Novel diubiquitin probes expand the chemical toolkit to study DUBs

Kulathu, Yogesh

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Expanding the chemical toolkit to study DUBs with novel diubiquitin probes

Yogesh Kulathu[1]*
[1]MRC Protein Phosphorylation and Ubiquitylation Unit, School of Life Sciences, University of Dundee, Dow Street, Dundee DD1 5EH, UK.
*Correspondence: ykulathu@dundee.ac.uk

Linkage-specific DUBs employ different mechanisms to recognize and cleave polyubiquitin chains of specific linkage types. In this issue of Cell Chemical Biology, Flierman et al. develop a new family of novel non-hydrolyzable diubiquitin probes that will be valuable tools to study how DUBs achieve specificity.

The posttranslational modification of proteins with one or several ubiquitin molecules (polyubiquitin) can alter the fate, activity, localization and protein-protein interactions of the modified protein. Polyubiquitin chains of 8 different linkage types can be generated and the type of linkage between the ubiquitins can determine the outcome of ubiquitylation. Thousands of cellular proteins are modified by ubiquitin and as a consequence ubiquitylation regulates a wide range of cellular processes in eukaryotes. Deubiquitinases (DUBs) are proteases that reverse this modification by hydrolysing the isopeptide bond between ubiquitin and the target protein, and thereby function as important regulators of ubiquitylation. How DUBs specifically recognize and hydrolyze different polyubiquitin modifications is poorly understood. In this issue, Flierman et al. describe novel ubiquitin probes that will be valuable tools to study how DUBs recognize ubiquitin chains of different linkage types.

There are ~100 DUBs encoded in the human genome that can be classified into five different families – Ubiquitin C-terminal Hydrolase (UCH), Ubiquitin Specific Proteases (USPs), Ovarian Tumor (OTU), Josephins and JAB1/MPDN+/MOV34 (JAMM). With the exception of JAMM family DUBs which are metallo-proteases, the remaining DUBs are thiol proteases. Ubiquitin probes or ubiquitin suicide probes as they are commonly referred to have been valuable tools in studying DUBs. These probes contain a thiol-reactive chemical group or warhead at the C-terminal end of ubiquitin that reacts with the active site cysteine of DUBs (**Figure 1**). Such probes have contributed greatly to the study of DUBs. For instance, several OTU family DUBs were discovered using these probes (Borodovsky et al., 2002). These probes have also been valuable tools to obtain crystal structures of DUBs in complex with ubiquitin at the distal or S1 site (Eletr and Wilkinson, 2014). However, these probes provide limited information on how DUBs process polyubiquitin chains.

In addition to cleaving ubiquitins off modified proteins, DUBs can also cleave between ubiquitin moieties within a polyubiquitin chain to edit the ubiquitin signal. Some of these DUBs display remarkable preference for cleaving polyubiquitin chains of certain linkage types. DUBs employ different strategies to recognize substrates (**Figure 1**). DUBs that rely only on interactions on the S1 site tend to be linkage promiscuous and will cleave polyubiquitin chains of all linkage types. In contrast, DUBs such as OTULIN and AMSH rely on additional interactions on the proximal S1' site to cleave Met1 and Lys63 ubiquitin chains, respectively in a linkage selective manner. To study these linkage-selective DUBs, diubiquitin-based probes carrying an electrophilic group between the two ubiquitins have been developed to capture the DUB with ubiquitins positioned in the S1 and S1' sites (**Figure 1D**) (McGouran et al., 2013; Mulder et al., 2014).

A third mechanism has recently been recognized for DUBs such as OTUD2 (Mevissen et al., 2013) and the coronavirus DUB SARS PLpro (Békés et al., 2015), which harbour an additional S2 binding site that enables these DUBs to bind to longer ubiquitin chains and cleave off ubiquitin at the proximal end. How ubiquitin binding away from the catalytic site at the S2 pocket determines substrate selectivity is poorly understood. This is because linkage-specificity in DUBs is generally investigated using qualitative gel-based cleavage assays using diubiquitin chains of different linkages as substrates (**Figure 1B**). Such assays and the available chemical probes do not provide any information about the role of the S2 site in driving linkage preferences in DUBs. To be able to study DUBs that use S2 pocket binding to modulate DUB activity and specificity, Flierman et al. design a set of novel probes based on non-hydrolyzable diubiquitin that target the S1 and S2 sites (**Figure 1E**).

The ubiquitins in the developed diubiquitin probes are connected via a non-hydrolyzable triazole linkage, a good mimic of the native isopeptide bond. Importantly, this toolset comprises diubiquitins of all 7 possible isopeptide linkage types to profile linkage specificity. When equipped with a highly reactive propargylamide warhead at the C-terminus and a fluorescent TAMRA label at the N-terminus these probes can be used to label DUBs in cell extracts, and such profiling reveals subsets of DUBs that are labelled by these probes in a linkage-specific manner. How many DUBs rely on S2 site interaction to fine tune their activity is unknown and these probes will be useful tools to identify them. Most USP DUBs will cleave diubiquitins of all linkage types and so they are largely thought of as linkage promiscuous. Whether some of the USP family DUBs use additional S2 site interactions to achieve some linkage selectivity towards longer chains can now be investigated using these probes.

