How E.Coli is involved in discovery of ubiquitylation cascade

TJC 25.10.2016

Valeria Eckhardt

Overview

Introduction ubiquitylation

Paper I: Synthetic biology approach to reconstituting Ub in bacteria

Paper II: A bacterial genetic selection system for Ub casc. discovery

Summary & Conclusion

Introduction ubiquitylation

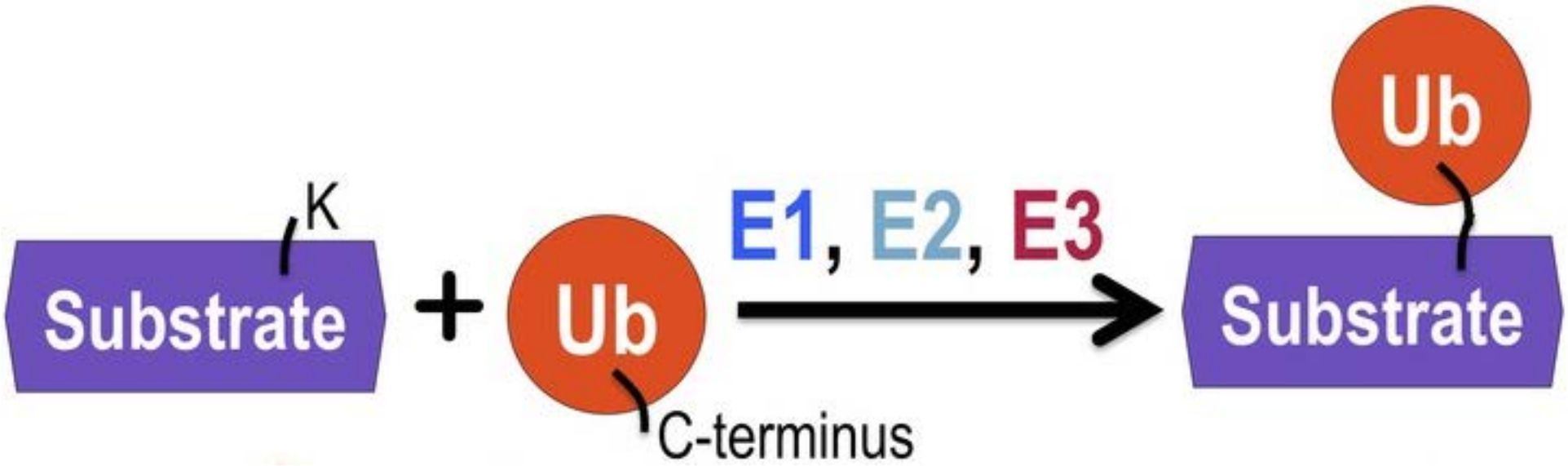
Ubiquitylation is involved in

- Protein degradation (proteasome)
- Protein trafficking
- Signalling functions
- DNA remodelling and repair

Malfunctions of Ub signals underlie

- cancer
- metabolic and infectious diseases
- neurodegeneration

Introduction ubiquitylation: Mechanism



E1, E2, E3 ligate the carboxyl terminus of Ub to a lysine residue on selected protein targets -> isopeptide bond

