Ubiquitination of STING on Lysine 224 Facilitates Cytosolic DNA Activated Host Defense

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UBIQUITINATION OF STING ON LYSINE 224 FACILITATES CYTOSOLIC DNA ACTIVATED HOST DEFENSE

By

Guoxin Ni

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UBIQUITINATION OF STING ON LYSINE 224 FACILITATES CYTOSOLIC DNA ACTIVATED HOST DEFENSE

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Conserved structures among microbial species, known as pathogen associated molecular patterns (PAMPs), are recognized by host pattern recognition receptors (PRRs), which then initiate a serial of innate immune signaling transductions to protect the host from pathogen invading. A recently identified endoplasmic reticulum-associated protein STING (stimulator of interferon genes) has been shown to be an essential component of the innate immunity, especially with regard to the cytosolic DNA sensing pathway.

Cytosolic DNA species derived from invading microbes or leaked from the nuclear or mitochondrial compartments of the cell can potently trigger the induction of host defense genes by activating STING-dependent pathway. Posttranslational modifications of STING, such as phosphorylation, palmitoylation and ubiquitination, have been shown to play critical roles in regulating STING activity. However, previous studies about STING ubiquitination frequently appears conflicting and thus need to be further clarified.

By using a mass spectrometry and mutagenesis approach, we identified lysine 224 as the most critical ubiquitination site of STING. We demonstrated that after association with cyclic dinucleotides (CDN’s), STING-dependent trafficking which is required to
deliver Tank binding kinase 1 (TBK1) to interferon regulatory transcription factors (IRF) such as IRF3, was reliant upon the K63-linked polyubiquitination on K224. The inhibition of K224 ubiquitination specifically prevented IRF3 but not NF-κB activation, additionally indicating that STING trafficking is not required to activate the latter signaling pathway. siRNA-based screening further identified mitochondrial E3 ubiquitin protein ligase 1 (MUL1) as an E3 ligase able to catalyze the K63-linked polyubiquitination of STING on K224. Suppression of MUL1 expression in murine cells inhibited DNA-induced STING trafficking, which lead to impaired IRF3 activation and type I IFN production. Collectively, these findings demonstrate the critical role of K224 ubiquitination in STING function and provide molecular insight into the mechanisms governing host defense responses.
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Chapter 1: Introduction

1.1 Innate immunity

1.1.1 Overview of innate immunity

Living organisms are constantly threatened by pathogens from the environment and therefore have evolved sophisticated immune system to recognize and eliminate invading pathogens. The mammalian immune system consists two interrelated branches: innate immunity and adaptive immunity. While the adaptive immune system are highly specific but temporally delayed, the innate immune system responses much faster and therefore serves as the first line of host defense against invading pathogens (1). The innate immune system is comprised of a limited number of germline-encoded pattern recognition receptors (PRRs), which recognize structures conserved among microbial species named as pathogen-associated molecular pattern (PAMPs). PAMPs are essential components for the survival of the microbial species and are therefore difficult for them to alter (1-3). Following detection of PAMPs, PRRs activate a series of signaling cascades which eventually lead to the induction of multiple pro-inflammatory cytokine genes including type I interferon (IFN) genes to establish an antiviral state. Meantime, some PRRs can also activate both T-cell and B-cell mediated adaptive immune responses to completely eliminate invading pathogens (4, 5).

Type I IFNs (IFNα, IFNβ) are the most fundamental and critical cytokines among all the genes induced by PRRs during a course of pathogen infection. Upon secretion, type I IFNs bind to IFNα/β receptors (IFNAR) on cell surface through an autocrine and paracrine pattern and initiate a signaling cascade through the JAK-STAT (Janus kinase - Signal transducer and activator of transcription) pathway, leading to the transcriptional
induction of hundreds of IFN stimulated genes (ISGs) in not only infected cells but also neighboring uninfected cells (6, 7). These ISGs-encoded proteins involve in enhancing pathogen detection, viral RNA digestion and translational inhibition, which rapidly establish a remarkable antiviral state within the host to effectively against invading viruses, bacteria fungi and parasites (8, 9). Additionally, type I IFN signaling also plays an important role in modulating the adaptive immune response (10, 11).

1.1.2 Innate immune sensing of cytosolic nucleic acids

A variety of PAMPs, such as lipopolysaccharides (LPS), lipoproteins, flagellin, and zymosan, are only found in microbes but not in the host, allowing the host to distinguish non-self from self through PRRs. On the other hand, genomic materials such as DNA/RNA from microbes can also serve as PAMPs and activate host immune response (2, 3). Although recognition of nucleic acids by PRRs sometimes may make it difficult to differentiate non-self from self and might introduce the risk of causing autoimmune diseases, the fact that microbial nucleic acids could serve as PAMPs greatly expands the repertoire of pathogens detectable by the host immune system since all microbes use DNA/RNA as genetic materials (3). According to the structure similarity and PAMPs recognized, PRRs which recognize nucleic acids can be generally divided into three groups: Toll-like receptors (TLRs), retinoic acid-inducible gene I-like receptors (RLRs) and cytosolic DNA receptors. The nucleic acids-detecting TLRs localize on the endosomal membrane and recognize various forms of nucleic acids from bacteria and viruses, while RLR family members recognize pathogen-derived RNA in the cytosol. The third group of receptors are most recently identified receptors which have been shown to
detect DNA from both pathogens and self in the cytosol, although the function of them have not been very well characterized.

TLRs are the first identified PRRs and also the best characterized receptors (12). TLRs are type I transmembrane proteins with an ectodomain containing leucine-rich repeats that mediate PAMPs recognition, a transmembrane region, and cytosolic Toll/IL-1 receptor (TIR) domains that activate downstream signaling pathways. Among the 10 TLRs identified in human, four of them detect nucleic acids: TLR3, TLR7, TLR8, and TLR9. All of these four TLRs are sequestered in the endoplasmic reticulum (ER) in unstimulated cells and rapidly traffic to endolysosomes via Golgi apparatus after ligand stimulation (13). This translocation is regulated by the ER-resident protein UNC93B1 (14, 15). TLR3 was originally identified as recognizing synthetic double-stranded RNA (dsRNA), polyinosinic-polycytidylic acid (poly I:C) (16). It was also reported to detect genomic RNA from reovirus, respiratory syncytial virus, encephalomyocarditis virus (EMCV), and West Nile virus (WNV) (13). TLR7 and TLR8 share phylogenetical similarity and both recognize imidazoquinoline derivatives such as resiquimod (R-848) as well as single-stranded RNA (ssRNA) derived from RNA viruses such as vesicular stomatitis virus (VSV), influenza A virus and human immunodeficiency virus (HIV) (17-19). TLR9 recognizes unmethylated cytidine-phosphateguanosine (CpG) DNA motifs that are frequently present in bacteria and viruses but are rare in mammalian genomes (20). After detection of nucleic acids, TLR3 activates the adaptor protein TRIF (TIR-domain-containing adaptor-inducing interferon β), which then leads to the activation of transcription factor NF-κB (nuclear factor kappa-light-chain enhancer of activated B cells) and IRF3 (interferon regulatory factors 3), while TLR7, TLR8 and TLR9 utilize MyD88
(myeloid differentiation primary response gene 88) to activate NF-κB and IRF7. Both signaling pathways eventually activate the transcription of type I IFN genes and pro-inflammatory cytokine genes (13).

Although TLR3 detects dsRNA in the endosome, it is “blind” to microbes that have successfully invaded the host cytoplasm. Besides, TLR3 is not expressed in most non-immune cells such as epithelial cells and fibroblasts. Therefore, recognition of dsRNA in the cytosol is mainly depending on the RLR family which contains three members: retinoic acid-inducible gene I (RIG-I), melanoma differentiation factor 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2). All three RLRs comprise a central DEAD box helicase/ATPase domain and a C-terminal regulatory domain. In addition, RIG-I and MDA5, but not LGP2, contain two caspase recruitment domains (CARDs) in the N-terminus, which mediate signaling to downstream adaptor proteins (21). Due to the lack of CARD domain, LGP2 has been suggested to act as a negative regulator of RIG-I/MDA-5 signaling (22). However, other groups reported that LGP2 plays a positive role in MDA5 signaling (23, 24). Both RIG-I and MDA5 respond to the synthetic dsRNA analog poly I:C, but with different length dependency. RIG-I recognizes relatively short dsRNA (<1 kb), while long fragments (>4 kb) are preferred by MDA5 (25). In addition, dsRNA with a triphosphate group at the 5’ end, which is lacking in host mRNAs, greatly enhances its type I IFN-inducing activity through RIG-I (26, 27). Recognitions of invaded virus are also differentiated between RIG-I and MDA5. RIG-I specifically recognizes Newcastle disease virus (NDV), Sendai virus (SeV), VSV, influenza virus, and Japanese encephalitis virus, while Piconaviruses including EMCV, Mengo virus, and Polio virus are recognized by MDA5. Some viruses such as reovirus,
WNV, and Dengue virus (DENV) are recognized by both MDA5 and RIG-I (28-32). Activated RIG-I and MDA5 induce downstream signaling by binding to an adaptor protein MAVS (mitochondrial antiviral signaling protein, also known as IPS-1, CARDIF, or VISA) via a CARD-CARD-mediated interaction (33-36). The engagement of MAVS by RLRs causes a conformational change that leads to the formation of large aggregates of MAVS and thereby results in a large-scale amplification of the signaling cascade (37). MAVS then activates the kinases TBK1/IKKe (TANK-binding kinase 1/IκB kinase-ε), responsible for the activation of IRF-3 and IRF-7, and IKKα/IKKβ, responsible for NF-κB activation. These transcription factors translocate to the nucleus and induce the expression of the genes encoding type I IFNs, as well as other antiviral genes (1, 21).

TLR9 is the first identified PRR that recognizes double-stranded DNA (dsDNA) in the endosomal lumen (Figure 1.1). However, TLR9 is expressed preferentially in plasmacytoid dendritic cells (pDCs) and only responses to unmethylated CpG DNA motifs. Besides, cytosolic dsDNA is still able to induce innate immune response in TLR9-deficient cells, indicating the existence of additional DNA receptors (38). Later, a PYHIN family member AIM2 (absent in melanoma 2) was reported to regulate DNA-mediated inflammatory responses by four independent groups (39-42). AIM2 directly binds to DNA through its HIN200 domain and then assembles the inflammasome with ASC (apoptosis-related speck-like protein containing a CARD). Formation of the AIM2 inflammasome leads to the activation of caspas-1 which mediates cleavage of the pro-interleukin-1β (Pro-IL-1β) to release IL-1β to the extracellular space. Although AIM2 inflammasome is essential for DNA-triggered IL-1β secretion, it’s completely dispensable for type I IFN production in response to cytosolic dsDNA. The search for
**Figure 1.1 Recognition of cytosolic DNA**

Unmethylated CpG DNA from bacteria or virus could be detected by TLR9, which then activate NF-κB and IRF7 via MyD88 to induce inflammatory cytokines and type I IFNs. Some AT-rich DNA could be recognized by RNA polymerase III as a template to synthesize ssRNA bearing 5’-triphosphate and double-stranded secondary structures, which then activate the RIG-I-MAVS pathway. A majority of cytosolic DNA could be recognized by cGAS, which transduce signals to STING by producing cGAMP. STING recruits and activates NF-κB and TBK1-IRF3 to induce inflammatory cytokines and type I IFNs. In addition to inducing type I IFNs, cytosolic DNA could also activate the AIM2 inflammasome, leading to caspase-1 activation and IL-1β maturation. P, phosphorylation.

