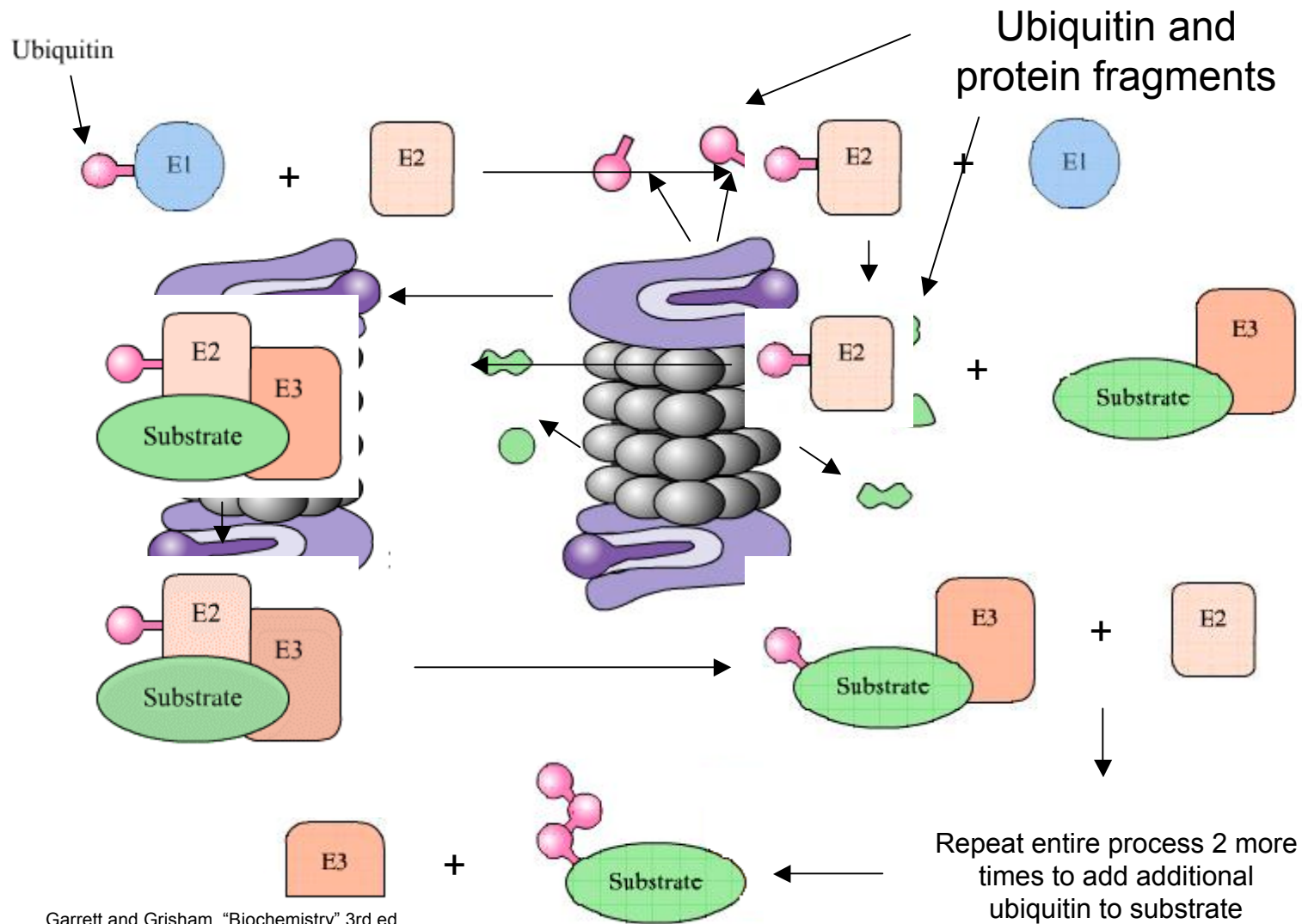


# Ubiquitin Pathway

- Two types of proteasomes in eukaryotic cells: 20S and 26S
- The 26S is about 45nm in length > 20S length
- The 26S proteasome is composed of the 20S and two 19S regulators (or caps), which have ATPase activity
- 4 stacked rings ( $\alpha_7\text{-}\beta_7\text{-}\beta_7\text{-}\alpha_7$ ) form a barrel shaped structure



