INNATE IMMUNITY

A chain reaction

New research published in *Cell* adds to the evidence indicating a crucial role for protein ubiquitylation in regulating immune responses. But in this example, free polyubiquitin chains in the cytoplasm — rather than the direct ubiquitylation of immune factors — are important for activating innate antiviral immunity. Chen and colleagues show that free polyubiquitin chains are an endogenous ligand of the innate pattern recognition receptor for viral RNA known as retinoic acid-inducible gene I (RIG-I).

RIG-I binds 5′-triphosphate viral RNA and signals through mitochondrial antiviral signalling protein (MAVS), TANK-binding kinase 1 (TBK1) and the transcription factor interferon regulatory factor 3 (IRF3) to induce the expression of type I interferons (IFNs). Several ubiquitin ligases have been shown to regulate this pathway, but the precise mechanism was unclear.

The authors set up a cell-free system to investigate the RIG-I pathway in more detail: when RIG-I was purified from HEK293 T cells infected with Sendai virus and mixed with mitochondria (containing MAVS) and cytoplasmic extracts (containing TBK1) from uninfected cells, together with *in vitro* synthesized IRF3, IRF3 activation (as indicated by its dimerization) was observed. However, when RIG-I was purified from infected cells lacking the ubiquitin E3 ligase TRIM25 or when RIG-I from uninfected cells was incubated with ATP and 5′-triphosphate RNA in the absence of ubiquitin ligases, IRF3 dimerization did not occur. So in this cell-free system, as *in vivo*, ubiquitylation is required for RIG-I signalling.

Activation of the RIG-I signalling pathway by RNA and ATP *in vitro* required TRIM25 and the E2 ligases UBC5 and UBC13, which are known to specifically synthesize lysine 63 (K63)-linked polyubiquitin chains. Ubiquitin proteins wih a lysine substitution at K63 were inactive in the cell-free system. Previous studies have shown that RIG-I can undergo K63-linked ubiquitylation at K172 and that K172R mutation of RIG-I impairs the induction of type I IFNs. But in the cell-free system, RIG-I with intact K172 but mutated lysine residues at five other positions could activate IRF3 despite being defective for ubiquitylation. In addition, deubiquitylation of RIG-I did not affect IRF3 activation. These results indicate that ubiquitylation of RIG-I itself is not required for signalling.

Instead, the authors showed that the amino-terminal portion of RIG-I can activate IRF3 even in the absence of RNA, dependent on the presence of ubiquitylation components. This involved the synthesis of free K63-linked polyubiquitin chains that were shown to bind to the N-terminal caspase-recruitment domains (CARDs) of RIG-I but did not require ubiquitylation of RIG-I itself. K63-linked polyubiquitin chains containing more than two ubiquitin moieties, but not ubiquitin monomers or K48-linked chains, could potently activate the N-terminal portion of RIG-I *in vitro*. Importantly, endogenous unanchored K63-linked polyubiquitin chains can be isolated from human cells and they potently stimulate RIG-I. Binding of K63-linked polyubiquitin chains to full-length RIG-I required both RNA and the ATPase activity of RIG-I; RNA binding was required to precede polyubiquitin binding for RIG-I activation.

The authors therefore suggest a two-step model of RIG-I activation in which RNA binding activates the ATPase activity of RIG-I and induces a conformational change that exposes the N-terminal CARDs for polyubiquitin binding. This results in additional conformational changes and/or oligomerization, which activates downstream signalling through MAVS.

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ORIGINAL RESEARCH PAPER Zeng, W. *et al.* Reconstitution of the RIG-I pathway reveals a signaling role of unanchored polyubiquitin chains in innate immunity. *Cell* **141**, 315–330 (2010)