Study of different types of ubiquitination

Rudi Beyaert (rudi.beyaert@irc.vib-ugent.be)
VIB – UGent Center for Inflammation Research
Ghent, Belgium

VIB Training
Novel Proteomics Tools: Identifying PTMs
October 5, 2017
Provinciehuis, Leuven
Overview

- Introduction
  - Ubiquitin
  - Ubiquitination machinery
  - Types of ubiquitination

- Simple biochemical methods
  - Is protein X ubiquitinated?
  - What type of ubiquitination is it?

- Troubleshooting
Ubiquitin

- 76 Amino Acids
  - Compact, globular protein, 8.5 kDa
  - Highly conserved (3 aa difference from yeast to man)
  - Expressed in every eukaryotic cell

- Post-translational modification on proteins
  - Attaches on Lysine residues or N-terminal Methionine
  - Multiple functions
Ubiquitin

Adapted from Komander et al., EMBO Rep., 2009

Lys-side chain

Gly75

Gly76

Ubiquitin

Lys-linkage (isopeptide bond)

Met1

Gln2

Met1-Ub linkage (peptide bond)

Adapted from Komander et al., EMBO Rep., 2009
Ubiquitin

The Nobel Prize in Chemistry 2004
“for the discovery of ubiquitin-mediated protein degradation”

- Aaron Ciechanover
- Avram Hershko
- Irwin Rose

- The discovery of ubiquitin-mediated protein degradation
- Cells give a chemical "kiss of death" to proteins that need to be destroyed.

A. Ciechanover et al., Biochem Biophys Res Comm 81 (1978)
The ubiquitination machinery

- E1 (activating)
- E2 (conjugating)
- E3 (ligase)
- Ubiquitin, ATP

Deubiquitylating enzymes (DUBs)

Ubiquitin-binding domains: Recognize Ub signals and translate them into cellular responses

- Proteasomal degradation
- Signal transduction
- Endocytosis
- Vesicle and protein trafficking
- DNA repair
- Autophagy
- Cell cycle
- Inflammation
- Cancer
- ...
- ...
- ...
- ...
- ...
Types of ubiquitination

David Komander
Different ubiquitin chains have different architectures

Heride et al., Current Biology 2014

Clague et al., Physiological Reviews 2013
The ubiquitin code

Different types of ubiquitination are recognized by specific ubiquitin binding domains (UBD) in other proteins (Ub receptors)
Ubiquitination is key in NF-kB signaling

Adapted from Verstrepen et al., Biochem. Pharmaco. 80, 2009-20, 2010
Phosphorylation versus Ubiquitination

**Phosphorylation**
- Protein
- Kinase
- SH2
- P-Ser/Thr
- P-Tyr
- Phosphatase

**Ubiquitination**
- Protein
- Ub
- E3-Ligase
- UIM
- K48 K6 K27 K33
- K63 K11 K29 linear

**Complexity**
- 2
- 8
New complexity in the ubiquitin code

Ubiquitination - questions

- Is my protein ubiquitinated?
- What type of ubiquitin chain? Which residue?
Is protein X ubiquitinated?

- Ubiquitination increases MW of protein X (immunoblotting)
  - Ubiquitin = 8.5 kDa; but mostly a high MW smear instead of defined bands
    - differences in polyubiquitin chain length
    - heterogeneously ubiquitinated at multiple sites
    - different chain types lead to distinct motilities
  - Can also reflect other types of PTMs
    - treat with DUB before loading

Emmerich C and Cohen P, BBRC, 2015
Is protein X ubiquitinated?

- In most cases a smear is not visible; reasons?
  - Protein ubiquitination is rapidly reversed by DUBs → add DUB inhibitors (N-ethylmaleimide/iodoacetamide + EDTA/EGTA) to the lysis buffer or work in cells in which DUB expression is silenced (M1-Ub)
  - (K48)polyubiquitinated protein is rapidly degraded by the proteasome → add proteasome inhibitors to the cells (e.g. MG132)
  - Only small fraction of protein is modified → requires enrichment of ubiquitinated proteins

Emmerich C and Cohen P, BBRC, 2015
Is protein X ubiquitinated?

