

Working out coupled monoubiquitination

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Ubiquitin receptors that bind ubiquitinated proteins through ubiquitin-binding domains have key roles in various cellular processes. These receptors are often themselves monoubiquitinated, referred to as coupled monoubiquitination. Now, coupled monoubiquitination has been shown to involve monoubiquitination of a ubiquitin ligase and its subsequent interaction with a ubiquitin receptor.

Protein ubiquitination — the covalent attachment of the small protein ubiquitin to a lysine residue in a substrate protein — occurs in a three-step process involving the sequential action of ubiquitin activating (E1), conjugating (E2) and ligase (E3) enzymes¹. Ubiquitination can involve a modification with a single ubiquitin (monoubiquitination), several single ubiquitin molecules (multiubiquitination) or the attachment of ubiquitin chains (polyubiquitination)². Because of this versatility, ubiquitination regulates a variety of cellular processes, including protein degradation, signal transduction, membrane traffic, DNA repair, chromatin remodelling, peroxisome biogenesis and viral budding^{3–5}. Although ubiquitination may regulate protein function through conformational changes, the most common mode of regulation by ubiquitination involves specific ubiquitin receptors that recognize the ubiquitinated protein and thereby control downstream biochemical processes. These ubiquitin receptors contain one or more conserved ubiquitin-binding domains (UBDs), of which more than a dozen have been identified to date^{6,7}.

Curiously, several ubiquitin receptors, including proteins that control endocytic membrane traffic, have been found to be monoubiquitinated themselves. This type of modification, known as coupled monoubiquitination, requires the presence of a UBD, and most known UBDs have been found to sustain this process^{7–11}. This striking finding has raised two questions: what is the function of coupled monoubiquitination, and how does it occur? Recent studies have addressed the first question: ubiquitin receptors that are monoubiquitinated engage in intramolecular interactions between monoubiquitin and their UBDs and in this way become autoinhibited⁸. Thus, coupled monoubiquitination

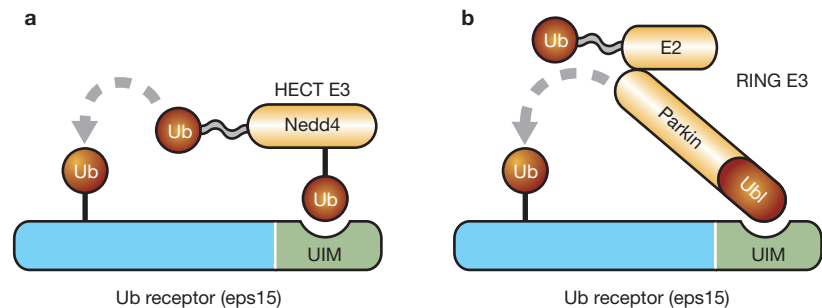


Figure 1 Schematic representation of two mechanisms for coupled monoubiquitination. **(a)** The E3–isopeptide model for coupled monoubiquitination. A monoubiquitinated HECT-type ubiquitin ligase (Nedd4) interacts with the ubiquitin-interacting motif (UIM) of the ubiquitin receptor (for example, eps15) and transfers thiolester-conjugated ubiquitin from itself to the ubiquitin receptor, which becomes monoubiquitinated. **(b)** In an analogous manner, the ubiquitin-like (Ubl) domain of a RING-type ubiquitin ligase (Parkin) interacts with the UIM of the ubiquitin receptor (for example, eps15) and mediates its monoubiquitination¹⁶ by transferring the ubiquitin from the E2 conjugating enzyme to the substrate. Although both HECT and RING ubiquitin ligases catalyse isopeptide bond formation (indicated in black) between ubiquitin and the target, HECT ubiquitin ligases form a catalytic intermediate wherein ubiquitin is linked through a thiolester bond (indicated in grey) to the active site cysteine of the ligase. In contrast, RING-type ubiquitin ligases transfer ubiquitin directly from the E2-conjugating enzyme to the substrate without an intervening catalytic intermediate.