Additional cytosolic DNA sensors that can induce type I IFN production first led to the identification of DNA-dependent activator of IFN-regulatory factors (DAI, also known as DLM-1, ZBP1) (43). DAI was found to interact with dsDNA both *in vitro* and *in vivo*, and knockdown of DAI reduced herpes simplex virus 1 (HSV-1)-induced IFNβ
expression in the murine fibroblast cell line L929. However, DAI deficient mouse embryonic fibroblasts (MEFs) are still capable of producing type I IFNs in response to cytoplasmic DNA, suggesting that the role of DAI in DNA sensing is redundant (44). Another cytosolic protein named RNA polymerase III (Pol III) was reported to detect AT-rich B-form dsDNA poly(dA:dT). RNA Pol III uses poly(dA:dT) as templates to synthesize single-stranded RNA which bears 5’-triphosphate and double-stranded secondary structures. As previously described, this form of RNA can be detected by RIG-I to induce type I IFN production through MAVS (45, 46). The employment of RNA Pol III as a DNA sensor may allow the host to take advantage of the RIG-I-MAVS pathway to defend against certain bacteria and DNA viruses. Nevertheless, RNA Pol III still could not account for non-AT-rich DNA recognition, suggesting that another more general cytosolic DNA-sensing system exists.

In a cDNA-expression-based screening to identify genes that could activate IFNβ, a protein named stimulator of interferon genes (STING, also known as TMEM173, MITA, ERIS and MPYS) was identified as a critical signaling molecule in the innate immune response to cytosolic dsDNA (47-50). STING is an ER-resident protein which can activate transcription factors IRF3 and NF-κB, leading to robust type I IFNs production in the presence of intracellular DNA species. IFNβ production is completely abolished in STING deficient cells after dsDNA stimulation or DNA virus infection. STING knockout mice are extremely susceptible to lethal infection by HSV-1, indicating the essential role of STING in host defense against DNA virus in vivo (51). STING can be activated by directly binding to cyclic dinucleotides including cyclic diguanylate monophosphate (c-di-GMP) and cyclic diadenylate monophosphate (c-di-AMP), which
are bacterial second messengers found in diverse bacteria but not higher species (52, 53).

A more recent study found that mammalian cells are also able to produce cyclic dinucleotides (CDNs) in response to cytosolic DNA. In this study, a cytosolic protein named cyclic GMP-AMP synthase (cGAS, also known as MB21D1) was identified as a DNA sensor which produces cyclic GMP-AMP (cGAMP) from ATP and GTP after binding with DNA (54). cGAMP in turn functions as a second messenger to bind and activate STING (55). Cells from cGAS-deficient mice exhibit a complete loss of DNA-induced type I IFN production, indicating its essential role in DNA sensing pathway (56).

Because cGAS and STING are expressed in most cell types including non-immune cells like fibroblasts and endothelial cells and recognize DNA from diverse origins, the cGAS-STING pathway is now considered the major DNA sensing pathway of the host defense. The detailed mechanisms and regulations of this pathway will be described later.

1.2 STING, immunity and disease

1.2.1 STING and the immune sensing of DNA

STING was first reported as a major histocompatibility complex class II (MHC II)-interacting protein, but the biological role of this interaction remains unclear (47). A cDNA library-based screening study by Ishikawa et al later identified STING as a critical component of intracellular DNA-mediated innate immune response (48). This work was supported by studies from two other groups independently (49, 50). Studies using STING-deficient mice further confirmed the essential role of STING in response to cytosolic DNA as well as bacterial second-messenger such as c-di-GMP (51, 52). STING is indispensable in activating the host innate immune response to various pathogens including bacteria, virus and parasites (48, 51, 57-62), and STING-dependent signaling is
required for the activation of adaptive immunity in response to DNA vaccines (51). Recent studies found that STING also plays an important role in the development of various autoimmune diseases and inflammation-associated cancer (63). All of these studies have established STING as a central player in a variety of innate and adaptive immune responses as well as inflammation-associated disease.

STING is an endoplasmic reticulum-associated protein which contains 379 amino acids in human. Human and mouse STING proteins share 81% similarity and 68% identity. Several forms of human STING with a different single nucleotide polymorphism (SNP) have been reported. For example, 20.4% of the populations express a form of STING protein with a triple R71H-G230A-R293Q (HAQ) SNP, while 13.7% have an R232H substitution, 5.2% have a G230A-R293Q (AQ) substitution and 1.5% have a R293Q substitution (64, 65). Putative STING orthologs have been identified from diverse species, including Drosophila, zebrafish and Xenopus (66). STING has a broad tissue distribution, and is widely expressed in a variety of normal cell types, including most immune cells and some non-immune cells such as epithelial cells, endothelial cells and fibroblasts (48-50). Interestingly, STING protein is not detectable in most transformed cell lines with only a few exceptions, such as HEK293, THP-1 and HTB-178 cells, although the reason remains unclear.

The human STING protein contains a signal peptide and four transmembrane domains in the N-terminal region which anchor it to the ER membrane (Figure 1.2) (48). STING protein does not contain any known functional domain, and no family member of STING has been identified yet. Crystal structure studies using the carboxy-terminal region of STING confirmed that STING exists mainly as a dimer even without ligand
Figure 1.2 STING protein architecture

Human STING is a 379 amino acids-long protein which contains a signal peptide and four transmembrane regions (1-4; yellow) at the N-terminus and a long C-terminal domain (CTD, a.a 139-379), which has been crystallized. The CTD includes the dimerization domain (DD, a.a 155-180; blue) and the CTT (a.a 340-379; orange), which is abundantly phosphorylated.

binding (67-71). Dimer formation is mediated by a hydrophobic α-helix that encompasses approximately residues 153-190. The STING dimer resembles a butterfly with a deep crevice between the two monomers, which can naturally accommodate the cyclic dinucleotide ligands. Interestingly, several groups reported that STING dimer did not show obvious structural rearrangement after c-di-GMP binding (67-70), while another group suggested the opposite (71). In addition, two groups reported that STING dimer underwent significant conformational change after 2’3’-cGAMP binding (72, 73). Therefore, whether ligand binding will lead to conformational rearrangement of STING dimer still needs to be carefully clarified, as this may answer the question how the downstream signaling of STING is activated.

STIGN plays a central role in the immune response to cytosolic DNA species. STING deficiency in a variety of cell types, such as macrophages, DCs and fibroblasts, abolished IFNβ production after dsDNA stimulation or DNA virus infection (51). STING deficient mice were extremely susceptible to lethal infection by some DNA virus such as HSV-1 (51). After activation by cytosolic DNA, STING undergoes a dramatic trafficking
process from the ER to the Golgi complex and eventually assembles into concentrated foci that contain the kinase TBK1 (51, 74). The nature of this relocalization process is not entirely clear, but might be related with non-canonical autophagy (75). The STING-TBK1 complex recruits and activates transcription factor IRF3 and NF-κB, which then

**Figure 1.3 STING-dependent innate immune signaling**

Cytosolic DNA of self or microbial origin could activate cGAS and other putative DNA sensors, which then transduce signals to the ER-resident adaptor protein STING. CDNs produced by certain bacteria (diGMP or diAMP) or by the host synthase cGAS, which produces cGAMP in the presence of ATP and GTP, could bind and activate STING, which then forms a complex with TBK1 and translocates from ER to the perinuclear endolysosomal compartments via an autophagy-like process. This complex then activates transcription factors IRF3 and NF-κB to induce the production of antimicrobial chemokines and cytokines, such as type I IFNs. P, phosphorylation.
translocate into the nucleus to initiate transcription of innate immune genes (Figure 1.3) (63, 76, 77). While the downstream signaling of STING is well understood, how STING is activated in response to DNA remains less clear. One possible model is that STING senses DNA directly. This model is supported by a report showing that STING interacted with both ssDNA and dsDNA and in vitro and in vivo (78). However, this does not rule out the possibility that other additional proteins may also be required to pass the signals from cytosolic DNA to STING. Actually, several proteins have been proposed to function as DNA sensors in the past five years, which are discussed below.

IFI16 (interferon gamma-inducible protein 16) is the first proposed DNA sensor which activates STING pathway (79). IFI16 and its mouse ortholog p204 are members of the PYHIN protein family that contains an N-terminal PYRIN domain and two HIN-200 DNA-binding domains, which enable IFI16 to bind DNA. Although predominantly a nuclear protein, a small portion of IFI16 was found to co-localize with HSV-1 and HCMV genomic DNA in the cytoplasm in primary human macrophages (80). IFI16 was seen to interact with STING after DNA stimulation in THP-1 cells and when was overexpressed in 293T cells (81). Knockdown of IFI16 or p204 partially decreased dsDNA or HSV-1-induced IRF3 phosphorylation and IFNβ production (81). In addition, IFI16 was reported to recognize HSV-1 or Kaposi’s sarcoma–associated herpes virus (KSHV)-derived DNA in the nucleus (82, 83). Despite of those studies, there is still no clear mechanism for how IFI16 interacts with and activates STING. Furthermore, some groups failed to find any roles of IFI16 in DNA-induced IFNβ production using IFI16 siRNA (54, 78). Therefore, further investigation of the role of IFI16 in DNA sensing pathway is required, preferably in IFI16 knockout cells.
DDX41 (DEAD-box helicase 41) is another putative sensor of cytosolic DNA which was identified in a RNAi screening including 59 members of the DExD/H RNA helicases family in a mouse DC line (D2SC cells) and human THP-1 cells (84). Knockdown of DDX41 markedly reduced type I IFN production in response to the synthetic B-form dsDNA poly(dAT:dTA) and poly(dGC:dCG) as well as HSV-1 infection, but did not affect interferon induction after dsRNA poly I:C stimulation or RNA virus (influenza A virus) infection. Although DDX41 shows homology to RNA helicases, it does not bind poly I:C, while instead interacts with both AT-rich and GC-rich dsDNA. DDX41 was seen to colocalize with STING in the cytoplasm and co-immunoprecipitate with STING. Furthermore, a following study from the same group proposed that, in addition to its role in sensing cytoplasmic DNA, DDX41 could also function as the “main” direct sensor for c-di-GMP and c-di-AMP (85). The conclusion was based on the data that c-di-GMP bound to recombinant DDX41 with a slightly lower dissociation constant (5.7 µM) than STING (~15 µM). However, this model is conflicting with previous report that STING is the direct sensor of cyclic dinucleotides (52). Besides, the binding affinity between c-di-GMP and STIGN measured in this study was lower than other studies (2.5-5 µM) (67-71). Thus crystallographic and biochemical analysis of a DDX41-c-di-GMP complex is needed to confirm this binding. Although these data suggest that DDX41 plays an important role in recognizing cytoplasmic DNA and CDNs, other groups found that knockdown of DDX41 did not significantly affect IFNβ production after DNA stimulation or DNA virus infection (54, 78). Therefore, it will be necessary to confirm the data from knockdown experiments using DDX41 knockout mice or cell lines.
In a recent study using biochemical purification and quantitative mass spectrometry, a protein termed cyclic GMP-AMP synthase (cGAS) was identified as a sensor for cytosolic DNA (54). cGAS contains a conserved motif found in the nucleotidyltransferase (NTase) family, which also includes adenylate cyclase and 2’5’-oligoadenylate synthase (OAS1). After binding DNA, cGAS catalyzes the synthesis of cyclic GMP-AMP (cGAMP) from ATP and GTP, which in turn serves as a second messenger to interact and activate STING (55). Overexpression of cGAS activated the transcription factor IRF3 and induces IFNβ production in a STING-dependent manner. cGAS deficient cells exhibited complete loss of type I IFN production in response to DNA stimulation or DNA virus infection, indicating a nonredundant role of cGAS in the DNA sensing pathway (56). Subsequent studies focused on the structure nature of cGAMP found that unlike the canonical 3’,5’ phosphodiester linkages in bacterial CNDs, the cGAS product contains mixed phosphodiester linkages: one noncanonical 2’,5’ phosphodiester linkage and a canonical 3’,5’ linkage between GMP and AMP (c[G(2’,5’)pA(3’,5’)]) (72, 86–88). Crystallographic study showed that cGAMP bound to STING with a \( K_d \) of \(~4\) nM, which was several hundred-fold lower than that of bacterial c-di-GMP (72, 73). These data probably explain the fact that cGAMP induces IFNβ production more potently than bacterial CDNs (55). As a small molecule, cGAMP can spread from producing cells to neighboring cells through gap junctions, and subsequently establish an innate immune response (89). This intercellular communication of cGAMP provides a rapid response mechanism that protects uninfected neighboring cells during pathogen invasion. Another study showed that cGAMP boosted antigen-specific T cell
activation and antibody production in mice, suggesting that cGAMP could be used as a vaccine adjuvant (56).