- **Enrichment of ubiquitinated proteins**
  - Immunoprecipitation (IP) with anti-protein X + immunoblotting for ubiquitin
    - needs cell lysis in stringent (1% SDS) conditions to eliminate detection of other interacting proteins that are ubiquitinated; 10x dilution of cell lysate needed for IP (needs native conditions)
    - sensitivity can be increased by using epitope tagged ubiquitin (caution!)
Overexpression of tagged Ub may render the system constitutive!

Polo et al, Nature, 2002
Commercial anti-Ub antibodies do not recognize all ubiquitin linkages equally!

Emmerich C and Cohen P, BBRC, 2015
Is protein X ubiquitinated?

- **Enrichment of ubiquitinated proteins**
  - Affinity purification of His-tagged ubiquitin via Ni\(^{2+}\)-affinity chromatography under denaturing conditions, followed by immunoblotting for protein X (caution: tagged Ub)
Is protein X ubiquitinated?

- **Enrichment of ubiquitinated proteins**
  - Affinity purification with TUBEs

TUBEs = ubiquitin traps = repeats of UBDs (with specificity for certain polyubiquitin-linkages)

TUBEs show almost a 1000-fold increase in affinity for polyubiquitin moieties over a single UBD

TUBEs protect against DUBs

TUBEs can be immobilized in different ways:
GST-TUBE, His-TUBE, Halo-TUBE, …
TUBE affinity purification

Step 1: 4 hr incubation
Step 2: 2 hr incubation
Step 3: 3 x 5 min wash
Step 4: Elution
TUBE affinity purification
Is protein X ubiquitinated?

- Global ubiquitin profiling using MS + enrichment for di-GlyLys containing peptides using diGly-Lys specific antibodies
- Determining the Lys acceptor site in the substrate (mutagenesis mostly does not work due to jumping sites)
D-GlyLys signature is not unique for ubiquitin!

- Need for ubiquitin enrichment by prior affinity purification of ubiquitinated proteins using e.g. anti-ubiquitin or Halo-TUBE

<table>
<thead>
<tr>
<th>UBL</th>
<th>C-term sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ubiquitin</td>
<td>LRLR-GG~K</td>
</tr>
<tr>
<td>ISG15</td>
<td>LRLR-GG~K</td>
</tr>
<tr>
<td>NEDD8</td>
<td>LALR-GG~K</td>
</tr>
<tr>
<td>SUMO1</td>
<td>QEQT-GG~K</td>
</tr>
<tr>
<td>SUMO2/3</td>
<td>QQQT-GG~K</td>
</tr>
<tr>
<td>FAT10</td>
<td>SYCI-GG~K</td>
</tr>
<tr>
<td>URM1</td>
<td>STLH-GG~K &amp; sulphur</td>
</tr>
</tbody>
</table>
What type of ubiquitination do I have?

- Use of epitope tagged **ubiquitin mutants** (caution)

Two complementary sets of ubiquitin mutants are available

**Figure B**
- WT Itoch

**Figure C**
- Single Lys substituted with Arg
- All Lys substituted with Arg except one
What type of ubiquitination do I have?

- Use of ubiquitin linkage specific antibodies

Emmerich C and Cohen P, BBRC, 2015
What type of ubiquitination do I have?