is likely to exert negative-feedback control in molecular machineries that require transient and consecutive interactions between the ubiquitin receptors and their ligands. Endocytic membrane trafficking, which involves a complex machinery consisting of several ubiquitin receptors, represents a prominent example of such regulation. This mechanism also applies to DNA repair, where the localization of translesion polymerases in or out of replication factories is controlled by their ubiquitin-binding ability, as well as their monoubiquitination⁷. But what about the mechanistic basis for coupled monoubiquitination? On page 1246 of this issue, Woelk *et al.* demonstrate that monoubiquitination of a ubiquitin ligase, and its interaction with the UBD in a ubiquitin receptor, is essential for the receptor's coupled monoubiquitination¹².

The authors used the endocytic regulator protein eps15, which becomes monoubiquitinated on epidermal growth factor stimulation of cells, as a model to elucidate the molecular mechanisms of coupled monoubiquitination. Eps15 contains two ubiquitin-interacting

motifs (UIMs) and coupled monoubiquitination of eps15 depends on the second UIM; however, the ubiquitination site is not within this region¹¹. By performing scanning alanine mutagenesis of the UIM, the authors identified key amino acids in the UIM that are required for coupled monoubiquitination. They found a strong correlation between the ubiquitin-binding ability of the UIM and coupled monoubiquitination, and concluded that an intact UIM–ubiquitin interaction is required for monoubiquitination of eps15.

Several models have been proposed in which the ubiquitin receptors recruit the ubiquitination machinery to promote coupled monoubiquitination^{5,6}. As binding of the UIM to ubiquitin is required for coupled monoubiquitination, Woelk *et al.* reasoned that the UIM of eps15 may bind ubiquitin attached to an E3 ligase, which would then monoubiquitinate eps15. Eps15 becomes ubiquitinated by the HECT domain E3 ligase, Nedd4 (ref. 11). HECT-family E3s form a transient thiolester bond with ubiquitin that is then transferred to the substrate. Several E3s, including Nedd4,

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are known to be modified with mono- and polyubiquitin themselves^{12,13}. To test their hypothesis, Woelk *et al.* used an *in vitro* ubiquitination system in which the substrate eps15 was added together with E1, E2 and E3 (Nedd4) enzymes and ubiquitin. Step-by-step, they could exclude a number of possible models, until only two remained. In the first model, called “the E3–thiolester model”, the UIM would bind to the ubiquitin linked through a thiolester to the catalytic residue of Nedd4, followed by Nedd4-mediated transfer of ubiquitin to eps15. In the second model, called “the E3–isopeptide model”, Nedd4 would itself be covalently modified with ubiquitin and the UIM would interact with the monoubiquitin modifying Nedd4, with subsequent transfer of a thiolester-linked ubiquitin from Nedd4 to eps15 (Fig. 1a). A key experiment to distinguish between these two models demonstrated that if Nedd4 was allowed to undergo isopeptide-linked monoubiquitination before addition of eps15 to the reaction mixture, the coupled monoubiquitination of eps15 was considerably enhanced. In contrast, the E3–thiolester model predicts that monoubiquitination of eps15 would have been unchanged under these conditions. On the basis of this, and other results that favour the E3–isopeptide model, they concluded that coupled monoubiquitination of eps15 is mediated by monoubiquitinated Nedd4, and is dependent on the interaction between the UIM of eps15 and monoubiquitin that is covalently attached to Nedd4 (Fig. 1a).

It remains to be determined whether eps15 undergoes exclusively monoubiquitination under these conditions, and to what extent polyubiquitinated Nedd4 may contribute to the coupled monoubiquitination of eps15. Woelck *et al.* also showed that a subset of other HECT E3 family members that undergo monoubiquitination enhance coupled monoubiquitination of eps15, whereas non-ubiquitinated HECT family members were unable to do so. These data demonstrate that E3 ubiquitination is required for coupled monoubiquitination of eps15 *in vivo* and provide a mechanistic explanation for why an intact eps15 UIM is necessary for this process.

