A feedforward loop of NLRC5 (de)ubiquitination keeps IKK–NF-κB in check

Yinling Hu

Cancer and Inflammation Program, Center for Cancer Research, National Cancer Institute, Frederick, MD 21701

Many receptors signal via adaptors to the IKK–NF-κB axis, transducing extracellular cues to transcriptional regulation. In this issue, Meng et al. (2015. J. Cell Biol. http://dx.doi.org/10.1083/jcb.201505091) reveal that the IKK regulator NLRC5 shapes NF-κB activity through a feedforward loop of NLRC5 ubiquitination and deubiquitination, highlighting a new pathway modulating IKK–NF-κB activity.

NF-κB is required for lymphoid cell and organ development, innate and adaptive immunity, and cell survival. The NF-κB family contains several members, which form heterodimers or homodimers that execute their functions as transcriptional factors in the nucleus. Regulation of NF-κB activation is crucial to many biological functions, and dysregulation of NF-κB activity has been involved in the pathogenesis of immune deficiency, infectious diseases, inflammation, and cancer (Liu et al., 2006; Pannicke et al., 2013). The IKK complex is central to the regulation of NF-κB activity (Ghosh and Karin, 2002). It is composed of the kinases IKKα and IKKβ, as well as of the regulatory subunit IKKγ (NEMO). After the activation of many receptors, IKK phosphorylates IκB, which physically retains NF-κB in the cytoplasmic compartment. This phosphorylation induces IκB degradation through the ubiquitin-proteasome machinery, allowing NF-κB translocation to the nucleus and its activation to regulate gene expression.

Multiple receptors, including TNFRs, IL-1R, Toll-like receptors (TLRs), nod-like receptors (NLRs or NLRCs), RIG-like receptors (RLRs), TCRs, and BCRs, depend on the IKK–NF-κB pathway to transduce extracellular signals to the transcriptional machinery. It is known that such diverse and important receptors require IKK–NF-κB; however, the regulatory mechanisms by which distinct receptors signal to IKK in different cell types are not fully understood. NLRs, TLRs, and RLRs specifically recognize pathogen-associated molecules, which provide the innate immune response as the first line of defense against invading microbes (Akira et al., 2001). NLRs are intracellular pattern-recognition receptors that feature a central nucleotide-binding and oligomerization domain. Although they were originally thought to initiate inflammasome formation, recent works showed that several NLR family members, including NLRC5, negatively regulate TLR and RLR signaling (Benko et al., 2010; Cui et al., 2010). Indeed, Cui et al., 2010 previously revealed that NLRC5 negatively regulates the NF-κB pathway by blocking the phosphorylation of IKKα and IKKβ in LPS-stimulated, TLR4-activated signaling in macrophages. In this process, NLRC5 competes with IKKγ to bind to IKKα and IKKβ, thereby inhibiting IKK and NF-κB activity. The exact mechanism by which NLRC5 regulates IKK and NF-κB activation and its regulation needs to be further investigated.

In this issue, Meng et al. build a mathematical model based on the competition for IKKβ binding between NLRC5 and IKKγ–NEMO to predict the role of NLRC5 in NF-κB signaling upon LPS stimulation. Interestingly, they observed that the experimental temporal dynamics of IKK–NLRC5 complex formation did not exactly overlap with the prediction from the mathematical model, suggesting that modulation of the NLRC5–IKK interaction, other than that based on competitive binding, might exist. The authors used coimmunoprecipitation analyses in HEK 293T cells expressing TLR4 and other cell types to delineate whether NLRC5 undergoes posttranslational modification after TLR4 activation. Meng et al. (2015) discovered that NLRC5 is ubiquitinated with K63 linkage. Remarkably, the levels of ubiquitinated NLRC5 inversely correlated to the levels of IKKβ–NLRC5 complex formation, suggesting that modification of NLRC5 affects its binding to IKKβ, which the authors confirmed in silico based on their initial model. Indeed, the researchers found that treatment with LPS recruits E3 ligases and NF-κB signaling adaptors TRAF2/6 into the complex with NLRC5 and IKKβ. SiRNA-mediated knockdown of TRAF2 or TRAF6 abolished NLRC5 polyubiquitination and increased the interaction of NLRC5 with IKKβ after LPS treatment. The authors therefore propose that NLRC5 is targeted for degradation through K63-linked ubiquitination at a specific site mapped by analyzing truncated constructs, i.e., lysine 1178. These results also suggest that TRAF2/6-mediated NLRC5 degradation removes the IKK–NF-κB negative regulator NLRC5, which allows IKKγ to replace NLRC5 in the complex with IKKα and IKKβ to activate NF-κB.