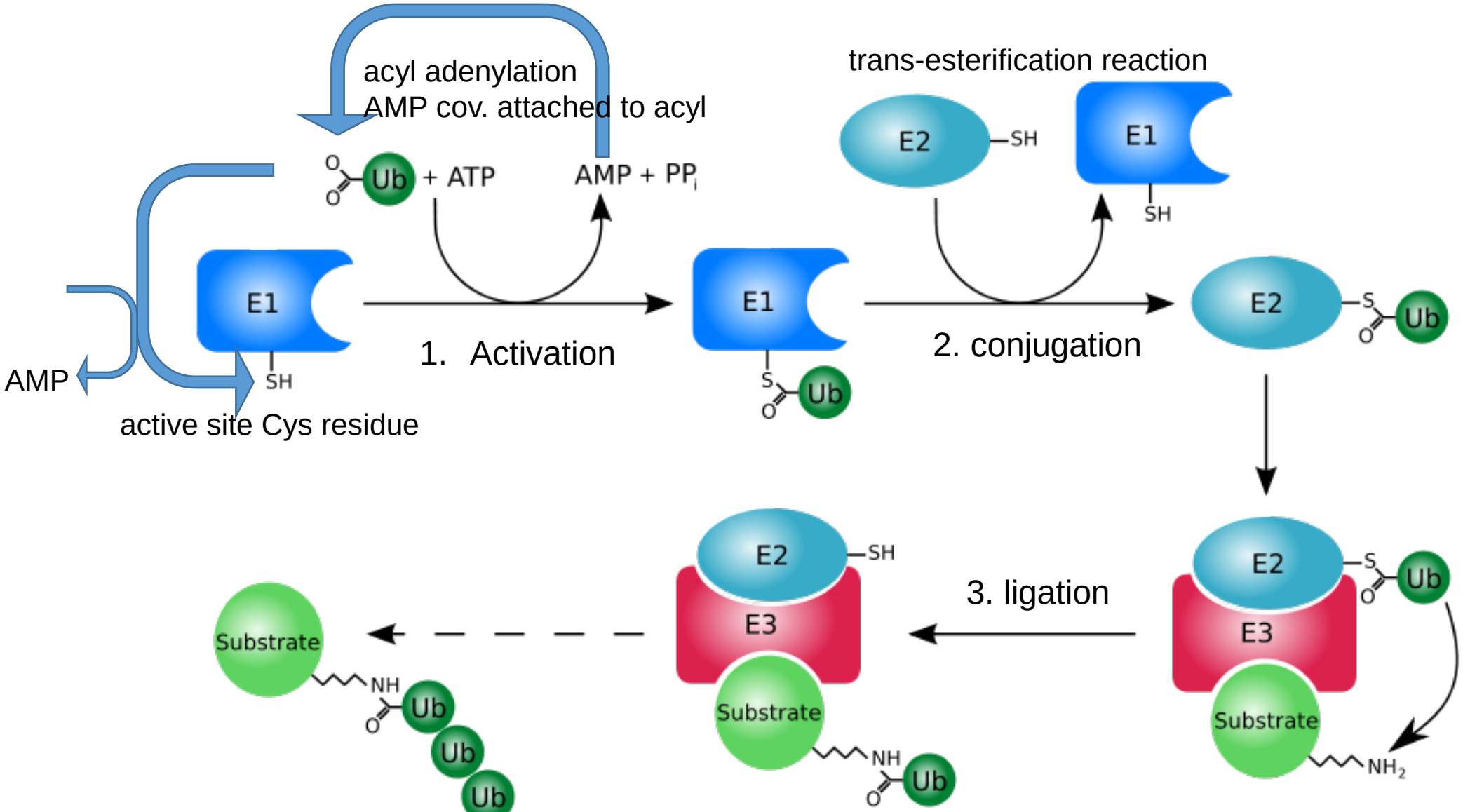
Kravets et al., 2016

Introduction ubiquitylation: Mechanism

Ubiquitin is

1. activated by E1
2. conjugated by E2
3. ligated by E3 to the target protein

Kravets et al., 2016



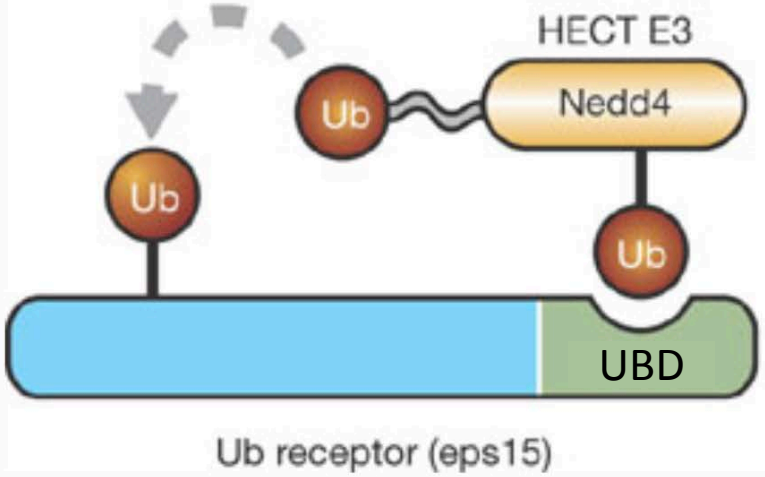
Isopeptide bond between
- Lysine of target protein
- C-terminal glycine of ubiquitin

http://docs.abcam.com/pdf/proteins/Ubiquitylation-system.pdf

2 E3 ligase types: HECT & RING

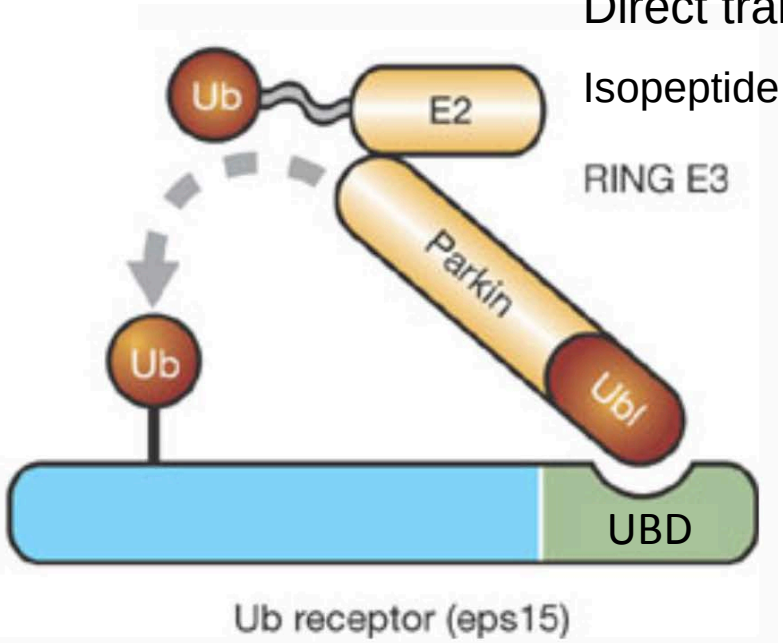
Ub transiently binds E3

thioester intermediate



Direct transfer fro E2 to substrate

Isopeptide bond



UBD = Ub-binding domain

Ub-Receptor

Regulated by mono-ubiquitylation

apo form: not ubiquitylated, active, binds ub target proteins

cis form: ubiquitylated, closed, inactive

Haglund & Stenmark, 2006

Paper I

Synthetic biology approach to reconstituting the ubiquitylation cascade in bacteria

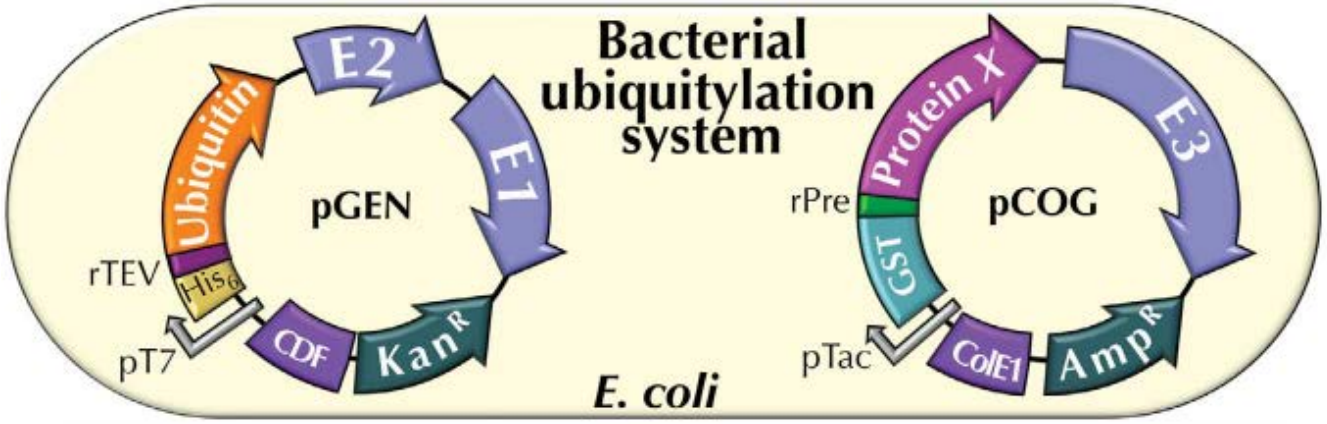
Tal Keren-Kaplan[1], Ilan Attali[1], Khatereh Motamedchaboki[2], Brian A Davis[3], Neta Tanner[1], Yael Reshef[1], Einat Laudon[1], Mikhail Kolot[1], Olga Levin-Kravets[1], Oded Kleifeld[4], Michael Glickman[5], Bruce F Horazdovsky[3], Dieter A Wolf[2] and Gali Prag[1,*]

BO Journal VOL 31 | NO 2 | 2012

Reconstruct the entire eukaryotic ubiquitylation system in E.coli

Co-express substrates and Ub with E1, E2, E3

Build a synthetic bacterial ubiquitylation system

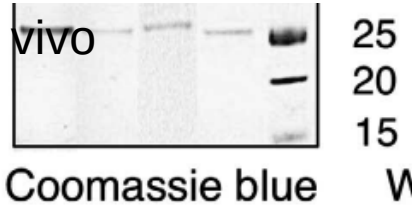


Co-express affinity-tagged substrates and Ub with E1, E2, E3

Modular system:
>20 plasmids with different E2s and Ub mutants
> 30 plasmids with cognate substrates and E3