This observation raises the question of whether similar mechanisms apply for coupled monoubiquitination of UIM-containing proteins other than eps15, and for monoubiquitination of UBD-containing proteins, in general. Interestingly, HECT-type ubiquitin ligases have been implicated in

coupled monoubiquitination of other ubiquitin-binding proteins. For instance, AIP4 and Nedd4 mediate monoubiquitination of the UIM-containing protein Hrs^{9,14}, and yeast Rsp5 monoubiquitinates the CUE (coupling of ubiquitin conjugation to ER degradation; a UBD)-domain-containing protein Vps9 (ref. 15). In addition, a recent report showed that the RING-type ubiquitin ligase Parkin mediates monoubiquitination of eps15 (Fig. 1b). Parkin directly interacts with the UIM domain of eps15 through its ubiquitin-like (Ubl) domain¹⁶. Thus, present knowledge indicates that monoubiquitination of UIM-containing proteins can be performed by both HECT- and RING-type E3 ubiquitin ligases by distinct mechanisms that involve interaction with monoubiquitin inducibly attached to an E3 ligase (Fig. 1a) or a Ubl domain present in the E3 ligase (Fig. 1b). Therefore, the dynamic regulation of this process, as well as the contributions and redundancy of different E3 ligases in monoubiquitinating eps15 and other UBD-containing proteins, warrant further investigation.

Another outstanding question is, what determines the specificity of the interaction between the ubiquitin ligase and the UIM-containing protein? Traditionally, E3 ligases are known to mediate the specificity of the ubiquitination reaction by directly interacting with the substrate¹. According to the E3–isopeptide model, however, any ubiquitin-binding protein could, in principle, become monoubiquitinated by any monoubiquitinated ubiquitin ligase. In the case of eps15, the authors found that Nedd4 efficiently promoted the coupled ubiquitination more than any other monoubiquitinated HECT E3 ligases tested. Therefore, it is likely that there are additional determinants that ensure the specificity and efficiency of the reaction, such as additional binding surfaces between the ligase and the substrate, other than the covalently attached ubiquitin on the E3 ligase.

An important and unanswered question about the mechanism of coupled monoubiquitination is: why do ubiquitin receptors tend to be monoubiquitinated rather than polyubiquitinated? Is this a characteristic of the E2–E3 complex or do UBDs block the formation of a polyubiquitin chain once the first ubiquitin has been attached? In support of the second scenario, structural data from UBDs in complex with ubiquitin suggest that they indeed partially mask the lysines important for ubiquitin chain formation^{2,6}. The recent discovery

of intramolecular interactions between the UBD and attached monoubiquitin in the same protein also supports this model⁸. Indeed, an intramolecular interaction between the UIM and covalently attached ubiquitin has been shown to restrict ubiquitin chain extension on the transcription factor Met4 by shielding the terminal ubiquitin molecule in the chain¹⁷. Met4 is normally modified with a polyubiquitin chain of about four ubiquitin molecules, and the intramolecular interaction protects it from the otherwise expected interaction with, and degradation in, the proteasome. Prevention of the intramolecular interaction, on the other hand, leads to further chain elongation and subsequent proteasomal recognition and degradation of Met4 (ref. 17). It will be exciting to determine whether protection by the UBD broadly specifies monoubiquitination or ubiquitin chain length of ubiquitin-binding proteins. Additionally, the mechanisms that determine monoubiquitination in general need to be defined, as proteins that seemingly do not have UBDs can also be monoubiquitinated^{4,18}.

These new findings have made an important contribution to our understanding of the biochemical basis for coupled monoubiquitination. However, it remains to be determined whether this is a general mechanism *in vivo* and/or whether there are additional mechanisms. Future challenges will include investigating the spatial and temporal requirements, and functions of coupled monoubiquitination *in vivo*. □

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