Interestingly, the in silico analyses by Meng et al. (2015) also suggested that deubiquitination of NLRC5 might be involved in NF-κB signaling regulation by restoring the pool of unmodified NLRC5. The authors tested various ubiquitin-specific proteases (USPs), which belong to a subclass of deubiquitinases (DUBs), for their ability to bind NLRC5 and enhance its interaction with IKKβ in vitro. They show that three USPs—USP14, USP18, and USP22—fulfilled these criteria, but focused on USP14, as USP18 and USP22 could also inhibit IKKβ activation directly in the absence of NLRC5. The
Despite its thorough characterization of NLRC5 modification, this work does not further dissect the downstream partners and effectors of NLRC5 necessary for NF-κB activation, and in particular, it raises questions as to which IKK subunit is required to mediate its effects. Indeed, treatment with certain stimuli such as TNF, which activates TNFR1, induces the phosphorylation of both IKKα and IKKβ (Xia et al., 2013), whereas LPS stimulation, which activates TLR4, mainly induces phosphorylation of IKKβ, but not of IKKα (Cui et al., 2010; Meng et al., 2015). These results suggest that the mechanism connecting TLR4 to the IKK complex differs from that linking TNFR1 to the same complex. Tools are available to investigate this possibility, as, for instance, Western blotting allows the separation of IKKα, IKKβ, and their phosphorylated forms thanks to their different molecular weights, although the antibody used in this work to identify phosphorylated IKK recognizes both IKKα and IKKβ. In addition, other experimental set-ups may limit the interpretation and robustness of the conclusions, including artificial effects of IKKα and IKKβ ectopic overexpression, which might mask cell type–specific phenotypes. Lastly, several lines of evidence point to different biological activities for IKKα and IKKβ, including their different knockdown phenotypes and the differences in which molecules or pathways can rescue them (Pasparakis et al., 2002; Liu et al., 2008). For example, in T cells, deletion of IKKβ, but not of IKKα, causes apoptosis, reducing T cell numbers (Sentftleben et al., 2001; Schmidt-Supprian et al., 2003; Balkhi et al., 2012; Chen et al., 2015). Similarly, Tnfr1 knockout rescues the lethality of Ikkβ−/− mice and the skin phenotype of mice lacking IKKβ in their keratinocytes (Pasparakis et al., 2002), but it neither rescues the lethality of Ikkα−/− mice nor the skin phenotype of mice lacking IKKα in their keratinocytes (Li et al., 1999a; Liu et al., 2008). IKKα-inducible deletion in keratinocytes is known to cause spontaneous skin tumors, and it can be rescued in the Egfr heterozygous (Egfr+/−) genetic background (Liu et al., 2008). The phenotype of IKKβ deletion on skin tumorigenesis remains to be examined, but keratinocyte-specific deletion of p65, a major NF-κB target for IKKβ, inhibits chemical carcinogen-induced skin carcinogene-

![Diagram of NLRC5 and IKK interactions](image-url)
sis (Kim and Pasparakis, 2014), which stands in contrast to the IKKα deletion phenotype. As dysregulation of IKKα and IKKβ has been implicated in several human diseases, it will be important to identify the differences in their biological functions and downstream signaling to design targeted therapeutics.

Regulation and timely termination of the immune responses triggered by NLRs, TLRs, and RLRs are crucial to prevent inflammation-associated diseases. Understanding the dynamic control of these pathways is therefore necessary to produce adequate therapeutics for inflammation-induced pathologies. Through computational and experimental approaches, this work takes an important step in delineating the temporal and dynamic modulation of NF-κB signaling, as well as its cell type specificity. The reversible ubiquitination of NLR5 shapes NF-κB activation by allowing efficient activation and termination of innate immune signaling; by creating a feedforward loop that sets a threshold for robust innate immune responses; and, lastly, by altering the cellular sensitivity to NLR5 ablation. This work opens the door to the future investigation of other dynamic modes of regulation of these pathways and of the impact of the intracellular (de)ubiquitination environment on inflammatory responses.

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