Adding to the STING

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STING (also known as MITA) is a central component in innate immunity against DNA virus. In this issue of *Immunity*, Wang et al. (2014) demonstrate that K27-linked polyubiquitination of STING (MITA) by the ER-associated E3 ligase AMFR is essential for STING (MITA)-mediated signaling and innate antiviral response.

Innate immune response provides the first line of host defense against invading pathogens and endogenous danger signals. Sensing of pathogen-derived nucleic acids via pattern-recognition receptors (PRRs) is a general strategy used by host cells to detect invading pathogens, which initiates a series of cellular signaling events and ultimately induces the production of type I interferons (IFNs), proinflammatory cytokines, and other antimicrobial effector proteins. Studies during the past decade have led to considerable advances in the mechanisms of viral RNA-triggered innate immune signaling. However, our understanding of innate immune response against exogenous DNA derived from invading pathogens and endogenous aberrant self-DNA is relatively limited. In the past years, several proteins have been reported to recognize microbial DNA, including TLR9, AIM2, DAI, RNA polymerase III, IFI16, DDX41, and LSm14A. Various studies have demonstrated that these proteins are important for innate immune responses against various DNA pathogens in particular cell or mouse models. However, it has been demonstrated that these proteins are not universally required for detecting microbial DNA in distinct cell types or in vivo. Recently, a nucleotidyltransferase family member, called cyclic GMP-AMP (cGAMP) synthase (cGAS), has been identified to detect cytosolic microbial or endogenous aberrant DNA in various cell types. Gene-deletion studies demonstrate that cGAS is essential for innate immune response against various DNA viruses in cells and mice, suggesting that cGAS is a widely used viral DNA sensor (Cai et al., 2014).

Although the cytosolic DNA sensors seem very divergent, the downstream signaling components are largely conserved. Various studies have established a central role for the adaptor protein STING, also known as MITA, in innate immune response against DNA viruses or bacteria (Ishikawa and Barber, 2008; Zhong et al., 2008). STING (MITA) is a transmembrane protein localized in the ER, mitochondria, and mitochondrial-associated membrane. Upon binding of cytoplasmic DNA, cGAS catalyzes the formation of the second messenger molecule cGAMP, which subsequently binds to STING (MITA). STING (MITA) then traffics from the endoplasmic reticulum (ER) to the Golgi apparatus and further to the perinuclear microsomes or punctuate structures, which in turn recruit the downstream kinase TBK1 and the transcription factor IRF3, leading to induction of type I IFNs. However, it remains unclear how STING (MITA) signals to TBK1. In this issue of *Immunity*, Wang et al. (2014) provide mechanistic insights into the modulation of STING (MITA)-TBK1 axis during DNA virus infection. They found that the ER-associated E3 ligase AMFR (also called gp78) mediated K27-linked polyubiquitination of STING (MITA) in response to viral or bacterial DNA. These K27-linked polyubiquitin chains then serve as a scaffold to recruit downstream TBK1 kinase, leading to efficient innate immune response against DNA virus.

To identify regulatory proteins of STING (MITA)-mediated signaling, Wang et al. (2014) performed biochemical purification experiments and identified AMFR as a specific component of the STING (MITA)-associated complex following infection with the DNA virus HSV-1. Functional analysis showed that depletion of AMFR impaired type I IFN production induced by cytosolic DNA and inhibited innate immune response to HSV-1 or *Listeria monocytogenes* infection. Biochemical experiments with various ubiquitin mutants indicated that AMFR acted as an ER-associated E3 ligase, which catalyzed K27-linked polyubiquitination of STING (MITA) in vitro and in mammalian cells. Interestingly, polyubiquitination of STING (MITA) induced by HSV-1 infection for 6–8 hr was dramatically impaired in *Amfr*$^{-/-}$ cells in comparison to their wild-type counterparts, suggesting that AMFR-mediated K27-linked polyubiquitination is a major form of polyubiquitination modifications for STING (MITA) following DNA virus infection, at least at the examined time points after infection. Using synthesized polyubiquitin chains, Wang et al. (2014) clearly demonstrated that K27- but not K63-linked diubiquitin chains directly interacted with the ubiquitin-like domain (ULD) of TBK1, which has previously demonstrated to be essential for TBK1 activation. Coimmunoprecipitation experiments indicated that recruitment of TBK1 to STING (MITA) following HSV-1 infection was impaired in *Amfr*$^{-/-}$ cells following HSV-1 infection. Very interestingly, HSV-1-induced translocation of TBK1 to perinuclear microsomes or punctuate structures was also impaired in *Amfr*$^{-/-}$ cells. Taken together, these results suggest that AMFR-mediated K27-linked polyubiquitination of STING (MITA) provides a crucial link for recruitment of TBK1 and activation of IRF3.