Several proteins, well known to be involved in the DNA damage response, have also been reported to activate the DNA sensing pathway in a STING-dependent manner. For example, the DNA-dependent protein kinase (DNA-PK) complex was found to bind cytoplasmic DNA and trigger the transcription of type I IFNs, cytokine and chemokine genes in a manner dependent on STING, TBK1 and IRF3 (90). Cells and mice lacking DNA-PKcs showed attenuated cytokine production in response to DNA stimulation or DNA virus infection. In addition, the DNA damage sensor meiotic recombination 11 homolog A (MRE11) was reported to serve as a cytosolic sensor for dsDNA that activates the STING pathway (91). Cells with a mutation of MRE11 gene derived from a patient with ataxia-telangiectasia-like disorder exhibited defective type I IFN production upon cytosolic DNA stimulation. These studies suggest a possible link between the DNA damage responses and DNA-induced innate immunity.

Although numerous studies have well established the essential role of STING in the innate immune response to cytosolic DNA, the role of STING in RNA sensing pathway remains poorly elucidated. Several groups have reported that STING interacts with key components of the RNA sensing pathway, such as RIG-I and MAVS (48-50, 92). In addition, RNA virus such as VSV was seen to replicate more rapidly in STING deficient MEFs than in wild type MEFs, and STING knockout mice infected with VSV were more sensitive to lethal infection compared to wild type mice (51). All these data suggest that STING might also play an important role in RNA-sensing pathway. However, STING seems to be dispensable for the induction of IFNβ in response to RNA
stimulation or RNA virus infection (92, 93). Therefore, how STING involves in regulating RNA virus replication is still open to question. One study has shown that STING was selectively required for STAT6-dependent response to RNA viruses, such as induction of CCL2, which is independent of the activation of IRF3-IFNβ axis (92). This study revealed a novel role of STING in the RNA sensing immune response, although more studies are required to further understand the underlying mechanisms.

1.2.2 Regulation of STING activity

After binding with CDNs, STING translocates from the ER through the Golgi apparatus to perinuclear regions via autophagosomal-like punctate vesicles which contains the kinase TBK1 (51). However, how this process is initiated and regulated remains unclear. Several studies have suggested that the trafficking of STING might be associated with the activation of autophagy (75, 94). STING has been shown to co-localize with several autophagy-related proteins such as ATG9A (autophagy related 9a), p62 and LC3 in DNA stimulated cells, while the co-localization of STING with other autophagy-associated protein including ATG14L, ATG5 and ULK1 (Unc-51 like autophagy activating kinase 1) was not observed in the same study (75). Electron microscopy revealed that the STING-positive puncta induced by dsDNA did not have morphological characteristics of the double-membrane-bound autophagosomal structures, indicating this is not a canonical autophagy-related event (75). Atg7-deficient cells that are defective in autophagy were seen to be defective in the STING-dependent induction of type I interferon (95), but study from another group reported that induction of interferon in both Atg7-deficient cells and Atg16L-deficient cells were not affected (75). On the other hand, ATG9a negatively regulates dsDNA-induced interferon production by
inhibiting the assembly of STING and TBK1 (75). Taking together, although STING trafficking seems to involve some autophagy-associated proteins, how this process is regulated by the autophagy system still awaits further evaluation. Nevertheless, several studies have demonstrated the importance of STING-dependent autophagy-like responses for innate defense during pathogen invasion. For example, bacterial DNA from *Mycobacterium tuberculosis* (*M. tuberculosis*) activated the STING-dependent pathway, which resulted in ubiquitination of bacteria and delivery of bacilli to autophagosomes via the p62 and NDP52-dependent selective autophagy (57, 96). A STING-dependent autophagy-like response was also observed during infection of α-herpesvirus (95).

In attempt to explore the regulatory mechanisms of STING signaling, several groups discovered that human STING protein was rapidly phosphorylated on several serine residues located in the C-terminal region. One early study reported that phosphorylation on serine 358 by TBK1 was essential for STING to mediate IRF3 activation (49). However, although the phosphorylation on S358 was confirmed by two other groups, the human STING S358A mutant only partially impaired the ability of STING to rescue IRF3 activation in STING-deficient cells (94, 97). By using mass spectrometry analysis, a recent study identified four phosphorylated serine residues on hSTING protein purified from dsDNA stimulated cells (94). Further investigation found that phosphorylation on one of these residues, serine 366, inhibited the ability of STING to activate IRF3 and the induction of IFNβ. Screening assay subsequently identified an autophagy-related protein kinase ULK1 that could specifically phosphorylate STING on S366. Knockdown of ULK1 by RNAi significantly increased dsDNA-induced IRF3 activation and INF-β production. This study further proved that activity of ULK1 was
regulated through AMPK-LKB1 axis, which was activated by dsDNA and/or CDNs. The negative feedback loop established through dsDNA/CDNs-ULK1-STING is critical for the host to prevent chronic STING over-activity, which may otherwise lead to some autoinflammatory diseases (94). However, another group suggested that TBK1 could also phosphorylate STING on S366, which was required to positively regulate STING activity (98). Therefore, whether phosphorylation on S366 positively or negatively regulates STING activity still awaits further investigation.

In addition to phosphorylation, ubiquitination has also been demonstrated to play critical roles in regulating STING activity. TRIM56 (tripartite motif containing 56) is the first identified STING ubiquitin E3 ligase which catalyzes K63-linked polyubiquitin chains on lysine residue 150, an event that is required for IFNβ promoter activation (99). Later, another E3 ligase TRIM32 was reported to catalyze K63-linked polyubiquitination on several lysine residues of STING (100). In both studies, ubiquitination of STING by TRIM56 or TIRM32 enhanced its interaction with TBK1, suggesting a requirement of ubiquitination for STING to recruit TBK1. Whereas TRIM56 and TRIM32 were suggested to positively regulate STING signaling, the ubiquitin ligase RNF5 (ring finger protein 5) was reported to negatively modulate STING activity (101). RNF5 was seen to catalyze K48-linked polyubiquitination of STING, which resulted in subsequent proteasomal degradation of STING and down-regulating of virus-induced immune response. Recently, the same group reported RNF26 as a new STING E3 ligase that catalyzed K11-linked polyubiquitination on lysine 150, the same residue targeted by RNF5 (102). This study suggested that RNF26 competed with RNF5 to catalyze K11-linked polyubiquitin chains on K150 instead of K48-linked polyubiquitin chains, thereby
stabilized STING and enhanced STING-dependent innate immune signaling. However, knockdown of RNF26 showed opposite effects on type I IFNs induction during the early phase and late phase of viral infection. The authors explained that it was because RNF26 also promoted autophagy-related IRF3 degradation, although exact mechanism was still unclear. By using co-immunoprecipitation and mass spectrometry analysis, Wang et al. identified the ER-resident autocrine motility factor receptor (AMFR, also known as GP78) as a STING-interacting protein (103). AMFR, together with insulin induced gene 1 (INSIG1) protein, catalyzed K27-linked polyubiquitination of STING on several lysine residues in response to cytosolic DNA. AMFR deficient cells exhibited significantly impaired type I IFN production in response to cytosolic DNA, and were more susceptible to HSV-1 or Listeria monocytogenes infection compared with wild type cells. The authors further demonstrated that K27-linked polyubiquitination of STING catalyzed by AMFR was essential for TBK1 recruitment, because TBK1 was seen to selectively interact with K27-linked, but not K63-linked, polyubiquitin chains.

Considering the essential role of STING in the anti-viral innate immune response, it is conceivable that pathogens may have evolved mechanisms to suppress STING activity in order to favor their survival. As a matter of fact, several pathogen-encoded proteins have already been found to negatively regulate STING activity. Sequence analysis indicates that NS4B protein encoded by flaviviruses yellow fever virus (YFV) and Dengue virus exhibits strong homology with the amino terminus of STING (amino acids 125-222). Overexpression of YFV NS4B in 293T cells inhibited STING-induced IFNβ promoter activity, probably due to the direct association between NS4B and STING (51). Later, Hepatitis C virus NS4B protein was found to inhibit STING-dependent innate
immunity by blocking the interaction between STING and TBK1 (104). Additionally, DENV NS2B3 protease complex was shown to cleave human STING protein at LRRG motif between the second and third transmembrane domain, which disrupted STING activity and attenuates virus-induced innate immune response (105, 106). Notably, DENV NS2B3 is not able to cleave mouse STING, which explains the fact that replication of DENV is more severely restricted in mouse cells. Based on a screening which overexpressed more than 80 KSHV ORFs in 293T cell, viral interferon regulatory factor 1 (vIRF1) was identified to inhibit cGAS-STING-dependent IFNβ production (107). Subsequent study found that vIRF1 directly bound STING and prevented it from interacting with TBK1, thereby inhibiting STING phosphorylation and IRF3 activation, which resulted in attenuated type I IFN induction. Recently, two oncogene proteins from DNA tumor viruses, including E7 from human papillomavirus (HPV) and E1A from adenovirus, have been demonstrated to potently inhibit the cGAS-STING pathway (108). E1A and E7 interacted with STING and subverted its activity through the LXCXE motif of these oncoproteins, which is also essential for blockade of the retinoblastoma tumor suppressor. As expected, ablation of these oncogenes in human tumor cells restored the cGAS-STING pathway. This study opened the possibility that STING may play an anti-tumor role during the carcinogenesis of certain virus-induced tumors, and thereby can serve as a therapeutic target in these cancers. In addition to viral proteins, proteins from certain bacteria can also negatively modulate STING function. One recent study found that the Shigella effector protein IpaJ potently inhibited STING signaling by blocking its translocation from the ER to ER-Golgi intermediate compartments (ERGIC), even in the context of dinucleotide binding (74). Interestingly, some retroviruses also take advantage
of host cellular component to escape from activating the STING-dependent signaling. Retroviral cDNA generated from retrovirus infection has been found to activate the cGAS-STING axis and initiate immune response (109, 110). Surprisingly, a host DNA exonuclease TREX1 was found to degrade the retroviral cDNA to prevent its recognition by cGAS, thus avoiding the activation of STING and downstream signaling (61). Suppression of STING activity by pathogens with various strategies mentioned above suggests that targeting of STING may represent a general mechanism by which pathogens have evolved to manipulate innate immune signaling.

