The Keystone Symposium on The Many Faces of Ubiquitin took place between 11 and 16 January 2009, at Copper Mountain, Colorado, USA, and was organized by S. Polo, C.D. Lima & V. Dixit.

Keywords: deubiquitinating enzyme; E3; proteasome; ubiquitin


See Glossary for abbreviations used in this article.

Introduction

Living in a competitive environment, cells constantly integrate many inputs from their surroundings into rapid and appropriate responses. Crucial to accomplishing accurate signalling is the ability of cells to alter the activity or abundance of regulatory proteins, which is often achieved by conjugating them to ubiquitin or UBLs. Not surprisingly, ubiquitin and UBLs are essential for proliferation and cell survival, and aberrations in the pathways that produce these modifications are linked to diseases such as cancer and neurodegeneration (Kerscher et al., 2006).

Initial studies revealed the role of ubiquitination in promoting protein degradation. It is now known that ubiquitination also has many non-proteolytic functions such as regulating protein interaction, intracellular localization and enzymatic activity (Kerscher et al., 2006). The many ways in which ubiquitin/UBLs control signalling originate from the possible modification of proteins with a single molecule of ubiquitin, more than 10 UBLs or distinct types of ubiquitin chains. A large set of cellular proteins recognizes these modifications, thereby deciphering the ubiquitin/UBL code and mediating downstream signalling events (Fig 1; Kirkin & Dikic, 2007).

To achieve the high specificity required for signalling, cells invest heavily in the enzymatic apparatus responsible for attaching ubiquitin/UBLs to proteins. In human cells, ubiquitination is achieved through an enzymatic cascade of two known E1 enzymes, approximately 60 E2 enzymes and 600–1,000 E3 enzymes (Kerscher et al., 2006). In addition, about 100 human DUBs remove ubiquitin from substrates (Song & Rape, 2008). UBLs often use a similar cascade, but with fewer enzymes at each step: for example, modification with SUMO requires one dimeric E1, one E2 and only approximately 10 E3s.

The bewildering complexity underlying ubiquitin-dependent signalling harbours great potential for important discoveries, as was confirmed at the recent Keystone Symposium on The Many Faces of Ubiquitin; this report discusses some of the highlights of this exciting meeting.

Keynote lecture

Thanks to her groundbreaking discoveries and enthusiasm for ubiquitin—a molecule that she once described to D. Finley (Boston, MA, USA) as being ‘infinitely seductive’—C. Pickart was a pioneer in the ubiquitin field for many years. Among many important findings, her laboratory showed that substrates have to be modified with a chain of at least four ubiquitin molecules to be recognized by the proteasome (Thrower et al., 2000). Honouring these contributions, the 2009 Cecile Pickart Memorial Lecture was presented by Finley, who discussed the orchestrated delivery, unfolding, deubiquitination and degradation of proteasomal substrates. An example of the coordination of several events is the human proteasomal subunit RPN13, which not only recognizes ubiquitinated cargo but also recruits and activates the DUB UCH17. This surprising dual function of RPN13 suggests that ubiquitin chains might be targeted for disassembly on docking at the proteasome. Finley also presented his work on yeast
proteasomes, which recruit and activate another DUB, Ubp6. Similar to UCH37, Ubp6 trims ubiquitin chains, which is thought to limit the residence time of ubiquitinated proteins at the proteasome. In addition, Ubp6 can inhibit substrate degradation on the same substrate, independently of its DUB activity. Ubp6 levels increase when ubiquitin levels fall, at which time its potent inhibition of the proteasome might be crucial, in order to preserve the remaining ubiquitin pools.