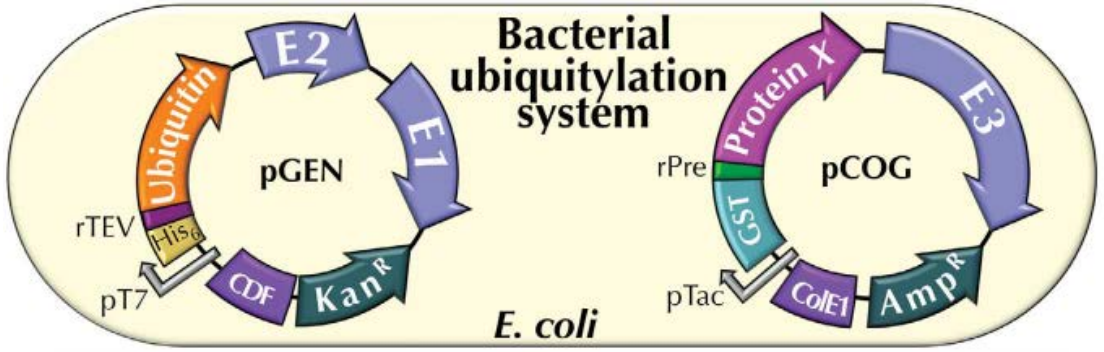
Do E3 ligases undergo auto-ubiquitylation in bacteria?

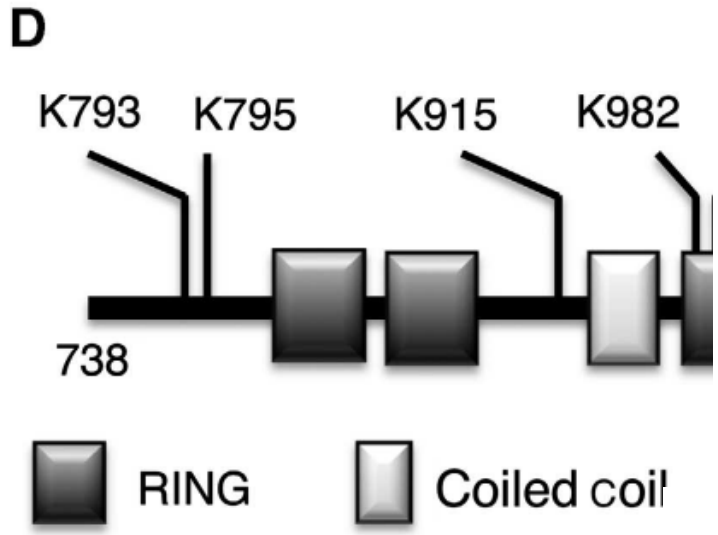
Most E3 ligases undergo auto-ubiquitylation when lacking a specific substrate in vivo



Mib = Mind bomb = RING-containing E3 ligase

His-Ub UbcH5b E2 E1 GST-Mib E3





Ub + wt Mib co-expression: laddering pattern
Mib underwent multi-monoubiquitylation/ polyubiquitylation
K48, K63 most abundant polyUb modifications
K0 = all known K residues mutated into Arg, pattern retained

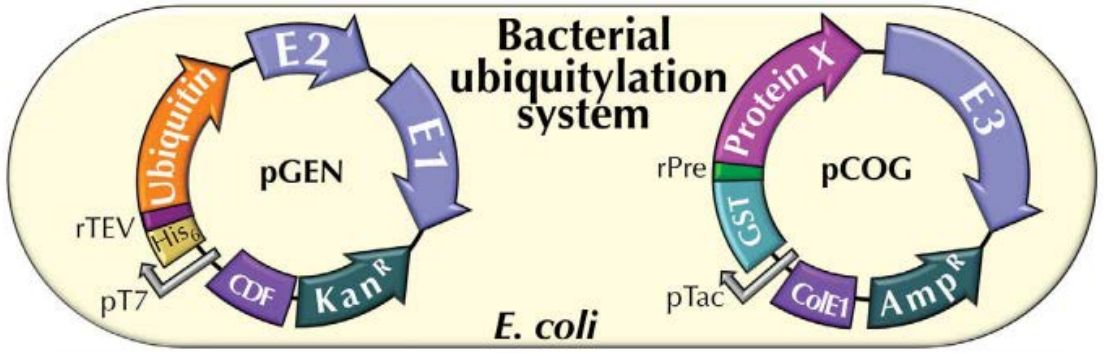
Other K residues ubiquitylated?

Do E3 ligases undergo auto-ubiquitylation in bacteria?

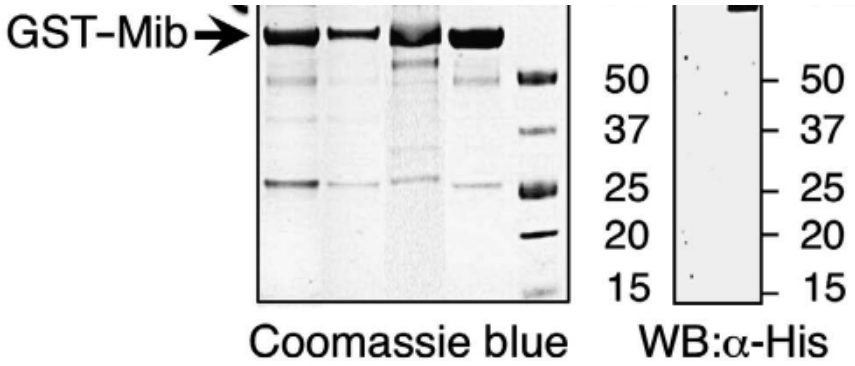
Most E3 ligases undergo auto-ubiquitylation when lacking a specific substrate in vivo