# Ubiquitin Pathway

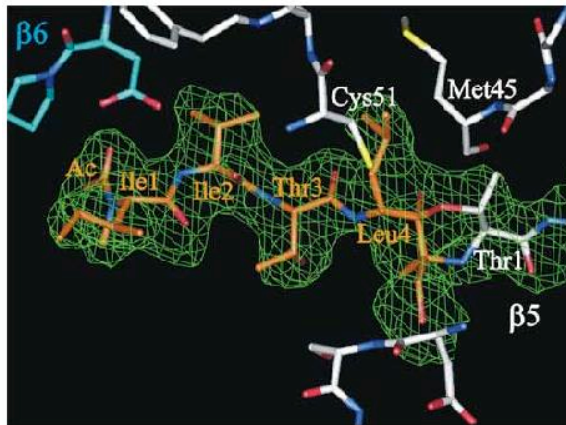


Garrett and Grisham, "Biochemistry" 3rd ed.,  
Brookscole, 2005, p.1036.

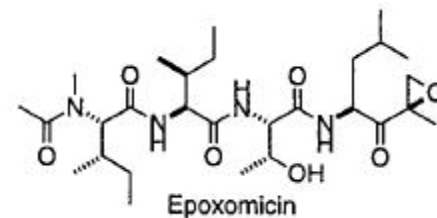


# Proteasome inhibition

- Epoxomicin is an antitumor agent of microbial origin
- Dr. Crew's and his colleagues demonstrated that epoxomicin covalently binds to specific catalytic subunits of a proteasome, thus inactivating it
- They believe that epoxomicin inhibits the chymotrypsin-like activity of the 20S proteasome
- Chymotrypsin is a serine protease that cleaves peptides on carbonyl side of aromatic residues (i.e. tyrosine, phenylalanine)



[www.yale.edu/cres/research/modes.html](http://www.yale.edu/cres/research/modes.html)



Crews, "Epoxomicin, a potent and selective proteasome inhibitor, exhibits *in vivo* antiinflammatory activity," *Proc. Natl. Acad. Sci.* Vol. 96, pp. 10403–10408, August 1999



# Exopomicin - Proteasome inhibition

- Epoxomicin induces accumulation of p53 and ubiquitinated proteins in cultured cells; In vivo proteasome function was inhibited.
- p53 mediates cellular responses to DNA damaging agents by inducing apoptosis. p53 is a relatively unstable protein and is easily degraded.
- Epoxomicin prevents I $\kappa$ B degradation, which in turn prevents activation of NF- $\kappa$ B DNA binding activity.
- NF- $\kappa$ B is a transcription factor protein associated with immune and inflammatory responses. It is found in the nucleus after immunostimulation.
- I $\kappa$ B is an inhibitor of NF- $\kappa$ B that keeps it in the cytoplasm. Epoxomicin prevents proteasome-mediated degradation of I $\kappa$ B. This demonstrates epoxomicin's anti-inflammatory ability.
- Irritant response assays to picrylchloride also demonstrated epoxomicin's anti-inflammatory ability.



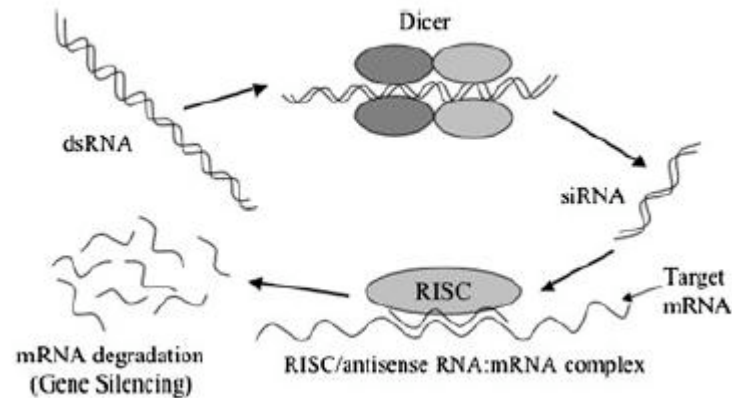
# Genomic analysis

- Traditional vs chemical genetic analysis
- Traditional – observation of a particular phenotype, identification of genes responsible for it, and manipulation of the gene sequence (i.e. knockout).
- Chemical – identify proteins responsible for a particular phenotype by using small molecules as probes to perturb signaling pathways.
  