The proteasome-bound ubiquitin ligase Hul5 counteracts Ubp6 and has E4 activity—which is the ability to add ubiquitin to pre-existing ubiquitin chains. Hence, Hul5 might help to preserve the ubiquitin chains of proteins that are already associated with the proteasome against DUBs such as Ubp6 and Uch37, thereby giving the proteasome more time to unfold substrates and initiate their degradation. Ubp6 and Hul5 constantly antagonize one another, thereby allowing for a dynamic regulation of protein degradation even after substrates have been targeted to the proteasome. Unlike Ubp6, Hul5 is not induced by ubiquitin stress, so the balance of Hul5 and Ubp6 can be tipped through feedback controls.

**New chains on the block**

Much of ubiquitin’s power as a cellular regulator originates from the fact that it can modify proteins in many different ways. Lys48-linked ubiquitin chains send substrates to the proteasome, whereas mono-ubiquitination or Lys63-linked chains usually trigger signalling events that are not related to proteolysis. Mass-spectrometry experiments have indicated that other lysine residues of ubiquitin are also used for chain formation in vivo (Peng et al., 2003); however, the function of these alternative chains has remained unclear. Several presentations at the meeting provided new insights into the functional consequences of ubiquitin chains with distinct topology.

Lys63-linked ubiquitin chains have an established function in activating the NF-κB transcription factor (Deng et al., 2000); however, most experiments addressing this role in human cells have relied on the overexpression of mutant ubiquitin. J. Chen (Dallas, TX, USA) presented a small-interfering RNA-depletion/rescue strategy that allows the exchange of wild-type ubiquitin with a mutant version at physiological levels. Confirming a large body of earlier work, his group found that Lys63-linked chains are essential for the activation of the IKK kinase after the stimulation of cells with IL-1β, but surprisingly not after stimulation with TNF-α. Accordingly, depletion of UBC13—the E2 responsible for assembling Lys63-linked chains—blocked IL-1β, but not TNF-α-dependent IKK activation. The activation of NF-κB by TNF-α depends on another E2 enzyme, UBCH5C, and on chains of unknown topology. The system developed by the Chen laboratory will be a powerful tool to study the role of the various chain types in human cells.

Emphasizing the complexity of ubiquitin-dependent NF-κB regulation, K. Iwai (Osaka, Japan) and I. Dikic (Frankfurt, Germany) revealed intriguing data supporting a role for new linear ubiquitin chains in NF-κB regulation. Iwai discussed the identification of the ubiquitin ligase LUBAC. As shown by mass spectrometry and linkage-specific antibodies, LUBAC assembles linear ubiquitin chains in which the carboxy terminus of ubiquitin is linked to the amino terminus of the previous ubiquitin. LUBAC associates with the IKK subunit NEMO and modifies it with linear chains, thereby triggering the activation of NF-κB. Accordingly, when a subunit of LUBAC is deleted in mice, activation of NF-κB is impaired and cells are sensitized to TNF-α-induced apoptosis. These findings were complemented by a structural analysis of the interaction between linear ubiquitin chains and NEMO presented by I. Dikic. NEMO strongly binds to linear di-ubiquitin using a conserved UBAN domain, as revealed by X-ray crystallography (Rahighi et al., 2009). The binding specificity of the UBAN domain originates from the recognition of a new surface in the proximal ubiquitin, which is accessible in linear di-ubiquitin but not in ubiquitin dimers linked through Lys63. If the NEMO residues that are required for binding the new ubiquitin surface are mutated, the activation of NF-κB is impaired and cells are sensitized to TNF-α-induced apoptosis.

Lys11-linked ubiquitin chains are abundant in vivo; however, they have only recently been shown to promote the proteosomal