Mib = Mind bomb = RING-containing E3 ligase

His-Ub UbcH5b E2 E1 GST-Mib E3



Mass spec. of purified Mib E3 identified 5 new ub-K residues

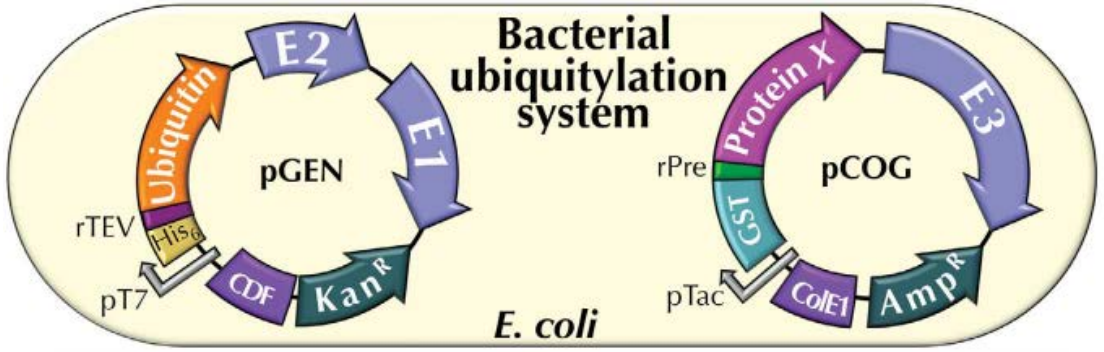


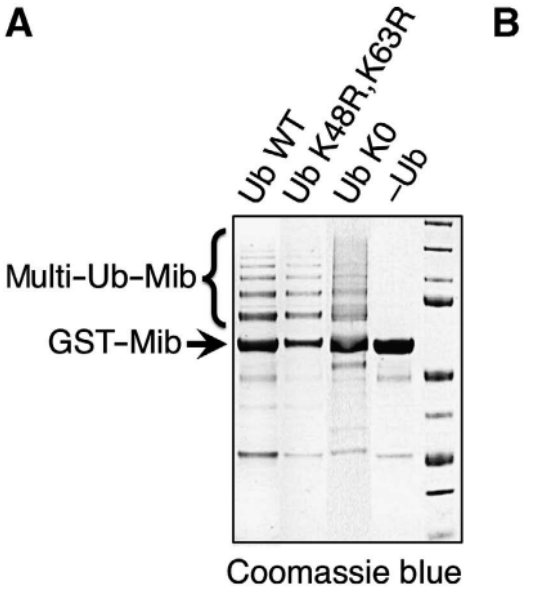
Do E3 ligases undergo auto-ubiquitylation in bacteria?

Most E3 ligases undergo auto-ubiquitylation when lacking a specific substrate in vivo

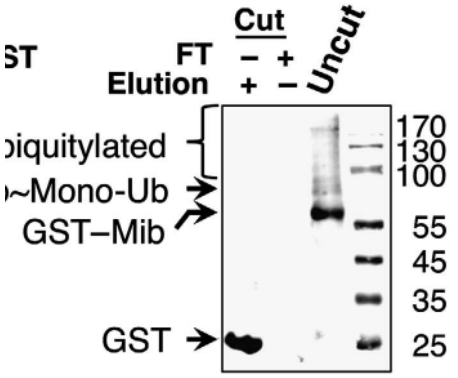
Rsp5 = HECT-containing E3 ligase: undergoes auto-ub.
contains a catalytic Cys: transfers Ub to substrate

His-Ub Uc4/Ubc5 E2 GST-yeast Rsp5 E3
E1





Same expression levels of ub components



Cys -> Ala mut:
Ligase fails to charge with Ub

Yes! Successful reconstitution of the entire ubiquitylation cascade for RING and HECT E3 ligases in E.coli

The reconstituted system faithfully recapitulates Rpn10 ub.

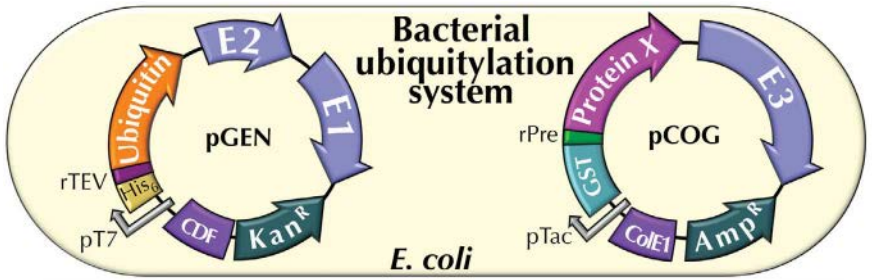
Rpn10 is ubiquitylated by Rsp5 in vivo in S.cerevisiae at K84

In vitro polyubiquitylated

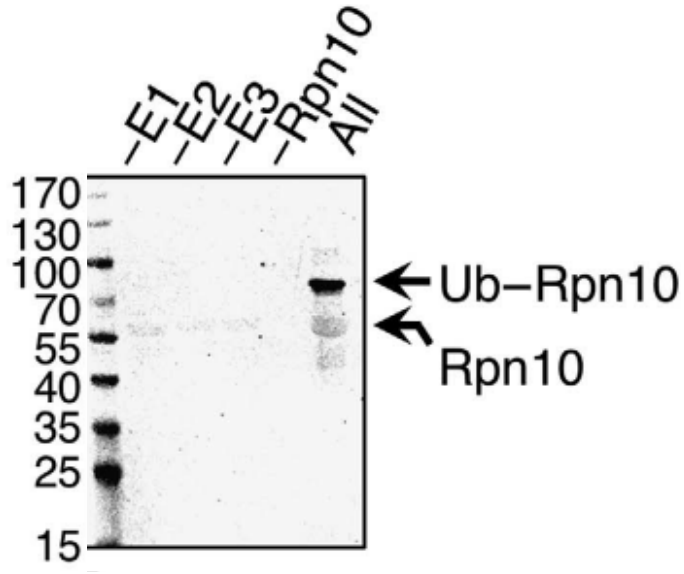
Test in E.Coli:

His-Ub Ubc5 E2 GST-Rpn10-Rsp5 E3

Uba1 E1



WB: label Rpn10 with anti-His ab (Ub)



Co-expression of all components are necessary for Rpn10 monoub.

Which K residue is monoub.?

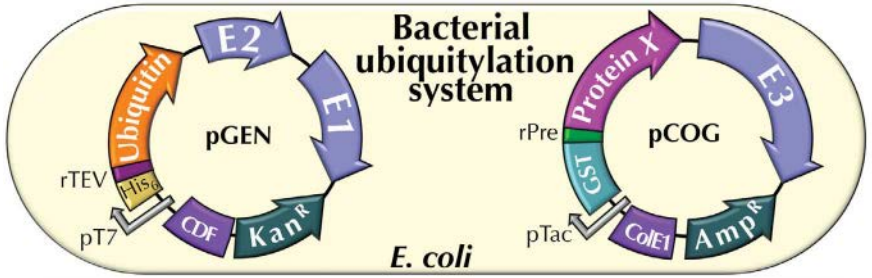
The reconstituted system faithfully recapitulates Rpn10 ub.