1.2.3 STING, autoinflammatory disease and cancer

Recognition of foreign DNA in the cytosol allows the host to effectively detect invading pathogens and then initiate a transient immune response to eliminate the invasion. However, DNA sensors of the STING pathway, such as cGAS, recognize DNA from various origins and do not seem to have sequence specificity, which indicates that these sensors may not be able to distinguish between self and foreign nucleic acids. Therefore, leaked self-DNA, probably from necrotic or inappropriately apoptosed cells under certain pathological conditions, might trigger sustained inflammatory response and cytokine production (63), which is known to play a key role in the development of autoimmune diseases (111). On the other hand, in order to avoid inappropriate immune response triggered by self-DNA, host cells have deployed several endogenous nucleases to keep the level of self-DNA under the threshold of receptor activation (3).

Deoxyribonuclease II (DNase II) is a lysosome-resident DNA endonuclease which degrades DNA from apoptotic cells engulfed by phagocytes. Deficiency of DNase II resulted in accumulated self-DNA in the lysosome and leakage of DNA to the cytosol
Mice lacking DNase II died during embryonic development due to anemia (112), which was caused by overproduction of type I IFNs induced by undigested DNA accumulated in macrophages (113). When DNase II-deficient mice were crossed with mice deficient in type I interferon receptor, the resultant ‘double-mutant’ mice were born healthy. However, these mice developed polyarthritis because of production of inflammatory cytokines such as TNF-α (113, 114). IFNβ was still produced in the fetal liver of mice lacking both DNase II and TLR9, suggesting that the abnormal cytokine production induced by undigested DNA was not through the TLR9 pathway (115). Surprisingly, a recent study found that mice deficient in both DNase II and STING were rescued from not only embryonic lethality but also polyarthritis (116), indicating that STING pathway plays a crucial role in the pathogenesis of this self-DNA-evoked autoimmune disease.

Apart from DNase II, three-prime repair exonuclease 1 (TREX1; also known as DNase III), the major 3’ DNA exonuclease in mammalian cells, also contributes to preventing self-DNA-induced autoimmunity. Mutations in TREX1 gene has been reported in patients with Aicardi-Goutieres syndrome (AGS), an immune-mediated neurodevelopment disorder (117). TREX1 deficient mice were viable but had a much shorter lifespan, partially due to the development of inflammatory myocarditis (118). Further studies showed that endogenous retroelements or replication debris, which was supposed to be degraded by TREX1, accumulated in the cytosol in Trex1 knockout mice and activated STING-dependent DNA-sensing pathway, which resulted in sustained cytokine production and subsequent autoimmune disease (119, 120). Notably, Trex1−/−Sting−/− double knockout mice were completely rescued from early death, exhibiting
reduced cytokine levels and no sign of inflammatory myocarditis (121, 122). These studies suggest that STING-dependent DNA-sensing pathway has an important role in TREX1-related inflammatory diseases.

Mutations of STING itself can also lead to the development of autoinflammatory diseases. Recently, *de novo* and inherited mutations in *TMEM173* gene (which encodes STING) have been described to cause early-onset systemic inflammation, cutaneous vasculopathy, and pulmonary inflammation, which was named as STING-associated vasculopathy with onset in infancy (SAVI) (123, 124). This study identified three point mutations in exon 5 of *TMEM173* from six patients, which encode STING mutants (N154S, V155M, and V147L). Elevated transcription of type I IFNs and other cytokine genes was observed in peripheral whole blood from these patients. *In vitro* studies demonstrated that these STING variants exhibited a gain of function phenotype as HEK293T cells transfected with mutant constructs showed elevated IFNβ reporter levels, although the detailed mechanism was unclear. A later research found that these STING mutants constitutive translocated from the ER to the Golgi even without ligand binding (74), indicating that the disease-associated mutations might result in a conformational rearrangement of STING which mimics the ligand-binding status, thus constitutively activates STING. Notably, constantly upregulated cytokines in SAVI patient-derived cells was reduced by JAK inhibitors, thus offering a possible treatment for these STING-associated autoinflammatory diseases (123).

Chronic inflammation with sustained cytokine and chemokine productions not only cause autoinflammatory diseases but also promote tumor development, progression, and metastatic dissemination (125, 126). Therefore, some carcinogens, which cause DNA
damages in the cells, could induce persistent inflammation that eventually instigates tumor development. 7, 12-dimethylbenz[α]anthracene (DMBA) was previously reported to induce skin papilloma formation in mice by promoting pro-inflammatory cytokine production in a MyD88-dependent manner (127). However, how DMBA induces cytokines production was largely unclear. A recent study showed that DMBA-induced DNA damage lead to nucleosome leakage into the cytosol and triggered STING-dependent cytokine production by those self-DNAs (128). Moreover, STING deficient mice were found more resistant to DMBA-induced skin tumor development, indicating that STING-dependent signaling pathway plays a crucial role in facilitating DMBA-induced skin tumorigenesis. Although whether STING plays a role in the development of other inflammation-associated cancers remains unclear, understanding the involvement of STING in such events may facilitate design of new therapies for certain cancer.

Although the STING signaling facilitates DMBA-induced skin tumorigenesis, other evidences have showed that STING might have a crucial protective effect against cancer development in several tumor models. It was reported that STING signaling was recurrently suppressed in a wide variety of cancers, including melanoma and colorectal carcinoma (129, 130). Indeed, the protective role of STING in colorectal carcinogenesis was observed in an azoxymethane/dextran sulfate sodium (AOM/DSS)-induced colitis-associated cancer (CAC) model. STING-deficient mice treated with AOM and DSS showed defective ability to produce key cytokines that facilitates tissue repair and antitumor T cell priming, such as IL-1β, IL-18 and type I IFNs, thus displayed markedly increased tumor formation with accelerated kinetics (131-134). The antitumor role of STING was also explored in an inducible mouse glioma model, which was known to
exhibit increased type I IFNs production. However, this induction was significantly reduced in glioma-bearing STING-deficient mice. Moreover, tumors in STING-deficient mice showed more aggressive growth, leading to shorter mouse survival (135).

Spontaneous T cell responses against human cancers are believed to contribute to the control of tumor growth, and a preexisting T cell-inflamed tumor microenvironment has been explored as a predictive biomarker for immunotherapies in metastatic cancers (136, 137). Although how antitumor T cell responses are initiated by tumor cells is largely unknown, it is believed that type I IFN production by CD8α+ DC cells is involved in this process (138, 139). However, the molecular mechanism of tumor-associated type I IFN induction events was also unclear as the production of type I IFN could be mediated through several innate immune pathways. A recent study demonstrated that induction of type I IFN by tumor-derived DNA, which is responsible for subsequent antitumor CD8+ T cell priming, was dependent on the STING pathway, but not MyD88, TRIF or MAVS-associated pathways (140). Study form another group showed that the STING pathway was also essential for radiation-induced type I IFN production and tumor antigen-specific CD8+ T cell responses. STING-deficient mice failed to generate efficient antitumor T cell responses and exhibited more aggressive tumor growth (141).

Considering the important role of STING in facilitating antitumor T response, it is conceivable that STING agonist might be useful in potential cancer therapies. Vascular disrupting agents 5, 6-dimethylxanthenone-4-acetic acid (DMXAA) was previously shown to have antitumor activity in mouse model (142), but ultimately failed to improve frontline efficacy in clinical trial (143). Recent studies found that DMXAA directly bound and activated mouse STING but not human STING, explaining the lack of clinical
activity of this compound in human patients (73, 144). Since STING can be directly activated by CDNs such as cGAMP, several studies have found marked antitumor activity of these STING agonists in mouse models (141, 145). Moreover, modified CDNs have been generated and exerted significantly improved antitumor efficacy in several tumor models, including tumors resistant to PD-1 (programmed death 1) blockade (145, 146). In addition, STING agonists have also been shown to enhance the antitumor effects of vaccines in various tumor models (135, 147, 148).

Numerous studies have established the central role of STING in cytosolic DNA-mediated innate immunity as well as the subsequent adaptive immune activation. However, several questions about the underlying mechanisms of this signaling pathway remain to be answered. For example, although several studies have revealed the crystal structure of ligand-free and ligand-bound STING, how the ligand activates STING and downstream signaling remains unclear. The regulation of autophagy-like STING trafficking is another major question to be answered. Growing evidences have indicated the importance of STING pathway in the development of a variety of autoinflammatory diseases and cancers. Therefore, fully understand the function of STING will greatly facilitate the design of antitumor, anti-inflammation drugs and vaccines.

1.3 Ubiquitination and immune regulation

1.3.1 Overview of ubiquitination system

Ubiquitin is a 76-amino acid protein which is remarkably conserved among all eukaryotic species. Ubiquitin can be covalently attached via its carboxyl terminus to usually lysine residues in target proteins by a three-step enzymatic cascade catalyzed by three different types of enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating
enzyme (E2) and ubiquitin ligase (E3) (Figure 1.4) (149-151). The E1 enzyme first activates ubiquitin by forming a thiol ester bond between the catalytic cysteine of the E1 and C-terminus glycine of ubiquitin. Activated ubiquitin is transferred to an E2 enzyme, which then interacts with an E3 ligase that facilitates transfer of the ubiquitin to the ε-NH₂ group of a lysine residue or the first methionine residue to form an isopeptide bond on target proteins (149-151). The human genome contains two E1 enzymes, around forty E2 enzymes and hundreds of E3 ligases (152). E2 enzymes play a major role in determining the length and linkage type of ubiquitin chains formed on the substrates, while E3 ligases control the specificity in choosing the ubiquitination substrates (152). E3 ligases have been divided into two different classes: the RING (really interesting new gene)-domain E3 ligase and HECT (homologous to E6-associated protein carboxyl terminus)-domain E3 ligase. RING E3s make the majority of E3 ligase with more than 600 predicted members, while there are only ~60 HECT E3s in human (152).

**Figure 1.4 Ubiquitination cascade**

The ubiquitination cascade begins with activation of ubiquitin by the E1 enzyme and results in formation of a thioester bond between the ubiquitin C terminus and the E1 active site cysteine. Ubiquitin is then transferred to the E2 active site cysteine in a transthioesterification reaction. An E3 ligase catalyzes transfer of the ubiquitin from the E2 to the ε-NH₂ group of a lysine residue or protein N terminus.
A substrate protein can be modified on one lysine residue with a single ubiquitin (monoubiquitination) or with a chain of ubiquitins (polyubiquitination). In some cases, a protein can be modified with ubiquitin or ubiquitin chains on multiple lysine residues at the same time. In a polyubiquitin chain, ubiquitin molecules can be linked through one of the seven ubiquitin lysine residues (which are K6, K11, K27, K29, K33, K48 and K63) or through the N-terminal methionine residue (which generates linear chains) (153). Polyubiquitin chains usually contain only one linkage type. However, ubiquitin chains containing branches (two ubiquitin molecules linked to a single ubiquitin within a chain) or a mixture of different linkages also exist (153). K48- and K63- linked polyubiquitination are the most well-studied linkage types, whereas the physiological roles of other polyubiquitin chain types are still not clearly understood. Extensive studies have established the essential roles of K48-linked ubiquitination in proteasome-associated protein degradation, while K63-linked ubiquitination have been shown to play important roles in kinase activation, DNA repair and protein trafficking (154). Ubiquitinated proteins are recognized by hundreds of proteins containing ubiquitin-binding domains (UBDs), which interact with ubiquitin or ubiquitin chains on the substrates (155). Ubiquitination can be reversed by deubiquitination enzymes (DUBs), which hydrolyze linkages between ubiquitin and the substrates (156). Thus, the ubiquitin conjugation and deconjugation enzymes, together with ubiquitin-binding proteins, formed the ubiquitin-mediated regulation machinery of diverse cellular processes (157).