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**Glossary**

- A20 also known as tumour necrosis factor-α-induced protein 3
- APC/C anaphase-promoting complex/cyclosome
- ATPBD3 ATP-binding domain 3
- BRCA1 breast cancer 1, early onset
- CDC cell-division cycle
- CUL1 cullin 1
- DUB deubiquitinating enzyme
- FANCD2 Fanconi anaemia, complementation group D2
- FANCI Fanconi anaemia, complementation group I
- G2BR Ube2g2-binding region
- HECT homologous to E6-associated-protein carboxyl terminus
- HIF1α heat shock-inducible factor 1-α
- IKK inhibitor of nuclear factor-κB kinase
- IL-1β interleukin 1-β
- LUBAC linear ubiquitin chain-assembly complex
- MCL1 myeloid cell leukaemia sequence 1
- MERIT40 mediator of receptor-associated protein 80 interactions and targeting of 40 kDa
- MoaD molybdopterin-converting factor subunit 1
- Mule Mc1 ubiquitin ligase E3
- Nedd8 neural precursor cell expressed, developmentally downregulated 8
- NEMO nuclear factor-κB essential modulator
- NF-κB nuclear factor-κB
- Pup prokaryotic ubiquitin-like protein
- RAD60 radiation sensitive 60
- RANBP2 RAN-binding protein 2
- RAP80 receptor-associated protein 80
- RIP receptor-interacting protein
- RPN13 proteasome-remodeling particle 13 (non-ATPase like)
- RSP5 reverses suppressor of Ty phenotype 5
- SCF Skp1–cullin–F-box
- SENP SUMO1/sentrin specific peptidase 1
- SMAD4 mothers against decapentaplegic homologue 4
- SUMO small ubiquitin-like modifier
- TGF-β transforming growth factor-β
- Thi5 thiamine biosynthesis protein
- TIF1γ transcriptional intermediary factor 1γ
- TNF-α tumour necrosis factor-α
- TPR tetratrico peptide repeat
- tRNA transfer RNA
- UAF USP-associated factor 1
- UBA ubiquitin binding in ABIN and NEMO
- UBC ubiquitin-conjugating enzyme
- UBL ubiquitin-like modifier
- UCH37 ubiquitin carboxy-terminal hydrolase 37
- UIM ubiquitin-interacting motif
- Urm1 ubiquitin-related modifier 1
- USP ubiquitin-specific peptidase
The approximate number of enzymes in each protein family in the human genome is indicated in parenthesis. Ubiquitin-modifiers use a similar enzymatic cascade, but with fewer enzymes at each step. Ubiquitination has many different consequences, depending on the number of ubiquitins attached and their linkage; those discussed in more detail during the meeting are mentioned below the modified substrate. Ubiquitin can be removed from substrates by DUBs, which, as discussed at the meeting, often interact with E3 enzymes to allow dynamic signalling. DUB, deubiquitinating enzyme; HECT, homologous to E6-associated-protein carboxyl terminus; NF-κB, nuclear factor-κB; RING, Really interesting new gene.

E2 enzyme cooperates with UBCH10 and the APC/C to assemble Lys11-linked chains. UBCH10 modifies substrate lysine residues and catalyses early steps of chain formation, whereas the new factor specializes in extending the Lys11-linked chain. This reaction is particularly important for the modification of proteins bound to the mitotic spindle, pointing to another intracellular location—in addition to the endoplasmic reticulum—that seems to be a hotspot for the assembly of Lys11-linked ubiquitin chains.

**Building a chain**

Thanks to a flurry of elegant structural and biochemical studies, much progress has been made in our understanding of how ubiquitin chains with a specific topology are assembled. Ubiquitin-chain formation is a biochemical reaction of perplexing complexity, in which substrates, E2 enzyme and E3 ligases are tightly regulated. Several captivating aspects of chain formation were presented during the meeting; however, unfortunately, only a few examples can be mentioned within the scope of this report.

D. Morgan (San Francisco, CA, USA) discussed the dynamic interaction between substrates and E1s, using the yeast APC/C as an example. The APC/C recruits substrates by using two adaptor proteins, Cdc20 and Cdh1, which are recognized by TPR repeat-containing subunits of the APC/C. Although mutations in such TPR repeats diminish the binding of APC/C to Cdh1 and substrates, the processivity of chain formation is, surprisingly, not affected. This indicates that after the initial delivery to the APC/C, substrates are, at least in part, handed over from Cdh1 to core APC/C subunits (Matyskiela & Morgan, 2009). Therefore, the interaction of substrates with their E3 can be highly dynamic; how those interactions are orchestrated during chain formation is an exciting question for future experiments.