Rpn10 is ubiquitylated by Rsp5 in vivo in S.cerevisiae at K84

In vitro polyubiquitylated

Test in E.Coli:

His-Ub Ubc5 E2 GST-Rpn10-Rsp5 E3
Uba1 E1



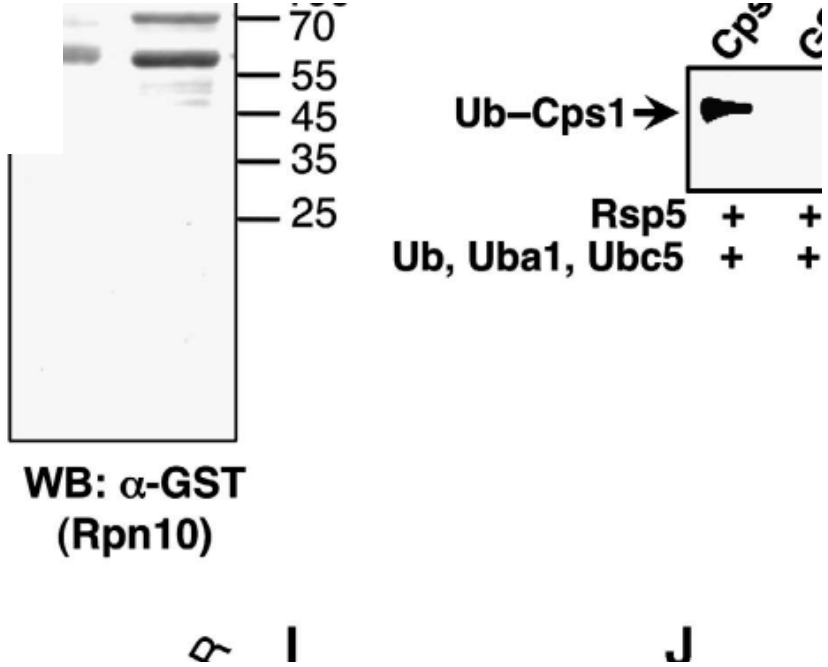
Sequential purification and isolation of Rpn10
In-vitro ubiquitylation assay
mass spectroscopy analysis

Found 4 K residues
K84 was found as the major site with 52%
as in vivo

Specificity and fidelity of the bacterial reconstituted ubiquitylation system

E2:E3 specificity
By **RING** containing E3 ligase

E1: Uba1
E2: UbcH5b vs. Cdc34
E3: Rsp5
Substrate: Mib

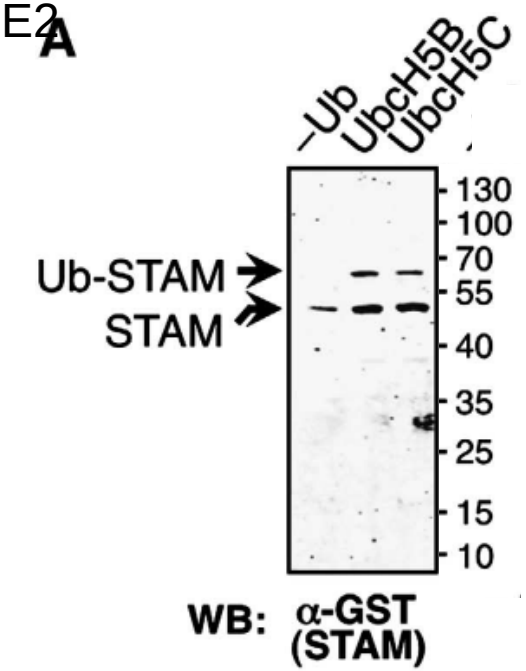


Mib is ubiquitylated by UbcH5b-E2 and Rsp5-E3
Cdc34: apo-Mib

E3 independent auto-ubiquitylation of Ub receptors

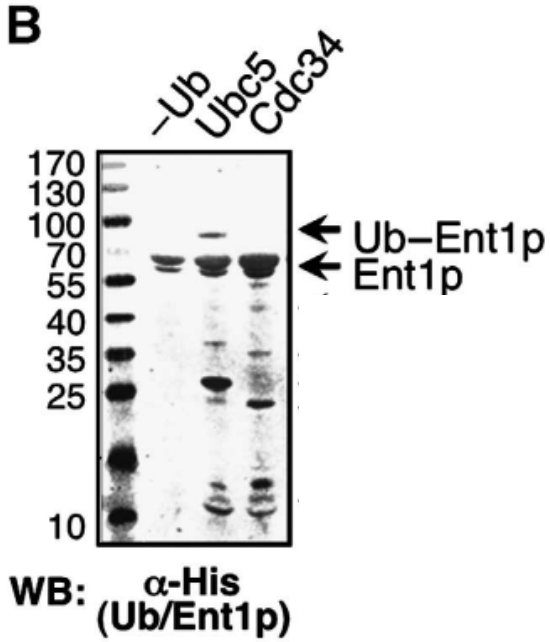
Many Ub receptors undergo auto-mono-ubiquitylation. Closed inactive conformation.
E3-independent Ub receptors

STAM Ub receptor + Ub + E1 + 2 E2 unspec. E2



STAM undergoes auto-monoubiquitylation with E2-UbcH5 B/C

Epsin-1 Ub receptor + Ub + E1 + spec./



Epsin-1 undergoes auto-monoubiquitylation with Ubc5, but not with Cdc34

E3 independent auto-ubiquitylation of Ub receptors is possible in the model system

Mapping the ubiquitylation sites on Vps9

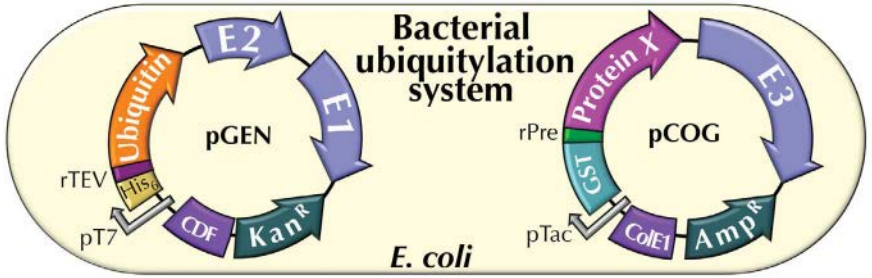
Ubiquitin receptor Vps9:

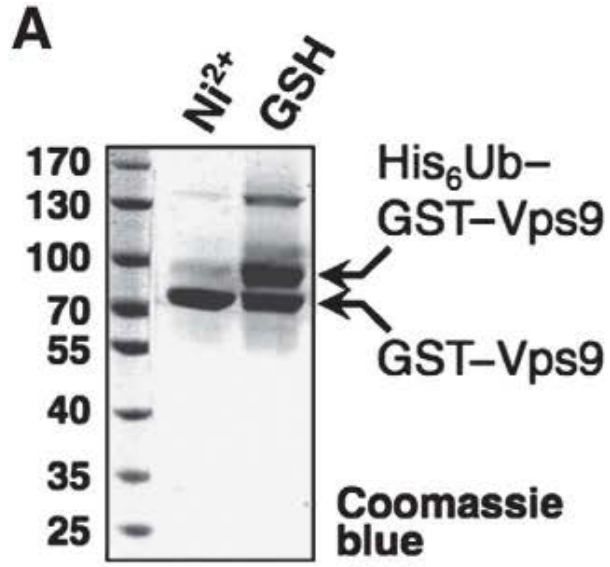
- Promotes trafficking of ub transmembrane cargo
- Ub binding and intramolecular mono-ubiquitylation are coupled

Test if Vps9 is ubiquitylated in the E.Coli bacterial system

2 sequential purification steps of Vps9
1. Ni^{2+}: His-Ub
2. GSH: Glutathione S-tranferase

His-Ub Uc4/5 E2 GST-Vps9-Rsp5 E3
Uba1 E1





Large quantities of mono-ub Vps9
Which K residues are ub.?