1.3.2 Ubiquitination in innate immunity

The innate immunity serves as the first line of defense to protect host from pathogen evasion. However, aberrant activation of the innate immunity can lead to
chronic inflammation which may potentially cause autoimmune disorders (158). Therefore, host has established elegant regulation systems, such as reversible posttranslational modification (PTM), to precisely modulate signal transductions during innate immune response. Ubiquitination is one of the most well studied PTM which plays important roles in fine-tuning innate immunity either by modulating activity or stability of key molecules in the innate immunity, or by serving as essential components in the signal transduction (158).

RIG-I is one the best characterized PRRs whose activity is delicately regulated by ubiquitination. TRIM25 is the first identified E3 ligase that binds the first CARD of RIG-I upon viral infection and then conjugates K63-polyubiquitin chains to residue K172 in the second CARD of RIG-I (159). This K63-linked ubiquitination is essential for RIG-I to activate downstream signals as it promotes the interaction between RIG-I and MAVS. Study with Trim25 deficient mice further confirmed that TRIM25 is essential for RIG-I-mediated interferon production and antiviral activity in response to RNA virus infection (159). Besides TRIM25, another E3 ligase Riplet was also reported to catalyze K63-linked ubiquitination on RIG-I and regulate RIG-I signaling (160). Moreover, a recent study reported that in vitro synthesized K63-linked, but not K48-linked or linear, polyubiquitin chains were able to interact with RIG-I and facilitated its ability to activate downstream signaling (160). On the other hand, several DUBs including CYLD, USP21 and USP3 have been identified to remove K63-linked ubiquitin chains from RIG-I, which prevents its excessive activation (161-163). Another negative regulation pattern of RIG-I activity is mediated by E3 ligase RNF125, which ubiquitinates RIG-I with K48-linked polyubiquitin chains and leads it for proteasomal degradation (164). The same study
showed that RNF125 did not only ubiquitinate RIG-I, but also catalyzed K48-linked ubiquitination on MDA5 and MAVS (164). Signaling downstream of MAVS also involve ubiquitination-related regulation. For example, the mindbomb E3 ligases (MIB1/2) have been proposed to activate TBK1 by catalyzing its K63-linked ubiquitination (165), while DTX4 catalyzes TBK1 with K48-linked ubiquitination for proteasomal degradation (166). IRF3 and IRF7 have been showed to be targeted for K48-polyubiquitination-dependent degradation by E3 ligase TRIM21 and RAUL respectively (167-169).

Signal transduction of the TLR pathways exhibits another example of how ubiquitination is involved in modulating innate immune responses. As mentioned previously, all the TLRs initiate signal cascades through two key adaptor proteins: MyD88 or TRIF (157). MyD88 recruits tumor necrosis factor receptor-associated factor 6 (TRAF6), which then catalyzes K63-linked polyubiquitination on IL-1R-associated serine/threonine kinases1 (IRAK1) and itself (170). This ubiquitination serves as scaffold for TAB2 and NEMO (IKKγ) binding, which then recruit TAK1 and IKKα/β respectively (171-173). NEMO has been reported to be ubiquitinated by TRIM23 with K27-linked polyubiquitination, which might facilitate the activation of IKK and TBK1 complexes (174). NEMO can also be targeted by LUBAC with linear ubiquitin chains, which leads to the stabilization of the TAK1/TAB and IKK complexes (175). After activation by TAK1/IKKs, IKKβ phosphorylates IκBα, leading to the recruitment of the SCFβTrCP E3 ligase which conjugates K48-linked ubiquitination on IκBα and targets it for proteasomal degradation (157, 176, 177). This releases the NF-κB subunits and allows them to translocate into the nucleus to turn on transcription of target genes. On the TRIF axis, receptor-interacting protein 1 (RIP1) is recruited to TRIF and undergoes K63-linked
polyubiquitination by the E3 ligase pellino 1 (178, 179). Ubiquitinated RIP1 then recruits TAB2 and NEMO, leading to NF-κB activation as on the MyD88 axis. A20 (also known as TNFAIP3) has been reported to remove the K63-linked ubiquitin chains from RIP1 through its ovarian tumor domain (OTU) and then catalyzes K48-polyubiquitination on RIP1, targeting it for degradation by proteasome (180). TRIF also recruits another E3 ligase, TRAF3, which modifies itself with K63-linked polyubiquitin chains (181). This ubiquitin chain serves as a scaffold to recruit NEMO, which subsequently recruits and activates the kinases TBK1 and IKKe, leading to IRF3 phosphorylation and subsequent type I IFN production (182, 183). Several DUBs have been identified to negatively regulate this pathway by removing the K63-linked ubiquitination from TRAF3, including deubiquitinating enzyme A (DUBA), ovarian tumor deubiquitination enzyme 1 (OTUB1), as well as the ubiquitincarboxyl-terminal hydrolase L1 (UCHL1) (184-186).

It’s not surprising that ubiquitination also plays important roles in the cytosolic DNA sensing pathway. As previously described, several E3 ligases have been reported to ubiquitinate STING and modulate its activity either by facilitating STING-TBK1 interaction or by mediating STING degradation (99-103, 187). Recently, cGAS was also found to be ubiquitinated by E3 ligase RNF185 with K27-linked polyubiquitination chains, which promotes its enzymatic activity (188).

1.3.3 **MUL1 and conventional functions**

Mitochondrial E3 ubiquitin protein ligase 1 (MUL1), also known as mitochondrial ubiquitin ligase activator of NF-kB (MULAN) (189), mitochondrial-anchored protein ligase (MAPL) (190), or growth inhibition and death E3 ligase (GIDE) (191), was first identified as an activator of NF-kB pathway in a luciferase reporter assay-
based large scale screening (192). MUL1 protein contains two transmembrane domains in the N-terminal region and a C-terminal RING domain which carries out the E3 ligase activity (189). MUL1 protein mainly locates in the outer mitochondrial membrane with its RING domain facing the cytoplasm, and can also be detected in the ER-mitochondria contact sites (189, 193). The two transmembrane domains have been shown to be critical for its proper subcellular localization and functions (189, 194). The expression of MUL1 mRNA and protein is observed in almost all human tissues (191). As a multifunctional E3 ligase, MUL1 has been reported to involve in various cellular process including protein ubiquitination, SUMOylation, mitophagy and apoptosis (195).

Figure 1.5 MUL1 protein architecture
The 352 amino acids human MUL1 protein contains two transmembrane domains (TM1 and TM2; blue) in the N-terminus and a C-terminal RING domain (RING; green), which carries out the E3 ligase activity.

As a member of the RING E3 ligase family, MUL1 has been reported to interact with several substrate proteins such as Mfn2 (mitofusin 2), AKT, p53 and ULK1 and catalyze their ubiquitination (196-199). MUL1 physically binds to Mfn2 and promotes its ubiquitination-dependent degradation, which is independent of the previous reported PINK1/parkin-regulated Mfn2 degradation (196, 200). This suggests that MUL1 plays an important compensatory function in cells lacking PINK1/parkin and therefore may serve as a potential therapeutic target for Parkinson's disease. MUL1 also ubiquitinates AKT and promotes its degradation, which suppresses cell proliferation and viability (197). An
earlier research also reported that overexpression of MUL1 induced cell apoptosis and slowed cell growth (191). These pro-apoptotic properties of MUL1 require its E3 ligase activity, although no substrate was identified in that study. In contrast, another group showed that MUL1 promoted ubiquitin-dependent degradation of p53, thus negatively regulated p53-mediated growth suppression and protects cells from apoptosis under various stress conditions (198). Therefore, the role of MUL1 in cell apoptosis still awaits further investigation. It has been shown that ULK1, an autophagy-initiating kinase, was ubiquitinated by MUL1 in selenite-treated cell, which indicated a role of MUL1 in regulating selenite-induced mitophagy (199). As an E3 ligase, MUL1 has been shown to interact with four different E2 conjugating enzymes (Ube2E2, Ube2E3, Ube2G and Ube2L3) in a yeast two-hybrid system (201). This study also found that the MUL1-Ube2E3 complex interacted with GABARAP, a member of the Atg8 family, indicating that MUL1 might play certain roles in autophagy.

In addition to its ubiquitin E3 ligase activity, MUL1 was reported to also act as a SUMO E3 ligase. Braschi et al found that MUL1 directly SUMOylated the mitochondrial fission GTPase dynamin-related protein 1 (DRP1) both in vitro and in vivo, which made MUL1 the first identified mitochondrial-anchored SUMO E3 ligase (202). The same group further reported that MUL1-dependent SUMOylation of DRP1 at the ER/mitochondria contact sites during cell death was involved in the stabilization of an ER/mitochondrial signaling platform, which was required for mitochondrial constriction, calcium flux, cristae remodeling, and cytochrome c release (193). Research from another group reported that MUL1 SUMOylated RIG-I and the SUMOylation of RIG-I competed with its polyubiquitination. Since polyubiquitination of RIG-I is essential for the
activation RLR signal transduction, MUL1 thus was suggested as a negative regulator of the RLR pathway (194).

MUL1 was first identified as an activator of the NF-κB pathway (192), and further studies confirmed its role in controlling activation of NF-κB (203). MUL1 has been shown to involve in the stress-induced mitochondrial hyperfusion related NF-κB activation. The capability of MUL1 to activate NF-κB depends on its RING domain, as the MUL1C339A mutant leads to significantly lower levels of NF-κB activation (203). In addition, MUL1 also plays a role in modulating ER stress-induced NF-κB activation, in an E3 ligase activity-dependent manner (204).