- Use of ubiquitin linkage specific TUBEs

<table>
<thead>
<tr>
<th>Protein/Peptide</th>
<th>UBD</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEMO</td>
<td>UBAN domain</td>
<td>M1 &gt; K63 chains</td>
</tr>
<tr>
<td>NEMO [D311N]</td>
<td>UBAN domain</td>
<td>No Ub-binding</td>
</tr>
<tr>
<td>TAB2</td>
<td>NZF domain</td>
<td>K63 chains</td>
</tr>
<tr>
<td>TAB2 [T674A/F675A]</td>
<td>NZF domain</td>
<td>No Ub-binding</td>
</tr>
<tr>
<td>Ubiquilin-1</td>
<td>UBA domain</td>
<td>All Ub chains</td>
</tr>
<tr>
<td>MultiDsk</td>
<td>UBA domain</td>
<td>All Ub chains</td>
</tr>
<tr>
<td>RAP80</td>
<td>dual UIM domains</td>
<td>K63 chains</td>
</tr>
<tr>
<td>TRABID</td>
<td>NZF domain</td>
<td>K29 and K33 chains</td>
</tr>
</tbody>
</table>

>20 different UBDs
What type of ubiquitination do I have?

- Use of poly-ubiquitin linkage specific TUBEs

Lane 1: GST
Lane 2: GST-S5a (UIM)
Lane 3: GST-TAB2 (NZF)
Lane 4: GST-S5a (UIM) + GST-TAB2 (NZF)
Lane 5: GST-NEMO (UBAN)
What type of ubiquitination do I have?

- Use of poly-ubiquitin linkage specific DUBs
What type of ubiquitination do I have?

- Ubiquitin chain restriction analysis (UbiCRIst): linkage specific DUBs are used as ‘restriction enzymes’ in ubiquitin sequencing
What type of ubiquitination do I have?

- Ubiquitin chain restriction analysis (UbiCRest): linkage specific DUBs allow analysis of ubiquitin chain architecture (linkage-type + topology)

adapted from Hospenthal et al., Nat Protoc. (2015)
What type of ubiquitination do I have?

- Ubiquitin chain restriction analysis (UbiCRest): linkage specific DUBs allow analysis of ubiquitin chain architecture

FLAG-TNFα purification of TNF receptor complex

Yogesh Kulathu
What type of ubiquitination do I have?

- Quantification of unique diGly signature peptides for each polyubiquitin linkage after trypsin cleavage
Troubleshooting

- **NEM** is better at preserving K63- and M1-Ub chains than IAA (unstable, destroyed by light)
- **IAA** may interfere with the identification of Ub sites by MS (IAA reaction with Cys leads to covalent 2-acetamidoacetamide with a MW of 114Da = that of diGly dipeptide (use NEM))
- **MG132** can have cytotoxic effects; increased ubiquitination may be due to cellular stress

- **Amount of ubiquitin in cells is very high** (~85µM in HEK293) → needs high amounts of anti-ubiquitin to IP all ubiquitinated proteins (first IP protein of interest and use anti-Ub for immunoblotting)
- **Antibody epitope may be blocked/masked by the presence of polyubiquitin chains** (use polyclonal Ab) (e.g. IL-1 induced degradation of IRAK1 is false!)
Troubleshooting

- Ubiquitin = globular protein; epitopes may not be accessible to antibodies due to insufficient denaturation during SDS-PAGE → signal strength can be enhanced by extra denaturation step (15 min boiling water, autoclaving, ...)
- Overexpression of tagged Ub may lead to ubiquitination of proteins that are normally not ubiquitinated
- N- or C-terminally tagged ubiquitin can not be used in case of M1-Ub (use of internally Strep-tagged Ub)
- Ubiquitin mutants may not fold equally well, have different affinity for UBDs, affect deubiquitination, ...
- GGK-specific monoclonal antibodies do not recognize the characteristic GGMQIFVK signature peptides resulting from tryptic cleavage of M1-polyubiquitin
Troubleshooting

- UBD linkage specificity is not 100%
- Also use immobilized Ub-binding defective UBD mutant to establish that the interaction with the ubiquitinated protein is specific
- GST-TUBE forms dimers, which can alter linkage specificity
Plasmids for ubiquitin research available at BCCM-LMBP plasmid collection at UGent-IRC

http://bccm.belspo.be/about-us/bccm-lmbp