Previously, several E3 ligases have been reported to ubiquitinate STING (MITA). The E3 ligase RNF5 catalyzes K48-linked polyubiquitination of STING (MITA) at the mitochondria following viral infection, and this negatively regulates innate antiviral response (Zhong et al., 2009). Conversely, TRIM32 and TRIM56 are demonstrated to catalyze K63-linked polyubiquitination of STING (MITA) and promote recruitment of TBK1 to STING

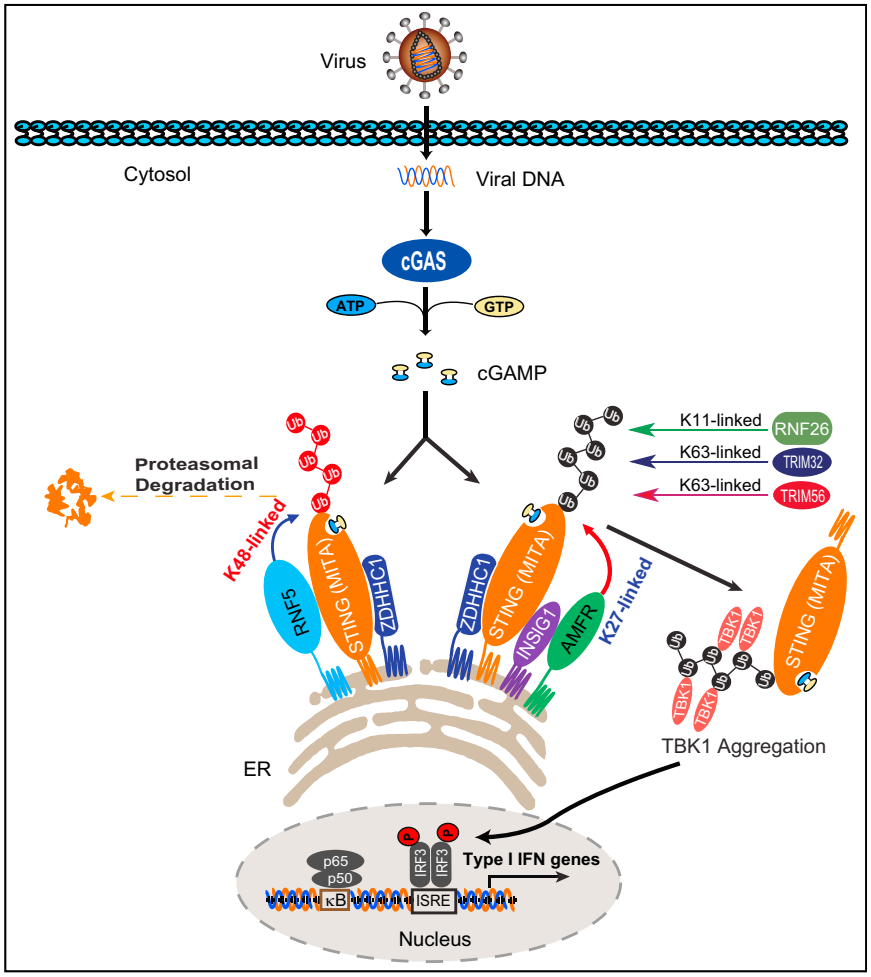

Figure 1. A Simplified Model on the Role of K27-Linked Polyubiquitination of STING (MITA) by the ER-Associated E3 Ligase AMFR in Innate Antiviral Response

The cytosolic sensor cGAS detects microbial DNA and catalyzes synthesis of secondary messenger cGAMP. cGAMP binds to STING (MITA) which is accompanied by the ER-associated modulator ZDHHC1, resulting in the recruitment of AMFR through INSIG. AMFR catalyzes K27-linked polyubiquitination of STING (MITA). Such K27-linked polyubiquitin chains serve as a platform to recruit TBK1, leading to activation of IRF3 and induction of type I IFNs. K11- and K63-linked polyubiquitination of STING (MITA) by RNF26, TRIM32, and TRIM56, respectively, might also contribute to recruitment and activation of TBK1. K48-linked polyubiquitination of STING (MITA) by RNF5 results in degradation of STING (MITA) and therefore negatively regulates STING (MITA)-mediated signaling.

(MITA), thereby positively regulating innate antiviral response (Tsuchida et al., 2010; Zhang et al., 2012). More recently, it has been shown that RNF26 plays an important role in innate antiviral response by catalyzing K11-linked and competing for K48-linked polyubiquitination of STING (MITA) (Qin et al., 2014). Mutagenesis has demonstrated that these E3 ligases target both overlapping and distinct lysine residues of STING (MITA). All these E3 ligases target K150 of STING (MITA). Additionally, AMFR also targets K137, K224, and K236, whereas TRIM32 also targets K20, K224, and K236. Interestingly, it has been demonstrated that RNF26 competes with RNF5 for targeting K150 of STING (MITA) with different linkage types of polyubiquitin chains. How STING (MITA) is coordinated and delicately regulated by these E3 ligases needs more investigation. These E3 ligases might be coordinated to regulate virus-triggered induction of type I IFNs in a spatial and temporal manner. It is also possible that some E3 ligases, such as AMFR, RNF26, TRIM32, and TRIM56, play redundant roles in STING (MITA)-mediated signaling through distinct mechanisms, or they function in a cell-type- and virus-class-specific context. In contradiction to previous reports (Tsuchida et al., 2010; Zhang et al., 2012), Wang et al. (2014) failed to detect polyubiquitination of STING (MITA) by TRIM32 and TRIM56 in mammalian overexpression system, though they did not further examine whether endogenous STING (MITA) is polyubiquitinated by TRIM32 and TRIM56 following viral infection. More rigorous studies are needed to resolve this discrepancy.