As a mitochondrial outer membrane protein, MUL1 involves in regulating several physiological processes of mitochondria, including mitochondrial dynamics, mitophagy and mitochondria-derived vesicles (MDVs) formation (195). Mitochondria are highly organized and dynamic organelles, which undergo fusion and fission under different physiological conditions to maintain healthy mitochondrial networks (205). Mfn2 and DRP1 are two critical players in this dynamic process: Mfn2 is considered essential for fusion while DRP1 is suggested as main regulator of fission (205). As mentioned earlier, MUL1 has been shown to promote ubiquitination-dependent degradation of Mfn2 (196). Meanwhile, MUL1 is able to SUMOylate DRP1 and enhances its activity (193, 202). Therefore, MUL1 can impede mitochondrial fusion by inducing Mfn2 degradation while promote mitochondrial fission by stabilizing DRP1. The quality and quantity of mitochondria are also regulated by mitophagy, an autophagy-like process which selectively degrades mitochondria following damage or stress (206). Several studies have demonstrated the roles of MUL1 in regulating mitophagy. It has been shown that
different muscle wasting stimuli upregulated MUL1 expression through a mechanism involving FoxO1/3 transcription factors (207). Upregulation of MUL1 promoted ubiquitination and degradation of Mfn2, which led to the activation of mitophagy in skeleton muscle (207). Another study reported that mitochondrial serine protease Omi/HtrA2 regulated the protein levels of MUL1 under normal conditions as well as in response to oxidative stress (208). Deficiency of Omi/HtrA2 resulted in accumulation of MUL1 in the cells, which caused a significant decrease of Mfn2 protein expression level, and thus increased mitophagy. In addition, MUL1 was seen to interact and ubiquitinate ULK1 in selenite-treated cells and thus regulated selenite-induced mitophagy in an ATG5 and ULK1-dependent manner (199). Mitochondria-derived vesicles (MDVs) have been shown to transport cargo from the mitochondria to the peroxisomes (190). Overexpression of MUL1 induced MDVs in a DRP1-independent but VPS35-dependent manner, as silencing of VPS35 markedly reduced the delivery of MUL1 to peroxisome (190, 209).
Primary wild type, *Sting*<sup>−/−</sup> and *Mul1*<sup>−/−</sup> MEF cells were prepared as described (Ishikawa and Barber, 2008). HEK293T cells, Vero cell and CRL-5800 cells were purchased from the ATCC. Platinum-E cells were purchased from Cell Biolabs. AmphiPack 293 cells and hTERT-BJ1 cells were purchased from Clontech. EA.hy926 cells were kindly provided by Dr. Blossom Damania. MEF cells, HEK293T cells, Vero cell, AmphiPack 293 cells, Platinum-E cells and EA.hy926 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin. CRL-5800 cells were cultured in RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin. hTERT-BJ1 cells were cultured in a 4:1 ratio of DMEM: Medium 199 with 10% FBS, 4mM L-glutamine and 1mM sodium pyruvate. All cells were maintained at 37°C in a 5% CO<sub>2</sub> tissue culture incubator subject to routine cleaning and decontamination. Brefeldin A, MG132 and lactacystin were purchased from Sigma. Poly I:C was purchased from American Bioscience. Interferon stimulatory DNA (ISD, 90-mer), used as dsDNA in this study was obtained from Sigma and reconstituted in ddH<sub>2</sub>O at 2 mg/ml, denatured at 70°C for 30 min, and allowed to anneal at room temperature before use. HSV-1 (KOS strain) was purchased from ATCC. HSV-1 γ34.5 was kindly provided by Dr. Bernard Roizman. HSV-1-Luc was kindly provided by Dr. David Leib. Anti-STING rabbit polyclonal antibody was prepared as described previously (Ishikawa and Barber, 2008). Other antibodies used in the study were obtained from following sources: anti-Ubiquitin (Santa Cruz, sc-8017), anti-β-actin (Sigma, A5441), anti-HA (Sigma, H9658), anti-HA (Biolegend, 923501), anti-FLAG (Sigma,
F1804); anti-FLAG (Sigma, F7425); anti-calreticulin (Abcam, ab14234); anti-IRF3 (Cell Signaling, 4302); anti-IRF3 (Santa Cruz, sc-9082); anti-phospho-IRF3 (Cell Signaling, 4947); anti-TBK1 (Abcam, ab40676); anti-phospho-TBK1 (Cell Signaling, 5483); anti-p65 (Cell Signaling, 8242); anti-phospho-p65 (Cell Signaling, 3033); anti-p38 (Cell Signaling 9212); anti-phospho-p38 (Cell Signaling, 4511); anti-MUL1 (Abcam, ab84067); anti-MUL1 (LS-c344409, LSBio); anti-MUL1 (Sigma, SAB2702071). Mouse and human Mul1 siRNA SMART pool were purchased from Dharmacon (LU-050675, LU-007062).

**Animals and treatment**

*Mull* ES cells were purchased from KOMP. *Mull* chimera mice were generated by Transgenic and Gene Targeting Mouse Model Core Facility at University of Miami on a C57BL/6 background. *Mull* chimera mice were further crossed to C57BL/6 mice to generate *Mul1*−/− mice. Mice were genotyped by standard PCR. All experiments were performed with Institutional Animal Care and Use Committee (IACUC) approval and in compliance with IACUC guidelines.

**Plasmid construction**

Plasmids (pcDNA3.1, Invitrogen) expressing human TBK1, STING or C-terminal hemagglutinin (HA)-tagged STING was generated in our lab as described before (Ishikawa and Barber, 2008). Plasmids encoding HA-ubiquitin, HA-ubiquitin-K48-only or HA-ubiquitin-K63-only were kindly provided by Dr. Edward Harhaj. pBabe-puro was purchased from Addgene. cDNA of MUL1 was obtained from Origene. pBabe-puro-STING was made by PCR amplification using AccuPrime Pfx SuperMix (Invitrogen) following standard cloning protocol. Point mutations introduced into STING and
ubiquitin were generated using the Quickchange II XL site-directed mutagenesis kit (Strategene) following manufacturer’s instruction.

**DNA transfection and RNA interference**

Plasmids, poly I:C or dsDNA transfection into 293T or MEF cells were carried out using Lipofectamine 2000 with Opti-MEM (Invitrogen) according to the manufacturer’s instruction. dsDNA or plasmids were transfected into hTERT-BJ1 cells using Lipofectamine 2000 or Lipofectamine LTX respectively. siRNA transfections were performed using Lipofectamine RNAiMAX (Invitrogen) with Opti-MEM.

**Immunoblot, immunoprecipitation, and ubiquitination assay**

Cells were boiled in SDS-sample buffer (60mM Tris.HCl pH6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) and separated by SDS-PAGE and then transferred to PVDF membrane (Millipore). The membrane was blocked in PBS-T (PBS with 0.1% Tween20) with 5% Blotting-Grade Blocker (Bio-Rad) and then incubated with primary antibody at 4°C overnight. The membrane was washed three times using PBS-T buffer, and then incubated with HRP-conjugated anti-rabbit or mouse IgG (Promega). The membrane was developed with Super Signal West Pico or Femto (Thermo) and then exposed to CL-XPosure film (Thermo). For immunoprecipitation, whole cell lysate were generated by lysing cells in RIPA buffer (50mM Tris.HCl pH7.6, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) with protease and phosphatase inhibitors (PMSF, Aprotinin, Leupeptin, Pepstatin (Gbiosciences), and Phosphatase inhibitor cocktail A (Santa Cruz Biotechnology)) at 4°C followed by centrifugation. The lysates were incubated with indicated antibodies overnight at 4°C and then added with 30 µl protein G beads for another 2h. The beads were washed three times
with RIPA buffer followed by addition of 2× sample buffer and boiling. For detection of STING ubiquitination, a similar immunoprecipitation procedure was performed except including an additional stringent wash using RIPA buffer containing 2M urea to remove unspecific binding of other ubiquitinated proteins.

**Virus infections and plaque assay**

MEF cells were seeded in 24-well plates and the next day washed with DMEM followed by infection with HSV-1 virus diluted in 100 µl DMEM at MOI of 0.1 or 1. Cells were incubated with virus for 1 hr at 37°C with gentle shaking every 15 minutes. After removal of the virus-containing media, the cells were washed with PBS and replenished with complete DMEM containing 10% FBS and antibiotics. Virally infected cells were then incubated at 37°C for an additional 24 hr post-infection and supernatants were collected for virus titration. To determine HSV-1 titers, the supernatants were diluted with DMEM at a 10-fold serial dilution and then infected Vero cells (ATCC) in 12-well plates as described above. After 1 h incubation and washed with PBS, the cells were layered with complete DEME containing 1% low melting agarose gel. The cells were incubated at 37°C for another 48 h, allowing for cell growth and plaque formation. Agarose gels were removed by heating in a microwave and cells were fixed with a solution containing 0.1% crystal violet and 30% methanol. Plaques in appropriate wells were counted and viral titers were calculated and expressed at pfu/ml.

**Mass spectrometry analysis**

HEK293T cells in twenty 15cm dishes were co-transfected with 5 µg pcDNA-hSTING and 10 µg pcDNA-HA-ubiquitin plasmids per dish using Lipofectamine 2000 for 30 h. After that, the cells were lysed in RIPA buffer (50mM Tris.HCl [pH7.6],
150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) with protease inhibitor and 20mM N-ethylmaleimide and then subjected to immunoprecipitation with mouse monoclonal HA affinity beads (Covance, AFC-101P) for 20 h at 4°C. After washing with RIPA buffer and one stringent wash with RIPA buffer containing 2M urea to remove nonspecific interacting proteins, HA-tagged ubiquitinated proteins were eluted with RIPA buffer containing 100 µg/ml HA peptide (Sigma). The whole elute was then divided into two portions evenly and followed by a second immunoprecipitation with protein A/G beads covalently conjugated with rabbit IgG or rabbit anti-STING antibody respectively for 20h at 4°C. Similarly, the beads were washed with RIPA buffer and one stringent wash with RIPA buffer containing 2M urea, and then ubiquitinated STING proteins were eluted with 100mM triethylamine (pH 11.5) and neutralized by 0.1M glycine (pH 2.7). 5% of elutes were subjected to SDS-PAGE followed by immunoblot and silver staining for quality control. The rest of elute was digested and peptides separated by a Waters nanoACQUITY with MS analysis on a AB Sciex 5600 Triple Tof mass spectrometer at Yale MS & Proteomics W.M. Keck Foundation Biotechnology Resource Laboratory

Immunostaining and confocal microscopy

The cells were seeded on poly-D-Lysine coated round coverslips (BD bioscience) and the next day transfected with indicated plasmids or dsDNA using lipofectamine 2000. The cells were fixed with 4% paraformaldehyde in PBS for 15 min and permeabilized with 0.2% Triton X-100 in PBS for 5 min. After blocking with 1% bovine serum albumin (BSA) in PBS-T for 30 min, the coverslips were incubated with appropriate primary antibodies in PBS-T containing 1% BSA. After washing three times with PBS, the coverslips were incubated with Alexa Fluor 488-goat anti-rabbit IgG (for STING, IRF3,
 Alexa Fluor 488-goat anti-mouse IgG (for HA), or Alexa Fluor-647 goat anti-chicken IgG (Calreticulin) (Invitrogen). After washing three times with PBS, the coverslips were mounted onto the glass slides with ProLong Gold anti-fade reagent (Invitrogen). Images were acquired under LSM510 confocal microscope (Zeiss) or SP5 confocal microscope (Leica).

**Reconstitution of Sting−/− MEFs and CRL-5800 cells**

pBabe-puro plasmids expressing human STING or STING mutants were transfected into Platinum-E retroviral packing cells using Lipofectamine 2000. Two days after transfection, the supernatants containing packed retrovirus were collected and centrifuged to get rid of floating cell debris. Sting−/− MEF cells were incubated with the supernatants in the presence of 10 µg/ml of polybrene (SIGMA) for 8 hr and then replenished with fresh medium. After another two days, puromycin (SIGMA) was added to the culture medium at a final concentration of 2 µg/ml to remove uninfected cells. Reconstitution of CRL-5800 cells was similar to that of MEF cells except that the retrovirus was packed in the AmphoPack 293 cells.

**In vivo 32P labeling**

Primary Sting−/− MEF cells were reconstituted with pBabe-puro, pBabe-hSTING or pBabe-hK224R as described above and cultured in phosphate free DMEM (Invitrogen) with 10% FBS for 2 h. After that cells were incubated with 0.2 mCi/ml 32P labeled orthophosphate (Perkin Elmer) for 1 h and then transfected with 5 µg/ml dsDNA for 9 h. The cells were lysed in RIPA buffer with protease and phosphatase inhibitors and immunoprecipitated with anti-STING antibody as described in “Immunoblot, immunoprecipitation, and ubiquitination assay” part. After washing five times with RIPA
buffer, the beads were boiled in 2×SDS-sample buffer and separated in a 7.5% acrylamide gel. The gel was fixed in fix solution (40% methanol, 10% acetic acid), dried and then exposed to BioMax Light Film (Kodak) for 6 days at -80°C.

**DNA microarray analysis and real-time PCR**

Reconstituted Sting−/− MEF cells were transfected with 5 µg/m dsDNA for 4 h and total RNA was then isolated by the RNeasy RNA extraction kit (QIAGEN). Preparation of cDNA and microarray analysis was performed at the Oncogenomics Core Facility, University of Miami. The Illumina Sentrix BeadChip Array (Mouse WG6 version 2; Affymetrix) was used for the analysis. Data analysis was performed at the Center of Computational Science, University of Miami.