The OTU family DUB OTUD2 contains an S2 site in addition to an S1 and S1’ site within its catalytic domain (Mevissen et al., 2013), and is mainly reactive towards K11-linked probes. To investigate the mechanisms in greater detail, Flierman et al. also develop diubiquitin fluorogenic substrates containing 7-amido-4-methylcoumarin (AMC) at the C-terminus to study kinetic parameters. The catalytic domain of OTUD2 shows a preference for cleaving K11-linked chains and in addition to the S1 and S2 binding pockets, OTUD2 uses S1’ interactions to achieve selectivity. OTUD3 is structurally similar to OTUD2 and also prefers K11 linked chains for the S1-S2 binding but in contrast to OTUD2, OTUD3 cleaves K6-linked diubiquitin bound across the S1 and S1’ sites. This suggests that OTUD3 may work on heterotypic chains containing K11 and K6 linkages.

From the first ubiquitin probes that were reported nearly 20 years ago, several new probes with improved design and application have been developed in recent years. These novel probes developed by Flierman et al. is an exciting addition to the existing toolkit that will allow us to study DUBs that use additional S2 site interactions. Mechanistic studies of DUBs are now possible thanks to these advances. At the same time, it also stresses the use of different probes to get a more complete picture of the mechanism used by a DUB. These nonhydrolyzable probes will also be invaluable tools for structural studies which will reveal the underlying molecular basis of how linkage specificity is driven by S2 site binding and whether S2 site interaction induces long range conformational changes in the catalytic site. These probes also lay the platform to design new probes to investigate S1, S2 and S1’ sites simultaneously (**Figure 1F**). One can envisage these probes to contain a non-hydrolyzable diubiquitin that binds at the S1-S2 sites and an electrophilic group between the S1 and S1’ ubiquitins to covalently trap the DUB.

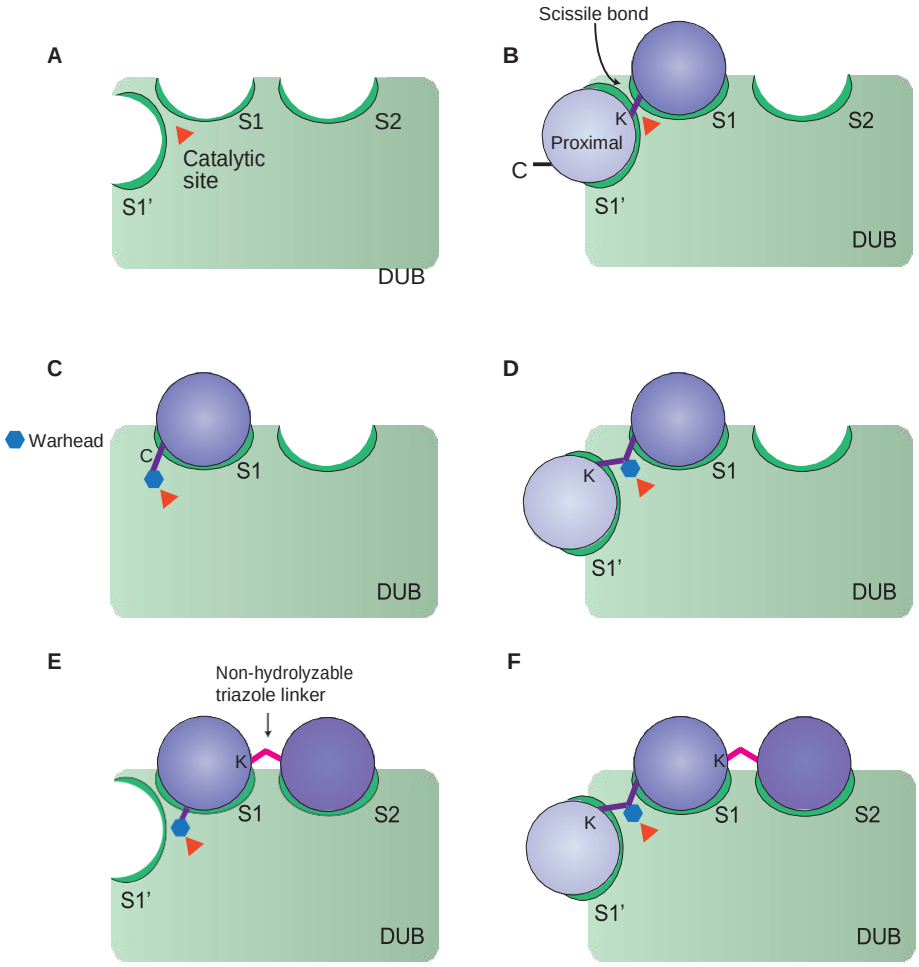

Figure Legends

(A) Schematic representation of the different ubiquitin binding sites in DUB catalytic domains discussed here: S1' (proximal), S1 (middle) and S2 (distal) of ubiquitin recognition employed by DUBs and the probes available to target them. (B) Mode of cleavage of diubiquitin substrates used in gel-based cleavage assays. (C) Monoubiquitin probe with a C-terminal reactive group targeting the active site cysteine. (D) Diubiquitin probes to capture S1-S1' interaction. (E) Non-hydrolyzable diubiquitin probes targeting the S1-S2 sites. (F) Model of probe to study putative S1, S2 and S1' sites in DUBs.

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