In addition to substrate recruitment, understanding the interactions between E2s and E3s is crucial to delineating the mechanism of chain formation. A point in case was made by R. Deshaies (Pasadena, CA, USA), who discussed the interaction of the E2 Cdc34 with the E3 SCF. The affinity of Cdc34 for SCF is slightly increased by modification of the cullin subunit of the SCF with the UBL Nedd8. More importantly, Cul1 neddylation induces conformational changes in SCF that bring the active site of Cdc34 into the proximity of substrate lysine residues (Saha & Deshaies, 2008). The Deshaies laboratory showed that after the transfer of the first ubiquitin, Cdc34 assembles ubiquitin chains with high processivity. This is due to a rapid binding and dissociation of Cdc34, which is made possible by the association of its acidic tail with a positively charged region on the cullin subunit. These findings point to a highly dynamic and tightly regulated interaction between E2 and E3 enzymes during chain formation.

The work from the Deshaies group and beautiful structural work from B. Schulman (Memphis, TN, USA) emphasize the importance of neddylation for chain assembly by cullin-dependent E3s. The neddylation of cullins was long thought to be promoted by only one E2 enzyme, UBE2M (Ubc12 in yeast); however, Schulman revealed a new NEDD8–E2, UBE2F (Huang et al., 2009). The canonical UBE2M catalyses the modification of most cullins, whereas UBE2F is specifically responsible for CUL5 neddylation. UBE2F is regulated differently than UBE2M, supporting the idea that neddylation is used to fine-tune ubiquitin-chain formation by different cullin-dependent E3s.

During chain formation, the RING-domain characteristic of many E3s is thought to activate bound E2s allosterically. A variation on this theme was put forward by A. Byrd (Bethesda, MD,
poor prognosis in multiple myeloma, indicating that aberrant deubiquitination and dependent degradation. On DNA damage, however, USP9X dissociates from RING fingers can participate in E2 activation, allowing for an intricate regulation of chain assembly.

Emphasizing the complexity of chain formation, various enzymes are, in some cases, required for chain initiation and elongation. J. Svejstrup (London, UK) reported that such a two-step mechanism underlies the DNA damage-dependent ubiquitination of RNA polymerase II. The HECT E3 RSP5 modifies lysine residues in RNA polymerase II; however, constant opposition by a DUB means that the modifications added by RSP5 are not extended into long chains. A cullin-dependent E3 ligase polyubiquitylates RNA polymerase II to trigger its proteasomal degradation following prolonged DNA damage.

Live and let die
As expected for a mechanism underlying quantitative signalling, the assembly of ubiquitin chains can be reversed by approximately 100 DUBs. Reminiscent of phosphatases, DUBs have long-led a wallflower existence; however, several presentations at the meeting showed that they have now gained their well-deserved limelight.

Despite much progress, most of the intracellular pathways controlled by DUBs are still unknown. To close this gap, W. Harper (Boston, MA, USA) presented proteomic approaches to identify hundreds of new binding partners of human DUBs. This work was facilitated by the development of a new software platform, dubbed CompPASS, which allows the identification of high-confidence interactors from immunoprecipitation experiments. This work allowed Harper to link DUBs to diverse processes such as RNA maturation, DNA repair and endoplasmic reticulum-associated degradation, emphasizing the importance of deubiquitination for cellular regulation. Interestingly, many DUBs also interact with E3s, suggesting that competition between ubiquitination and deubiquitination is a common mechanism to establish dynamic signalling.