- Examples of chemical genomic analysis:
- Post-transcriptional inactivation (gene silencing) – RNAi (RNA interference)
  - siRNA (small interfering RNA) prevent portions of mRNA translation thus disrupting gene expression
  - Disadvantage: not a complete knockout of gene of interest, highly stable proteins already synthesized are not effected, poor temporal control of protein expression
- Post-translational inactivation – protein destruction after being synthesized
  - Utilizes cell's own ubiquitin proteasome pathway
  - Can knock down long-lived proteins - “chemical knockout”



# Genomic analysis

- Post-transcriptional inactivation
  - Dicer enzyme – processes dsRNA (double stranded RNA) into siRNA.
  - RISC (RNA induced silencing complex) – protein activated with antisense siRNA that binds to complimentary mRNA sequence and cleaves the sequence.
- RNAi is catalytic and gene specific.

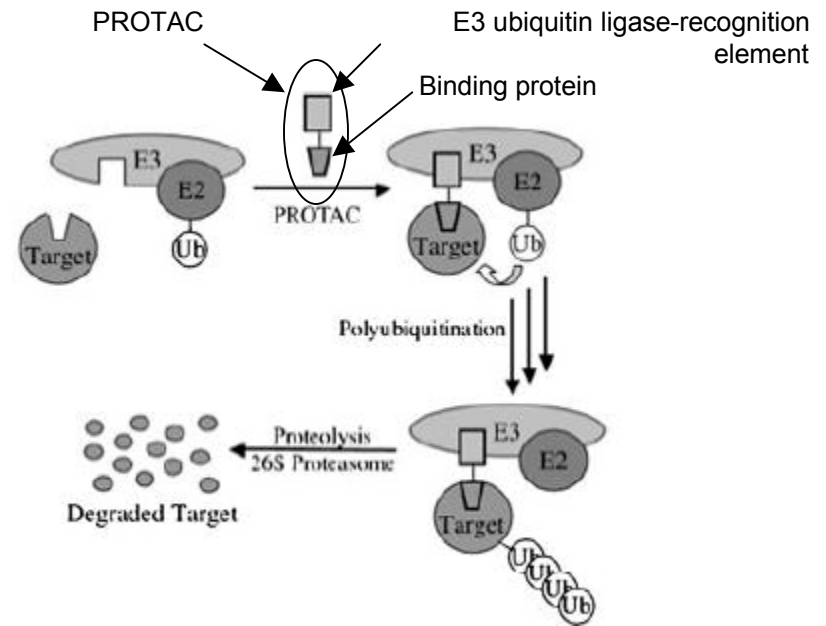


Crews, "Chemical Approaches to Controlling Intracellular Protein Degradation," ChemBioChem 2005, 6, 40–46.



# Induction of protein degradation

- Post-translational inactivation
- PROTACS (PROteolysis Targeting Chimeric moleculeS)
  - PROTACS consist of a ligand for the target protein, a linker moiety, and a ligand for the E3 ubiquitin ligase.
  - The PROTAC molecule is the link between the substrate and E3.
  - Once complexed, the target protein is ubiquitinated and degraded.



Crews, "Chemical Approaches to Controlling Intracellular Protein Degradation," ChemBioChem 2005, 6, 40–46.



