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CELL SIGNALING:

Anchors Away for Ubiquitin Chains

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It has become increasingly clear that cellular proteins can become conjugated with chains of the small protein ubiquitin without becoming degraded by the proteasome. This "noncanonical" process allows ubiquitinated proteins to regulate numerous processes including DNA repair and cell signaling. However, determining the mechanisms by which polyubiquitin chains affect such processes has been challenging, in part because of the unavailability of genetically engineered mice that lack ubiquitin and the partial redundancies of molecules that support ubiquitin chain assembly. Recent work now shows that rather than being assembled onto target proteins by the cell's ubiquitin conjugation machinery, ubiquitin is also present in cells as free, unanchored chains that can bind directly to a signaling protein involved in the immune response to viral infection (1). This raises the question of whether unanchored ubiquitin chains generally regulate other physiological functions.

The retinoic acid–inducible gene–I (RIG-I)–like family of cytosolic pattern recognition receptors play an essential role in initiating the host antiviral response by detecting nucleic acids derived from viral genomes. Upon binding to viral RNAs that bear a 5′-triphosphate, RIG-I signals to the transcription factor interferon regulatory factor 3 (IRF3), which upon activation elicits an antiviral program to restrict virus replication and spread. RIG-I signaling to IRF3 is mediated by mitochondria antiviral signaling protein (MAVS; also known as IPS-1, VISA, and Cardif). MAVS further recruits adaptor proteins and enzymes (TBK1 and IKKi) to phosphorylate IRF3, ultimately resulting in dimerization of the transcription factor and subsequent nuclear translocation. Zeng *et al*. (2) previously showed that noncanonical protein ubiquitination is essential for activating IRF3 via MAVS, but the connection between these events was not clear. Using an in vitro cell-free system, Zeng *et al*. (1) recapitulated RIG-I–IRF3 signaling, showing that virally activated RIG-I, together with a mitochondrial fraction (containing MAVS) and cytosolic extracts from cells (containing TBK1 and IKKi kinases), support IRF3 activation. They further demonstrated in this system that the binding of a viral RNA mimic to purified RIG-I was not sufficient to activate IRF3, but also required ubiquitination of RIG-I. The latter event required the E3 ubiquitin ligase TRIM25, an enzyme that functions in the cell's ubiquitin conjugation (Ubc) mechanism. TRIM25 was previously shown to potentiate RIG-I signaling by promoting its ubiquitination (3).

Two caspase recruitment domains (CARDs) at the amino terminus of RIG-I [RIG-I(N)] are necessary and sufficient to activate IRF3 independently of viral RNA (4). Because these domains are also the target of polyubiquitin chain addition (catalyzed by TRIM25) to stimulate RIG-I signaling, Zeng *et al*. examined constitutively active RIG-I(N) to tease out how RIG-I activation signals to MAVS in a manner that depends on ubiquitin. In agreement with previous studies (3), RIG-I(N) required ubiquitination to activate IRF3 in the in vitro cell-free system. The ubiquitin moiety consists of 76 amino acids, including 7 lysine residues. Chains of ubiquitin may arise through linkage at the lysine residue in position 63

of the polypeptide (Lys^{63}). When the authors incubated purified RIG-I(N) with preassembled Lys^{63}-linked polyubiquitin chains, activation of IRF3 was achieved, which suggested that RIG-I(N) did not require assembly of a ubiquitin chain onto it by the cellular conjugation machinery, but direct ubiquitin chain binding instead to activate IRF3. Thus, the CARDs of RIG-I serve as ubiquitin sensors that recognize unanchored Lys^{63}-linked polyubiquitin chains synthesized by TRIM25.

Zeng *et al*. (1) also found that viral RNA binding was a required prerequisite for RIG-I binding to unanchored polyubiquitin chains, supporting a two-step model in which viral RNA binding to the regulatory domain of RIG-I promotes a conformational change that allows unanchored polyubiquitin chains to bind to the CARDs (see the figure). Conceivably, RIG-I binding to ubiquitin may also control interactions with other effectors such as stimulator of interferon genes (STING), an endoplasmic reticulum protein that binds to both RIG-I and MAVS (5).

The powerful nature of the cell-free system employed by Zeng *et al*. (1) establishes a potent tool for analyzing cellular signaling. However, the authors noted some curious findings. Although the polyubiquitin chains can assemble onto RIG-I through the ubiquitin conjugation machinery, the reason for modification through this route is unclear, as it is dispensable for IRF3 activation. One possibility is that the polyubiquitin chains anchored onto RIG-I through the conjugation system somehow mark RIG-I for negative feedback regulation by recruiting deubiquitinases for signal termination (6).

Zeng *et al*. (1) also noted that the CARDs of either MAVS or the intracellular pattern recognition receptor NOD2 do not bind ubiquitin. Because NOD2 also uses MAVS to activate IRF3 in response to viral RNA (7), it is of interest to determine why ubiquitin regulates the RIG-I–MAVS signaling pathway but not the NOD2-MAVS pathway in response to viral infection. The authors, however, did observe that the CARDs of the RIG-I homolog MDA5 bind directly to polyubiquitin chains, which suggests that MDA5 and RIG-I may be regulated in a similar manner (1). A next step is to identify whether TRIM25 or another ubiquitin ligase synthesizes the unanchored polyubiquitin chains for MDA5.

TRIM25 has also been implicated in modifying proteins with the ubiquitin-like protein ISG15 (8). Conjugation of ISG15 onto RIG-I inhibits its signaling through a negative feedback loop (9). Thus, a more refined analysis is needed to understand the complex role of TRIM25 in antiviral signaling, perhaps achieved with a modified in vitro system containing both ubiquitination and ISGylation conjugation systems.

Do unanchored polyubiquitin chains play a pervasive role in regulating many signaling pathways? Indeed, Xia *et al*. recently reported that unanchored Lys^{63}-linked polyubiquitin chains play an important role in activating the TAK1 and IKK kinases in the NF-κB signaling pathway (10). Zeng *et al*. (1) estimate approximately 6000 molecules of unanchored Lys^{63}-linked polyubiquitin chains per cell (the authors studied a human embryonic kidney cell line), although RIG-I may sense as few as 15 molecules of unanchored polyubiquitin chains. It is likely that the number of unanchored poly ubiquitin chains in a given cell is determined by the balance of ubiquitin ligases and deubiquitinases, which in turn is regulated by specific signaling pathways. Unanchored polyubiquitin chains may also be regulated by localization and compartmentalization within the cell, adding yet another dimension of complexity.

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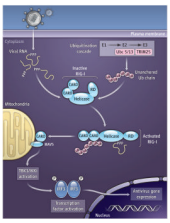

Figure 1. Unanchored in defense

During virus infection, viral RNA is detected by the regulatory domain (RD) of RIG-I, triggering a conformational change that allows an open conformation. The CARDs of RIG-I bind to unanchored Lys^{63}-linked polyubiquitin (Ub) chains. This facilitates RIG-I interaction with MAVS protein in the mitochondria and subsequent signaling to IRF3, which controls the expression of antiviral genes.