Mapping the ubiquitylation sites on Vps9

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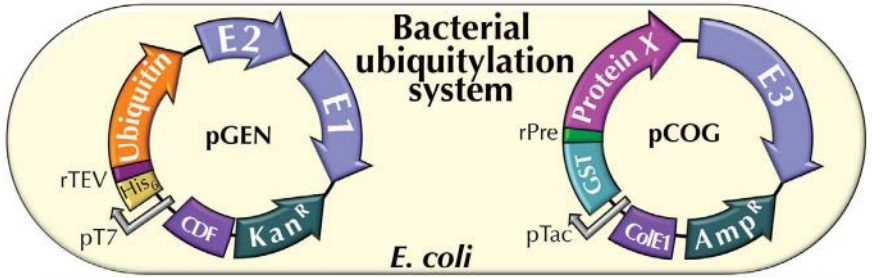
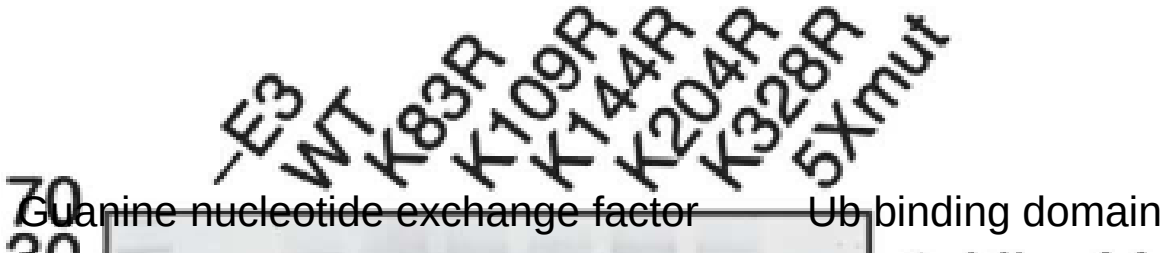
Test if Vps9 is ubiquitylated in the E.Coli bacterial system

Comprehensive mass spectroscopy of purified ub-Vps9

His-Ub Uc4/5 E2 GST-Vps9-Rsp5 E3

Uba1 E1





5 modified K residues

All but K328 within the GEF domain

Mapping the ubiquitylation sites on Vps9

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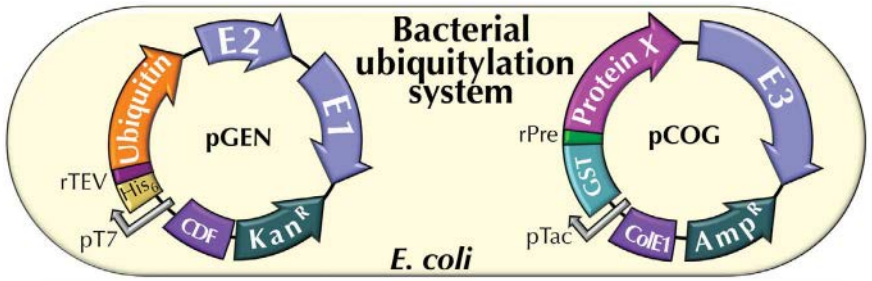
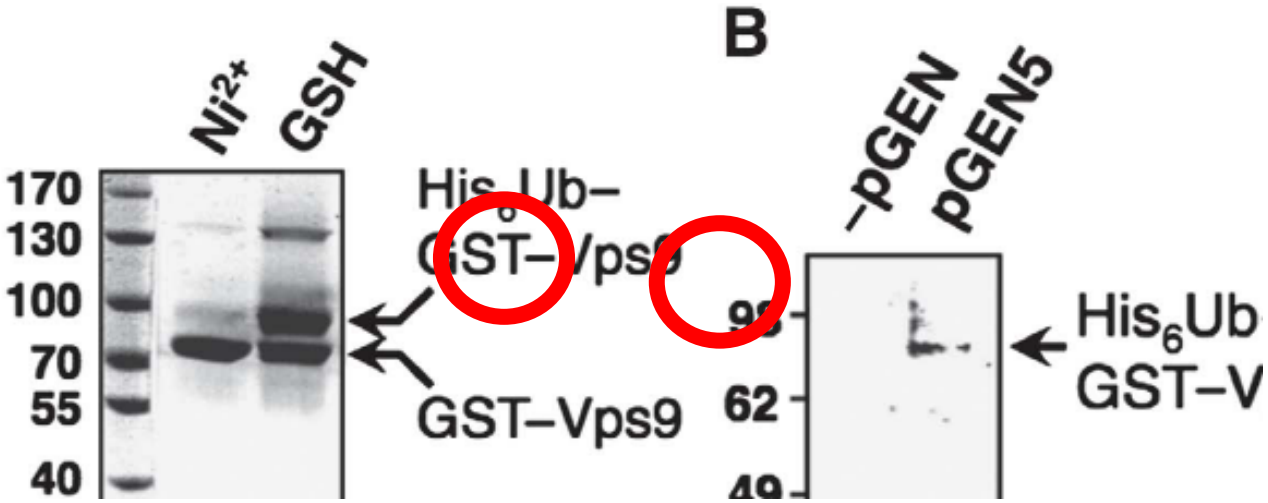
Uba1 E1



Comprehensive mass spectroscopy of purified ub-Vps9



5 modified K residues

All but K328 within the GEF domain

Mutate 5 K residues, individually or all together

Mapping the ubiquitylation sites on Vps9

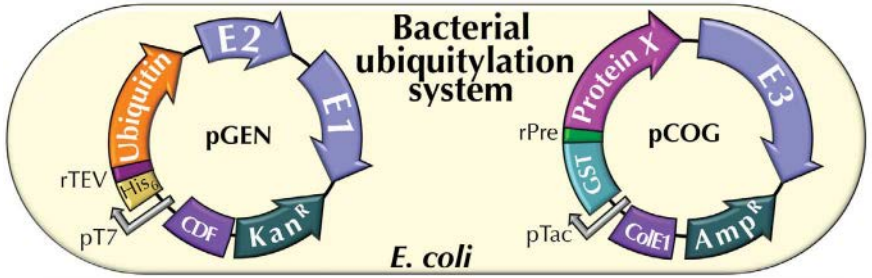
Ubiquitin receptor Vps9:

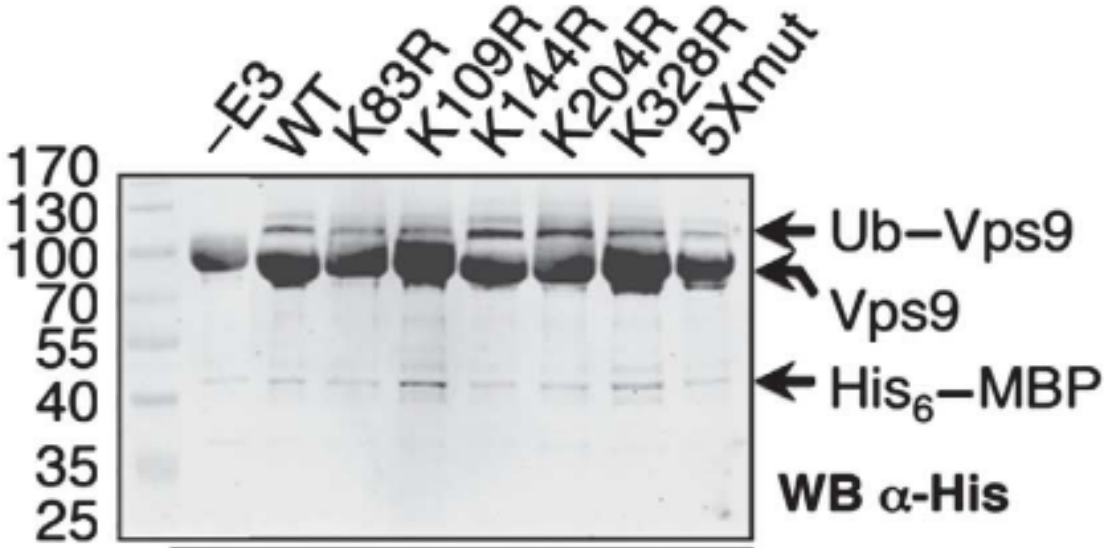
- Promotes trafficking of ub transmembrane cargo
- Ub binding and intramolecular mono-ubiquitylation are coupled

Test if Vps9 is ubiquitylated in the E.Coli bacterial system

His-Ub Uc4/5 E2 GST-Vps9-**Mut**-Rsp5 E3

Uba1 E1





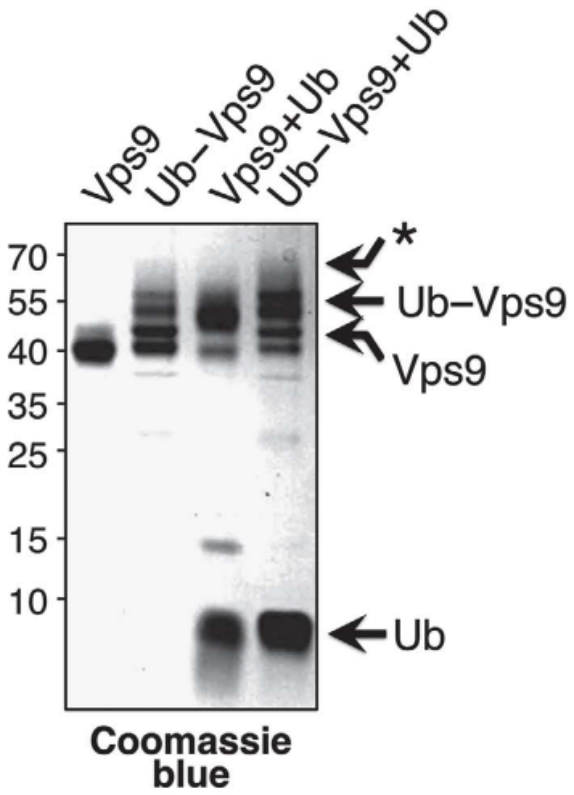
All mutants still undergo ubiquitylation
Quintuple mutant less ub.
-> Other K residues must become ub.

Direct evidence for cis and trans Ub binding by Vps9

Hyp:

ubiquitylated Ub receptor
cis conformation, inhibited
cannot bind ubiquitylated target proteins anymore

un-ubiquitylated Ub receptor
trans conformation, active
binds ubiquitylated target proteins



Cross-linking assay with purified Vps9, ubVps9, Ub

Apo-Vps9 binds Ub -> is active

Ub-Vps9 cis does not bind Ub
no band of the predicted migration distance of Ub bound to ubVps9 is seen
-> already ubiquitylated Vps9 is inactive

Summary Paper I

- Modular bacterial system to model eukaryotic post-translational ubiquitylation
  2 vectors pGEN and pCOG with > 20 / 30 different enzyme and substrate inserts allow various combinations

- Advantage of E.coli:
  easy to combine the vectors and transform into bacteria
  No deubiquitylases (DUBs) -> stable ubiquitylated products

- Chemical approaches to generate Ub produced only free Ub or Ub chains, with the reconstituted system conjugated E1, E2, E3 and native forms of ub proteins are obtained

- Identifcation, purification and characterization of ubiquitylated proteins:
  6 examples shown

Summary Paper I

1. RING- and HECT containing E3 ligases undergo auto-ubiquitylation in bacteria

2. The reconstituted system faithfully recapitulates Rpn10 ubiquitylation

3. E2:E3 specificity of the bacterial reconstituted ubiquitylation system by RING- and HECT containing E3 ligases is given

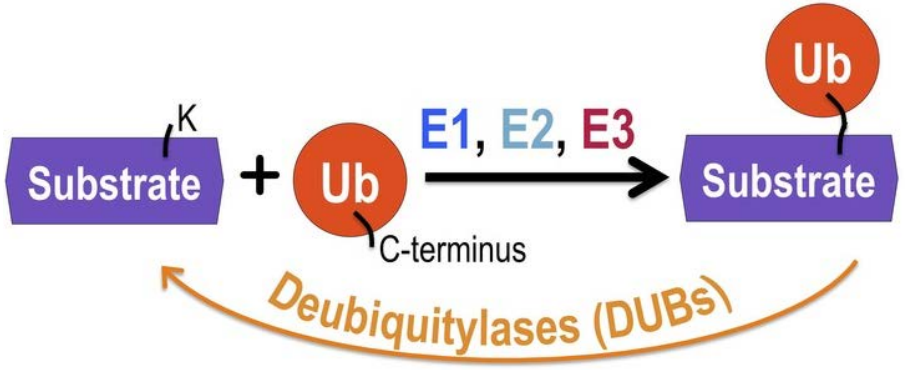
4. E3-independent auto-ubiquitylation of Ub receptors can be modeled