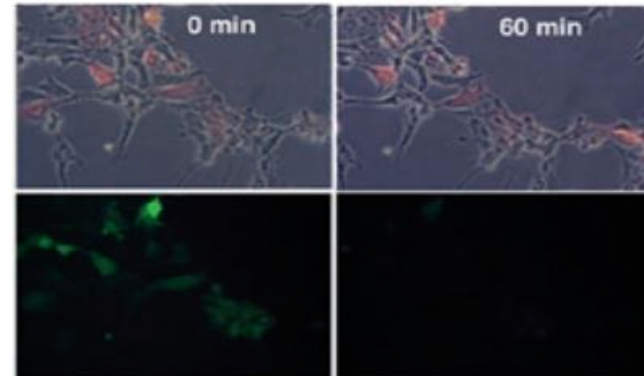
# Induction of protein degradation: PROTACS

- In Vitro: Microinjected into cells. I $\kappa$ B $\alpha$  phosphopeptide (I $\kappa$ B $\alpha$ pp) as the E3 recognition element has poor cellular permeability. The following PROTACS use I $\kappa$ B $\alpha$ pp with the base binding protein.
- PROTACS-1: ovalicin-based PROTAC; degradation of a stable protein, methionine aminopeptidase 2 (MetAP-2). Proof of principle experiment.
- PROTACS-2: estradiol-based PROTAC; induced degradation of ER (estrogen receptors), which are associated with breast cancer cells.
- PROTACS-3: dihydrotestosterone (DHT)-based PROTAC; induced degradation of AR (androgen receptors), which are associated with prostate cancer cells.
  
- In Vivo: Performed in cultured cells. To ensure membrane permeability a polyarginine molecular transporter along with hypoxia-inducible factor 1 $\alpha$  (HIF1 $\alpha$ ) as the E3 recognition element
- PROTACS-4: targets FKBP12 immunophilin
- PROTACS-5: DHT-based with HIF1 $\alpha$  instead of I $\kappa$ B $\alpha$ pp as used in PROTACS-3

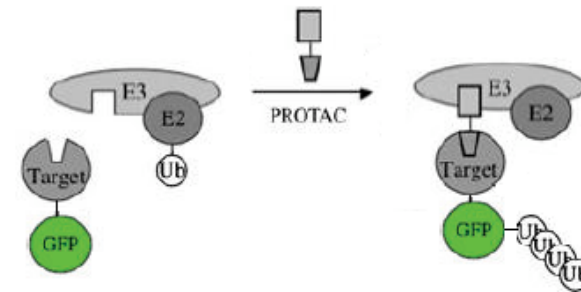


# Induction of protein degradation: PROTACS

- Slide demonstrating degradation of AR labeled with a fluorescent protein in HEK 293 cells.
- Uses of PROTACS:
  - Targeted chemical knockout
  - Proteolysis is not dependent on active site inhibition – only need exposed Lysine residues on target protein for ubiquitin to bind to.
  - Remove toxic or disease causing proteins – target proteins causing abnormal cellular growth.



Sakamoto et al., "Development of Protacs to target cancer-promoting proteins for Ubiquitination and Degradation," *Molecular & Cellular Proteomics* 2003, 2.12, 1350-1358.



Crews, "Chemical Approaches to Controlling Intracellular Protein Degradation," *ChemBioChem* 2005, 6, 40-46.



# Review

- Ubiquitin proteasome pathways are the most common type of cellular protein degradation.
- Epoxomicin is believed to inhibit 20S proteasome (and 26S) activity.
- Chemical genetic analysis uses both siRNA to silence genes and specific small molecules (PROTACS) to selectively target proteins for degradation.



# References

- Schneekloth, Crews, “Chemical Approaches to Controlling Intracellular Protein Degradation,” *ChemBioChem* 2005, 6, 40–46.
- Meng, Crews, et al., “Epoxomicin, a potent and selective proteasome inhibitor, exhibits *in vivo* antiinflammatory activity,” *Proc. Natl. Acad. Sci.* Vol. 96, pp. 10403–10408, August 1999.
- Sakamoto et al., “Development of Protacs to target cancer-promoting proteins for Ubiquitination and Degradation,” *Molecular & Cellular Proteomics* 2003, 2.12, 1350-1358.
- Garrett and Grisham, “Biochemistry” 3<sup>rd</sup> ed., Brookscole, 2005, p.1033-1037.
- [www.yale.edu/crews/research/modes.html](http://www.yale.edu/crews/research/modes.html)

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