**ELISA**

IFNβ concentration in the supernatants from transfected MEF cells were measured using an ELISA kit (PBL Interferon Source) according to the manufacturer’s instructions. Values are calculated from a standard curve derived from recombinant IFNβ provided in the kit and expressed as pg/ml±SD.

**E3 ligase screening**

A library containing siRNA to 369 genes (329 genes are from Mouse ON-TARGETplus siRNA Library Ubiquitin Conjugation Subset 3, 40 genes are from customized siRNA library, Dharmacon) were transfected into Sting−/− MEFs reconstituted with hSTING for 3 days and then transfected with 4 µg/ml dsDNA for 16 hr. IFNβ production was measured by ELISA and compared with cells transfected with non-targeting siRNA. Genes which reduce IFNβ production by more than 50% were selected
and further analyzed by immunoblot and ubiquitination assay to identify the E3s which significantly reduce IRF3 phosphorylation and STING ubiquitination.

**GST fusion protein purification**

Mul1 and Mul1-H319A coding sequence was cloned into pGEX-6P-1 vector and then transformed into E.coli BL21(DE3). Bacterial were cultured in LB medium with 0.2 mM IPTG for 3 hr before sonication in lysis buffer (20 mM Tris.Cl, pH 7.5, 1mM EDTA, 1% Triton X-100, 5mM DTT, protease inhibitor cocktail). Fusion proteins were precipitated with glutathione agarose (Pierce) overnight and washed with lysis buffer for five times before elution with high salt elution buffer (20 mM reduced L-glutathione, 100 mM Tris.Cl pH 7.5, 120 mM NaCl).

**In vitro ubiquitination assay**

Purified His-STING (152-379 aa, 500 nM) was mixed with GST-MUL1 (300 nM), UBE1 (100 nM), UBE2D1 (1 µM), ubiquitin (50 µM) (UBPBio) in a reaction buffer containing 20 mM Tris–HCl, pH 7.6, 50mM NaCl, 5 mM MgCl2, 2mM ATP, 1 mM β-ME and 5% glycerol. The reaction was carried out at 37°C for 1 hour and then subjected to SDS-PAGE followed by immunobloting with indicated antibodies.

**Statistical analysis**

Statistical significance of differences in cytokine levels, mRNA expression, and viral titers were determined using Student’s t-test (two-tailed). For all tests, a p value of < 0.05 was considered statistically significant.
Chapter 3: Ubiquitination of STING on Lysine 224 Is Essential for Its Activity

3.1 STING is ubiquitinated on lysine 224 with K63-linked polyubiquitin chains

3.1.1 Cytosolic dsDNA, but not dsRNA, induces STING ubiquitination

Previous studies in our lab and others have demonstrated that STING is a critical adaptor protein in the innate immune response to invading pathogens, especially in the intracellular DNA sensing pathway. Loss of STING dramatically reduces transfected dsDNA as well as DNA virus induced type I IFN production. Sting deficient mice are extremely susceptible to virus infection, including HSV-1 and VSV (51). However, the mechanism of how STING activity is regulated upon dsDNA stimulation was largely unclear. We noticed that both human and mouse STING proteins underwent a band shift and quickly degradation after dsDNA stimulation (Figure 3.1A, 3.1B). The shifted band was later demonstrated as phosphorylated STING (94). Considering that ubiquitination plays important roles in all three major protein degradation pathways in mammalian cells: the proteasome, the lysosome, and the autophagosome (210), we would like to investigate if ubiquitination is also involved in STING activation and degradation. Indeed, ubiquitination assay showed that STING was ubiquitinated after stimulation of dsDNA, but not dsRNA poly I:C, in human fibroblast cells (hTERT-BJ1) (Figure 3.1C). STING ubiquitination was also seen in primary Sting<sup>−/−</sup> MEFs reconstituted with human STING (hSTING) (Figure 3.1D), indicating that STING ubiquitination is a conserved process in both human and mouse. Consistently, STING was ubiquitinated only in DNA virus HSV-1, but not RNA virus VSV infected cells (Figure 3.1E). These data demonstrate that STING ubiquitination is specifically modulated by cytosolic DNA, but not RNA.
3.1.2 Lysine 224 is the major ubiquitination site of STING

In order to identify the ubiquitination site(s) in STING by mass spectrometry, we attempted to purify endogenous ubiquitinated STING protein from hTERT-BJ1 cells.

Figure 3.1 dsDNA, but not dsRNA, induces STING ubiquitination

(A, B) hTERT-BJ1 cells (A) or primary MEF cells (B) were transfected with dsDNA (4 µg/ml) for the indicated time periods, and cell lysates were immunoblotted (IB) with the indicated antibodies.

(C) hTERT-BJ1 cells were transfected with dsDNA (4 µg/ml) or poly I:C (4 µg/ml) for the indicated time periods. Cell lysates were immunoprecipitated (IP) with anti-STING antibody and then immunoblotted with the indicated antibodies.

(D) Primary Sting−/− MEF cells were reconstituted with hSTING using retroviruses. The cells were transfected with dsDNA (4 µg/ml) or poly I:C (4 µg/ml) for the indicated time periods. Cell lysates were immunoprecipitated with anti-STING antibody and then immunoblotted with the indicated antibodies.

(E) hTERT-BJ1 cells were transfected with dsDNA (6 hours) or infected with HSV-1 (MOI=10) or VSV (MOI=10) for the indicated time periods. Cell lysates were immunoprecipitated with anti-STING antibody and then immunoblotted with the indicated antibodies.
However, due to the extremely low abundance of endogenous ubiquitinated STING protein, we were not able to purify enough amounts of materials for the mass spectrometry analysis. Instead, we next employed a tandem affinity purification method and successfully purified ubiquitinated STING proteins from HEK293T cells transfected with STING and HA-tagged ubiquitin plasmids (Figure 3.2A and 3.2B. See description in Materials and Methods section). Mass spectrometry analysis indicated that STING was ubiquitinated on three lysine residues (K224, K236 and K338) (Figure 3.2C). Mass spectrometry also identified K48-linked and K63-linked polyubiquitin chains in the purified samples, indicating STING might be modified with these two types of polyubiquitination (Figure 3.2D). Of the three lysine residues, K236 appears to be highly conserved in mammals, while K224 is conserved only in primates (Figure 3.2E).

To validate the ubiquitinated sites identified by mass spectrometry, each of the nine lysine residues in hSTING protein was individually mutated to arginine and then transfected into HEK293T cells along with HA-tagged ubiquitin. Ubiquitination assay indicated that substitution of K224 markedly reduced STING ubiquitination (Figure 3.3A). To confirm this finding under a more physiologically relevant condition, we transduced wild type hSTING and hSTING variants into Sting−/− MEFs using retrovirus prior to treatment with dsDNA. This analysis further confirmed that only substitution of K224, but not other lysine residues, significantly reduced dsDNA-induced STING ubiquitination (Figure 3.3B and 3.3C). Interestingly, we also observed that substitution of K289 to K289R greatly increased dsDNA-induced STING ubiquitination, although the reason is not clear yet (Figure 3.3B and 3.3C). Collectively, these data indicate that lysine K224 is the major ubiquitination site of STING.
Figure 3.2 Ubiquitinated lysine residues of STING identified by mass spectrometry

(A) HEK293T cells were transfected with STING and HA-tagged Ub for 30 hours (hr). Cell lysates were precipitated with HA-affinity beads followed by a second precipitation with either rabbit IgG (I) or anti-STING antibody (II). Samples collected from critical steps during the purification process were immunoblotted with the indicated antibodies for quality control purpose.

(B) Purified Ubiquitinated STING proteins were visualized in the gel with silver staining. ST, anti-STING antibody.

(C, D) Ubiquitinated lysine residues of STING (C) and the polyubiquitin chain types (D) identified by mass spectrometry.

(E) Alignment of STING amino acid sequences. Highlighted amino acids indicate ubiquitinated lysine residues of hSTING, as detected by mass spectrometry.

3.1.3 STING is ubiquitinated with K63-linked polyubiquitin chains

Previous study reported that STING was ubiquitinated with K48-linked polyubiquitin chains after sendai virus infection, which led to its proteasomal degradation (101).
However, proteasome inhibitors MG132 or lactacystin did not significantly enrich ubiquitinated STING (Figure 3.4A and 3.4B), suggesting that STING might not be modified with K48-linked polyubiquitination. To further investigate the ubiquitination pattern of STING, we employed a panel of ubiquitin mutants, including those containing a single substitution of a corresponding lysine and those with all lysine residues mutated.

**Figure 3.3 Lysine 224 is the major ubiquitination site of STING**

(A) HEK293T cells were transfected with HA-tagged Ub along with STING or its mutants for 30 hr. Cell lysates were immunoprecipitated with anti-STING antibody and then immunoblotted with the indicated antibodies.

(B, C) Primary Sting−/− MEF cells reconstituted with hSTING or its mutants were transfected with dsDNA (4 µg/ml) for 6 hr. Cell lysates were immunoprecipitated with anti-STING antibody and then immunoblotted with the indicated antibodies.
Figure 3.4 Proteasome inhibitor does not enrich ubiquitinated STING

(A) hTERT-BJ1 cells were treated with MG132 (10 µM) for 1 hr followed with dsDNA (4 µg/ml) stimulation for the indicated time periods. Cell lysates were immunoprecipitated with anti-STING antibody and then immunoblotted with the indicated antibodies.

(B) hTERT-BJ1 cells were treated with Lactacystin (20 µM) for 4 hr followed with dsDNA (4 µg/ml) stimulation for the indicated time periods. Cell lysates were immunoprecipitated with anti-STING antibody and then immunoblotted with the indicated antibodies.

Figure 3.5 STING is predominantly ubiquitinated with K63-linked polyubiquitin chains.

(A) Schematic presentation of wild-type ubiquitin and its variants.

(B, C) HEK293T cells were transfected with STING along with HA-tagged Ub or its mutants for 30 hr. Cell lysates were immunoprecipitated with anti-STING antibody and then immunoblotted with the indicated antibodies.
to arginines except for the indicated one (Figure 3.5A). We noted that STING ubiquitination was greatly reduced when using ubiquitin Ub-K63R, whereas STING was normally ubiquitinated with other ubiquitin mutants (Figure 3.5B). Consistently, STING was ubiquitinated with the K63-only ubiquitin, but not with the K48-only ubiquitin (Figure 3.5C). These data indicate that K63-linked polyubiquitination is the major ubiquitination type of STING, although other types of STING ubiquitination may also exist when overexpressed in HEK293T cells. Taken together, these data indicate that STING is predominantly ubiquitinated on K224 with K63-linked polyubiquitin chains.

3.2 Ubiquitination on K224 is essential for STING activation

3.2.1. Ubiquitination on K224 of STING is required for IRF3 activity, but not NF-κB or AP-1 activity

Activation of STING by cytoplasmic DNAs leads to production of type I IFNs which establish an antiviral state in the cells to eliminate invading pathogens (48, 51). To evaluate the role of ubiquitination in STING activity, we reconstituted wild-type hSTING or its K to R mutants into Sting−/− MEFs followed with dsDNA or poly I:C stimulation. We found that cells reconstituted with hSTING-K224R failed to produce IFNβ upon dsDNA transfection, while IFNβ productions in cells reconstituted with other mutants were largely unaffected or only partially reduced, with the exception of K289R, which significantly increased IFNβ productions (Figure 3.6A). Accordingly, cells reconstituted with hSTING-K224R were also seen to facilitate HSV-1 replication, likely due to abolished viral DNA-induced type I IFNs production in these cells (Figure 3.6B). The DNA-induced STING-dependent type I IFN production is known to be regulated by transcription factor IRF3 (94). Not surprisingly, DNA-induced phosphorylation of IRF3,
Figure 3.6 Ubiquitination on K224 of STING is essential for dsDNA-induced type I IFN production

(A) Primary Sting−/− MEF cells reconstituted with hSTING or its mutants were transfected with dsDNA (4 µg/ml) for 16 hr, and IFNβ production was measured by ELISA.