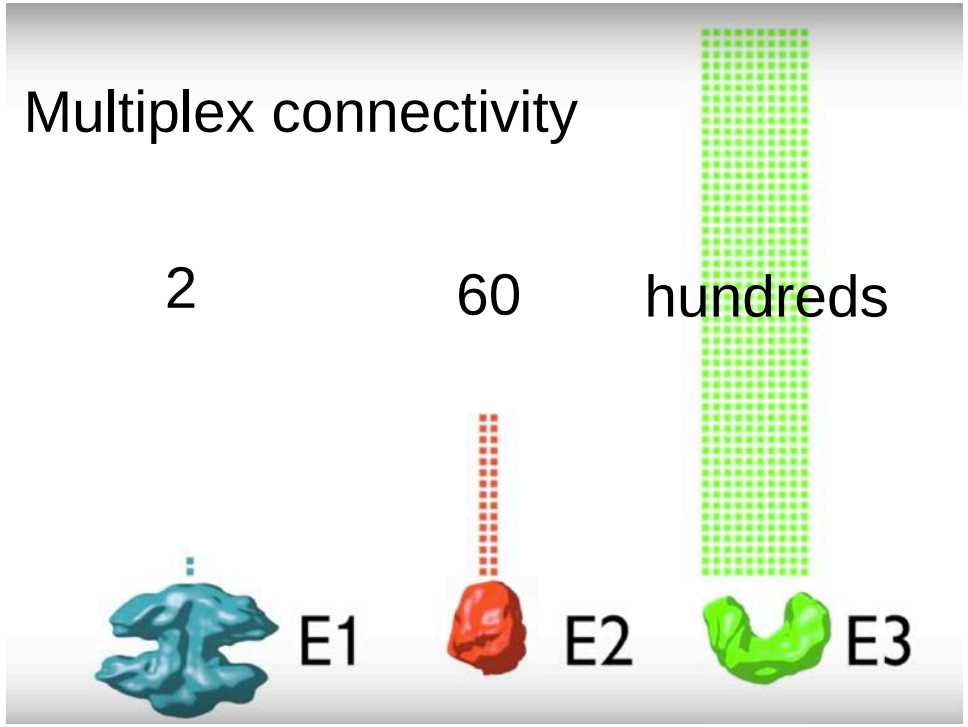
5. Mapping of five ubiquitylation sites on Vps9 is possible

6. Direct evidence for cis and trans Ub binding by Vps9 could be proven

Challenges in assigning specific associations of components along ubiquitin cascades

100 DUBs in humans





most of the Ub cascades, linking specific E1s, E2s and E3s to their cognate targets are still not known

Paper II

PUBLISHED ONLINE 3 OCTOBER 2016

Circumvent rapid dynamics of de- & ubiquitylation: E.coli lack DUBs

Genetic selection tool in E.coli to identify novel interactions and components of ub. cascades

Construction of a selection system for ubiquitylation

Modular architecture
Ub apparatus and substrate are co-expressed from two vectors

Dihydrofolate reductase split in to fragments:
N-terminus of murine nDHFR fused to N-terminus of Ub
C-terminus of cDHFR fused to N-terminus of substrate

Substrate ubiquitylation
DHFR assembles into functional enzyme
TRIM (trimethoprim) resistance

Assess functionality of the system

Ub Ubc4 E2 E1 Vps9 Ub receptor

Rsp5 E3

Selection

Demonstrate E3-substrate s

replace Rsp5 with non-cognate SIAH2

TRIM

TRIM

Complete bacteria grow in both conditions
Strains lacking a components cannot grow on selective media

SIAH2 promotes self-ub. but
bacteria only grow when cognate Rsp5 E3 expressed

Identification and characterization of E3s

Most E3 ligases undergo self ub.
Fused to cDHFR

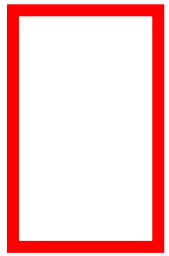
HECT E3 ligase Rsp5

RING E3 ligase SIAH2

All required ub. components needed to grow on non-permissive medium

Identification of a novel E3 ligase and its cognate E2s

Identify potential E3 in enterohaemorrhagic E.coli by blasting human CHIP E3 ligase against EHEC proteome
Found an uncharacterized sequence with 22% identity to CHIP domain helix-loop-helix-beta



Clone potential E3 ligase as fusion with cDHFR
Screen fusion agaist yeast library of E2
Readout: surviving bacteria on selective medium

Ubc4/5 are cognate E2 for putative E3 ligase

Identification and characterization of Ub binding domains = UBDs

Ub binding domains usually bind mono-Ub non-covalently, with low affinity
The system stabilizes UBD-Ub interactions, by forming a covalent bond between Ub and UIM = Ub interaction motif
The cDHFR was fused to UIM and co-expressed in the selection system without E3 ligase.
Detect E3 independent self ub.

Ub Ubc4-E2 E1 UIM

No E3

ENTH is a Ub binding domain

Challenge the system to detect a novel ultra-weak affinity UBD
Based on sequence homology they speculated ENTH could bind Ub
cDHFR-ENTH fusion

ENTH domains promote growth on selective medium
In E3-independent manner
-> ENTH directly binds Ub-E2 conjugase

Structural insight into Ub recognition by ENTH domains

Obtain high-resolution information on the ENTH-Ub interaction
Crystalized the complex

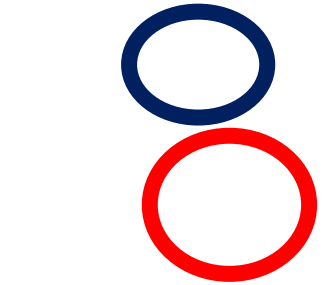
High-quality electron density maps

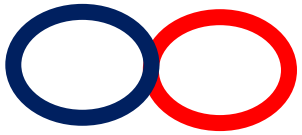
zebrafish ENTH

Yeast ENTH

Highly similar

superimposition of fish and yeast ENTH





zebrafish: W26 and L30 Ub binding residues
E42 and D45 electrostatic interact.
yeast: K36 and I40 Ub binding residues
E41 and E 44 electrostatic interact.

Genetic validation of ENTH-Ub interface

Evaluate contributions of specific residues involved in ENTH-Ub binding
Introduce point mutations at the predicted interface
Growth phenotype of complete Ub system and zebrafish ENTH point mutations

Monitor the growth rates of bacteria by timelapse scanning & quantify by Fiji-based timeseries analyzer

In yeast similar phenotypes, but less severe -> slight structural differences between ENTH complexes

Mutational analysis supports structual model

Alanine mutations or exchange acidic residues
-> growth-arrest phenotype

Summary Paper II

- The developed selection system serves as prototypical tool for genetic studies of Ub signals

- System can detect and quantify relatively minor differences in protein-protein interactions (zebrafish – yeast) along Ub pathways

- Limitations:
  - Nonfunctional expression of some E3s and their targets
  - Did not test different orientation fusions

- Tool should facilitate identification of genetic associations linking ubiquitylations enzymes to their substrates.

- Interesting for characterization of protein-protein interfaces along Ub cascade or for drug discovery: targeting cancer proteins for degradation

How E.Coli is involved in discovery of ubiquitylation cascade

Questions?