This opposition between DUBs and E3s was nicely illustrated in talks by V. Dixit (San Francisco, CA, USA) and S. Dupont (Padova, Italy). Dixit reported on the identification of the DUB USP9X as a crucial regulator of apoptosis that controls the abundance of the anti-apoptotic protein MCL1. MCL1 binds to both the E3 Mule and USP9X, and deubiquitination by USP9X protects MCL1 from Mule-dependent degradation. On DNA damage, however, USP9X dissociates from MCL1, thereby triggering rapid MCL1 degradation and apoptosis. High USP9X levels correlate with high MCL1 levels and poor prognosis in multiple myeloma, indicating that aberrant deubiquitination of MCL1 provides tumour cells with an important growth advantage. USP9X also functions in the TGF-β pathway, as discussed by Dupont. Dupont and co-workers had shown that an E3 enzyme, ectodermin/TIF1y, inhibits TGF-β signalling by ubiquitinating SMAD4. The monoubiquitination of SMAD4 blocks the formation of an active transcription factor complex between SMAD4 and receptor-activated SMADs (SMAD2 or SMAD3). By contrast, USP9X allows complex formation by deubiquitinating SMAD4, thereby initiating a new round of TGF-β signalling in cells.

The most impressive example of coordinating DUB and E3 activities is probably the bifunctional protein A20, which contains domains with E3 and DUB activity, and functions in downregulating the NF-κB pathway. As discussed by I. Wertz (San Francisco, CA, USA), A20 inactivates the signalling protein RIP by removing Lys 63-linked ubiquitin chains that are required for its activity. To ensure inactivation, A20 then adds Lys 48-linked chains to target RIP for proteasomal degradation. This model of ubiquitin-chain editing by A20 has now been confirmed by linkage-specific antibodies, which detect a loss of Lys 63 chains on RIP immediately preceding its modification with Lys 48-linked chains. Additional experiments using these powerful new tools suggested that chain editing is more widespread and can be found, for example, in response to interleukin signalling.

Ubiquitin is not everywhere: new findings on UBLs
Similar to ubiquitin, UBLs are transferred to substrates by a cascade of E1, E2 and E3 enzymes. The modification with UBLs seldom results in protein degradation, but rather alters signalling by changing protein interactions or localization. Several exciting talks about new modifiers, enzymes and substrates were presented at the symposium.

A new bacterial modification system reminiscent of ubiquitination and referred to as pupylation was discussed by H. Darwin (New York, NY, USA). By searching for interactors of a proteasomal ATPase in Mycobacterium tuberculosis, her laboratory identified a 64-amino-acid protein, Pup, which is present only in proteosome-containing bacteria. They showed that Pup covalently modifies a native proteosome substrate, FabD. Upon mutation of the modified lysine residue in FabD or the proteasomal ATPase, FabD accumulates, indicating that pupylation might function as a degradation signal. Interestingly, the Darwin laboratory also identified candidate enzymes that could catalyse the transfer of Pup to FabD, further extending the similarities between ubiquitination and pupylation.

Bridging prokaryotes and eukaryotes, C. Schlieker (Cambridge, MA, USA) presented work on the function of Urm1. He used a proteomic approach to show that a poorly characterized protein, ATPBD3, reacts with a Urm1-based suicide inhibitor, ATPBD3 and its binding partner UFP0432 both contain domains linked to RNA modification, and indeed further mass-spectrometry experiments revealed that Urm1 functions as a sulphur carrier required for the 2-thiolation of uracil nucleotides in tRNAs. Therefore, Urm1 functions as a sulphur carrier instead of modifying proteins, which is reminiscent of the prokaryotic MoaD and Thi5 proteins (Iyer et al., 2006).

The most intensively studied UBL is SUMO. Similar to ubiquitinisation, sumoylation is promoted by the action of E3s, which were discussed by F. Melchior (Heidelberg, Germany) and M. Boddy (La Jolla, CA, USA). Melchior presented new experiments on the interactions between the SUMO E2 UBC9 and the E3 RANBP2. Two distinct molecules of UBC9 associate with the RANBP2 complex: one has a structural role in stabilizing the interactions between members of the RANBP2 complex, whereas the other catalyses the sumoylation of target proteins. This mechanism should allow cells to modulate the E3 activity of RANBP2 depending on the levels of UBC9. Reminiscent of these tight E2/E3 interactions, Boddy discussed the RAD60 protein, which uses a well-conserved SUMO-like domain to bind to UBC9 and seems to function as a SUMO E3 during DNA repair. The regulated interaction between UBC9 and SUMO E3s will clearly be an important avenue for future research.