Previously, it has been shown that AMFR promotes the sterol-triggered degradation of the HMG-CoA reductase via binding to the ER membrane protein INSIG1 (Goldstein et al., 2006). Wang et al. (2014) found that INSIG1 was important for the association of AMFR and STING (MITA) following HSV-1 infection, suggesting that INSIG1 links AMFR to STING (MITA) following viral infection. Importantly, HSV-1-induced polyubiquitination of STING (MITA) as well as recruitment of TBK1 to STING (MITA) and translocation of TBK1 to the perinuclear microsomes or punctuate structures were impaired in *Insig*$^{-/-}$ cells. Consistently, *Insig1*$^{-/-}$ mice exhibited decreased IFN-β production and increased susceptibility to HSV-1 infection. These results support that the INSIG1-AMFR complex is important for innate immune response against DNA viruses. In similar experiments, Wang et al. (2014) found that both INSIG1 and AMFR were not required for Sendai virus-induced signaling, suggesting that the INSIG1-AMFR complex is specifically involved in innate immune response against DNA but not RNA viruses. Because previous studies have established that the INSIG1-AMFR complex is critically involved in cellular metabolism by mediating sterol-dependent degradation of HMG CoA reductase to suppress cholesterol biosynthesis (Goldstein et al., 2006), one intriguing question raised from the current study by Wang et al. (2014) is whether the INSIG1-AMFR complex serves as a link between innate immunity and metabolism.

In conclusion, the current study by Wang et al. (2014) provides an important link for understanding the molecular mechanisms of STING (MITA)-mediated innate immune response. On the basis of their results and previous published studies, we can draw a simplified model on STING (MITA)-mediated innate

immune response against cytosolic DNA (Figure 1). In this model, microbial DNA is detected by cGAS, which catalyzes synthesis of cGAMP from GTP and ATP. cGAMP binds to STING (MITA), which is also associated with an ER-associated modulator called ZDHHC1 (Zhou et al., 2014). At this point, AMFR is recruited to STING (MITA) by INSIG1, and then catalyzes K27-linked polyubiquitination of STING (MITA). The K27-linked polyubiquitin chains attached to STING (MITA) provide platforms for recruitment of TBK1, leading to activation of IRF3 and eventual induction of type I IFNs. In addition to AMFR, other E3 ligases, including RNF26, TRIM32, TRIM56, and RNF5, might also positively or negatively regulate STING (MITA)-mediated signaling.

The most recent works by Wang et al. (2014) and Qin et al. (2014) are significant because they provide the first evidences that K27- and K11-linked polyubiquitinations are important for innate antiviral response. It is possible that in addition to STING (MITA), additional components might also be regulated by these types of polyubiquitinations in innate antiviral response. In addition to questions mentioned earlier, some other outstanding questions are not answered in the current study. For examples, previous studies indicate that phosphorylation of STING (MITA) at S158 regulates the recruitment of TBK1 to STING (MITA), the relationship between this phosphorylation and AMFR-mediated K27-linked polyubiquitination of STING (MITA) is unknown. In addition, whether and how AMFR-mediated K27-linked polyubiquitination of STING (MITA) regulates the downstream NF-κB activation pathway needs to be investigated. Nevertheless, the study by Wang et al. (2014) provides an important link for understanding the molecular mechanisms of STING (MITA)-mediated signaling and intriguing clues for the roles of K27-linked polyubiquitination in innate immune response against DNA pathogens.

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A Swiss Army Knife for CTLs

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Granzyme B released by leukocytes cleaves multiple intracellular substrates required for target cell lysis. In this issue of *Immunity*, Prakash et al. (2014) demonstrate that granzyme B cleaves basement membrane proteins and promotes cytotoxic T cell diapedesis into inflamed tissue.

Interstitial migration and recirculation of cytotoxic T cells (CTLs) is a multistep mechanochemial process required for immune surveillance, CTL activation, and effector function (Friedl and Weigelin, 2008). Most tissues comprise interstitial spaces suited to support effective leukocyte migration; however, basement membranes consist of a network of laminins, type IV collagen, and linker proteins that interconnect to form a protein-dense layer that creates a viscoelastic barrier against migrating cells. Consequently, for passage of leukocytes from the vessel lumen into the tissue, migration through the vascular basement membrane, termed diapedesis, provides challenges and requires particular abilities (Nourshargh et al., 2010).

Using an interdisciplinary approach combining bioinformatics, biochemistry,