As it is the case with ubiquitin, SUMO is often rapidly removed from proteins to allow dynamic signalling. Desumoylation is catalysed by a family of enzymes known as SENPs, which were discussed by E. Yeh (Houston, TX, USA). By using knockout mice, she showed...
that SENP1 activates the transcription of erythropoietin by regulating the stability of the transcription factor HIF1α (Cheng et al., 2007). The sumoylation of HIF1α blocks the formation of a functional transcription factor complex, and facilitates HIF1α ubiquitination and degradation. These findings, and several talks on SUMO-regulated ubiquitin ligases that were presented at the meeting, illustrate the tight crosstalk between different modifications, which could further extend the ubiquitin/UBL code.

**Ubiquitin and disease**

The misregulation of ubiquitination enzymes has been linked to a range of diseases such as cancer and neurodegeneration. The progress made in identifying and dissecting the disease connections harbours great potential for the development of new therapeutics. Both the links between ubiquitin and disease, and the translation of our understanding of ubiquitination into new therapeutics, were the central theme of several talks at the meeting.

The rapid progress made in identifying ubiquitination enzymes linked to disease is exemplified by the tumour suppressor BRCA1, mutations in which lead to breast or ovarian cancer. BRCA1 functions in DNA repair, which requires its orchestrated recruitment to sites of DNA damage. R. Greenberg (Philadelphia, PA, USA) reported the identification of a new protein, MERIT40, which cooperates with RAP80 to recruit BRCA1 to centres of DNA repair (Shao et al., 2009). RAP80 recognizes ubiquitinated histones at sites of DNA damage through its UIM domains, before recruiting BRCA1. Interestingly, mutations in the UIM domains that interfere with ubiquitin recognition are found in familial breast cancer, further strengthening the links between the misregulation of BRCA1 and the disease.

Fanconi Anaemia (FA), which was discussed by A. D’Andrea (Boston, MA, USA), is one of the best understood diseases linked to deregulated ubiquitination. FA proteins function in DNA repair, and mutations causing the loss of FA activity predispose carriers to tumorigenesis. Targeting of the FA proteins to DNA is regulated by the reversible monoubiquitination of FANCD2 and FANCI, and D’Andrea reported much progress in the understanding of their ubiquitin-dependent chromosome loading. To be removed from chromatin, FANCD2 and FANCI have to be deubiquitinated by the DUB USP1 and its activator UAF. The D’Andrea laboratory has now created mice with homozygous USP1 deletions, and, importantly, these USP1-knockout mice display typical FA phenotypes, such as an increased sensitivity to DNA damage (Kim et al., 2009). Therefore, the addition and removal of ubiquitin from FANCD2/FANCI is equally important during DNA repair. These findings nicely emphasize the importance of dynamic ubiquitination for cellular signalling, which was a central theme of the meeting.

The ubiquitin–proteasome system as a target for drug discovery has been validated by the approval of proteasome inhibitors, such as bortezomib, for the treatment of multiple myeloma. How can our increased understanding of ubiquitination be translated into additional, potentially more specific, therapeutics? In the closing ‘Translational Lecture’, G. Draetta (Boston, MA, USA) addressed this question by describing the efforts leading to the discovery and development of bortezomib, and how it is now implemented in combination therapy. He then described the efforts undertaken at the Dana Farber Cancer Institute (Boston, MA, USA) to integrate basic science with drug discovery, encouraging scientists to endeavour in such projects. The multiple faces of ubiquitin, as highlighted by the symposium, certainly hold great promise for success.

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