(B) Primary Sting−/− MEF cells reconstituted with hSTING or its mutants were infected with HSV-1 (MOI = 0.1) for 24 hr, and the viral titer were measured by plaque assay.

(C) Primary Sting−/− MEF cells reconstituted with hSTING or its mutants were transfected with dsDNA (4 µg/ml) for the indicated time periods, and cell lysates were immunoblotted with the indicated antibodies. *, p<0.05.

which indicates activation of IRF3, was dramatically inhibited in cells reconstituted with hSTING-K224R (Figure 3.6C). This was in accordance with the defective IFNβ
production in these cells shown in Figure 3.6A. Collectively, these data indicate that ubiquitination on K224 is essential for STING to activate the TBK1-IRF3 pathway.

Type I IFN production is regulated by not only IRF3 but also other transcription factors including NF-κB and AP-1 (7). To evaluate whether ubiquitination on K224 is also required for dsDNA-induced NF-κB and AP-1 activation, we examined the phosphorylation status of p65 and p38, which are indicators of NF-κB and AP-1 activation respectively. As have been described before, dsDNA-induced TBK1 and IRF3

![Figure 3.7](image)

**Figure 3.7** Ubiquitination on K224 of STING is required for IRF3 activity, but not NF-κB or AP-1 activity

(A) *Sting*<sup>−/−</sup> MEF cells reconstituted with hSTING or its mutants were transfected with dsDNA (4 µg/ml) for indicated time periods, and cell lysates were immunoblotted with the indicated antibodies.

(B, C) Reconstituted *Sting*<sup>−/−</sup> MEF cells were transfected with dsDNA (4 µg/ml) for 6 hr, stained with anti-IRF3 (B) or anti-p65 (C) antibodies and imaged by confocal microscopy.
phosphorylation was significantly inhibited in Sting−/− MEFs reconstituted with K224R. However, phosphorylation of p65 and p38 was not affected in these cells in response to dsDNA stimulation (Figure 3.7A). Previous studies have shown that K150 was the major ubiquitination site of STING (99, 101). We noted that DNA-induced phosphorylation of TBK1 and IRF3 was partially reduced in Sting−/− MEFs reconstituted with K150R (Figure 3.7A), indicating K150 plays a redundant, but not essential role in regulating STING activity. Accordingly, immunofluorescence microscopy analysis indicated the translocation of the p65 subunit of NF-κB, but not the translocation of IRF3, into the nucleus of Sting−/− MEF cells when reconstituted with K224R and treated with dsDNA (Figure 3.7B, 3.7C). These data suggest that ubiquitination on K224 of STING is essential for dsDNA-induced activation of IRF3, but not NF-κB or AP-1.

To further substantiate that ubiquitination on K224 of STING is only required for dsDNA-induced IRF3 activity, we reconstituted Sting−/− MEFs with wild-type hSTING, K224R or K289R and treated the cells with dsDNA before carrying out a microarray analysis. This analysis confirmed that induction of type I IFNs as well as other cytosolic DNA-mediated, STING-dependent genes, including members of the IFIT family, was significantly suppressed in cells reconstituted with K224R (Figure 3.8A). Expression profiles of some genes were further validated by quantitative real-time PCR (Figure 3.8B). However, the expression of a number of genes such as Cxcl2 and Csf2 remained unaffected in response to cytosolic dsDNA, probably because their transcriptional activation only requires NF-κB or AP-1 but not IRF3, as does the transcription of type I IFNs (Figure 3.8C). Collectively, our data confirm that ubiquitination on K224 of STING is essential for dsDNA-mediated activation of IRF3, but not NF-κB or AP-1.
Figure 3.8 Ubiquitination on K224 of STING is required for IRF3 activity, but not NF-κB activity

(A) Reconstituted Sting−/− MEF cells were transfected with dsDNA (4 µg/ml) for 4 hr. Total RNA was purified and examined for gene expression with Illumina Sentrix BeadChip Array (Mouse WG6 version 2). Pseudocolors indicate transcript levels below, equal to, or above the mean (green, black, and red, respectively). The scale represents the intensity of gene expression (log2 scale ranges between -3 and 7).

(B) Real-time PCR was carried out with the indicated probes to confirm gene array analysis shown in Figure 3.8A. *, p<0.05. ns, not significant.

(C) Fold induction of selected genes in dsDNA treated Sting−/− MEF with hSTING, K224R or K289R (data from gene array analysis shown in Figure 3.8A). Promoter sequence of listed genes (-1,000 to +200) was obtained through DBTSS (http://dbtss.hgc.jp) and analyzed by TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) at threshold score 85.
3.2.2. *Ubiquitination on K224 of STING is required for its translocation, phosphorylation and degradation*

In the presence of cytosolic DNA, STING translocates from the ER to perinuclear vesicles, where it is degraded by the lysosomal compartments (51, 94). Blocking of STING trafficking by brefeldin A (BFA) also prevents dsDNA-induced STING phosphorylation, degradation, downstream signaling activation as well as type I IFNs production (51, 94), indicating STING trafficking is essential for its activity. Considering loss of ubiquitination renders STING defective to activate downstream signaling, we next

![Image showing ubiquitination on K224 of STING](image)

**Figure 3.9 Ubiquitination on K224 of STING is essential for dsDNA-induced STING trafficking**

Reconstituted primary *Sting*<sup>−/−</sup> MEF cells were transfected with dsDNA (4 µg/ml) for 9 hr. Cells were stained with the indicated antibodies and imaged by confocal microscopy.
Figure 3.10 Loss of ubiquitination inhibits dsDNA-induced STING translocation

(A) Reconstituted Sting−/− MEF cells were transfected with dsDNA (4 µg/ml) for 9 hr, stained with the indicated antibodies and imaged by confocal microscopy. Cells treated with chloroquine (50 µM) or brefeldin A (0.05 µg/ml) 1 hour before dsDNA stimulation were included as controls.

(B) HEK293T cells were transfected with TBK1 along with STING or its variants for 24 hr. Cell lysates were immunoprecipitated with anti-STING antibody and immunoblotted with the indicated antibodies.
investigated whether ubiquitination was involved in regulating STING trafficking. We observed that among all the nine K to R STING variants, only the ubiquitination-defective mutant K224R lost the ability to translocate in response to dsDNA stimulation (Figure 3.9), indicating that ubiquitination is required for initiating STING trafficking.

We also noted that inhibition of STING trafficking by K224R mutation mimicked the effect of BFA treatment. While treatment with chloroquine, a lysosomal inhibitor which prevents STING degradation (94), did not block STING trafficking (Figure 3.10A). It is known that TBK1 interacts with STING and co-translocated with STING to perinuclear vesicles in response to cytosolic DNA (48, 51). We confirmed that K224R variant interacted with TBK1 normally as wild-type STING (Figure 3.10B). However, dsDNA-induced TBK1 translocation was also prevented in cells reconstituted with K224R (Figure 3.10C), due to defective trafficking ability of K224R. Plasmids are exogenous dsDNA which are able to trigger STING trafficking when transfected into cells. However, K224R did not translocate when plasmids expressing K224R were transfected into hTERT-BJ1 cells (Figure 2.10D). These data further confirm that ubiquitination on K224 is essential to initiate STING trafficking in both mouse and human cells.

Since ubiquitination is required to initiate STING trafficking, it should be a relatively early event during STING activation. Previous study showed that BFA inhibited STING phosphorylation and degradation (94). We observed that BFA also prevented STING ubiquitination (Figure 3.11A). Considering BFA inhibits protein transport from the ER to the Golgi apparatus, this indicates that STING ubiquitination probably occurs during

(C) Reconstituted Sting−/− MEF cells were transfected with dsDNA (4 µg/ml) for 3 hr, stained with anti-TBK1 antibody and imaged by confocal microscopy.

(D) hTERT-BJ1 cells were transfected with HA-tagged hSTING or mutants for 36 hr. Cells were stained with the indicated antibodies and imaged by confocal microscopy.
Figure 3.11 Loss of ubiquitination inhibits STING phosphorylation and degradation.

(A) hTERT-BJ1 cells were incubated with ethanol or brefeldin A (0.05 µg/ml) for 1 hr and then transfected with dsDNA (4 µg/ml) for the indicated time periods. Cell lysates were immunoprecipitated with anti-STING antibody and then immunoblotted with the indicated antibodies.

(B) Reconstituted Sting⁻/⁻ MEF cells were transfected with or without dsDNA (4 µg/ml) for 6 hr. Cell lysates were immunoprecipitated with anti-STING antibody and then immunoblotted with the indicated antibodies. 4S-4A stands for S345/358/366/379A.

(C) Reconstituted Sting⁻/⁻ MEF cells were transfected with dsDNA (4 µg/ml) for 9 hr, stained with indicated antibodies and imaged by confocal microscopy.

(D) Reconstituted Sting⁻/⁻ MEF cells were incubated with ³²P-labeled phosphate for 30 min and then transfected with or without dsDNA (4 µg/ml) for 9 hr. Cell lysates were immunoprecipitated with anti-STING antibody and then analyzes by gel electrophoresis followed by autoradiography or immunoblotted with anti-STING antibody.

(E) Reconstituted Sting⁻/⁻ MEF cells were transfected with dsDNA (4 µg/ml) for the indicated time periods, and cell lysates were immunoblotted with the indicated antibodies.
the ER to the Golgi transport. Previous study in our lab demonstrated that STING was phosphorylated on four serine residues after dsDNA stimulation and phosphorylation on S366 negatively regulated STING activity (94). We next investigated the relationship between STING ubiquitination and phosphorylation. We observed that both STING S366A mutant and the phosphorylation-defective mutant, in which all the four phosphorylated serine residues were substituted to alanine, could be normally ubiquitinated in the presence of cytosolic dsDNA (Figure 3.11B). The phosphorylation-defective mutant was also able to traffic normally in response to dsDNA (Figure 3.11C). These data indicate that STING ubiquitination and trafficking does not require phosphorylation. On the other hand, phosphorylation of the ubiquitination-defective mutant K224R was significantly inhibited (Figure 3.11D), indicating that ubiquitination of STING occurs before phosphorylation and probably is a prerequisite for phosphorylation. In addition, degradation of K224R after dsDNA stimulation was found to be markedly inhibited comparing with wild-type hSTING (Figure 3.11E). We also noted that the hyper-active mutant K289R degraded more quickly than wild-type hSTING, presumably due to its increased ubiquitination, as has been shown in Figure 3.3. Taken together, our data indicate that ubiquitination on K224 is critical for the initiation of STING trafficking. Loss of ubiquitination prevents STING translocation as well as the consequential phosphorylation and degradation.

3.2.3. **K224R substitution does not affect STING dimer formation or its interaction with CDNs**

Our data demonstrate that ubiquitination on K224 is essential for STING function as K224R substitution not only abolishes STING ubiquitination but also its activity. To
make sure the functional defect of K224R is caused by loss of ubiquitination, but not the possible conformational change of STING structure due to the substitution itself, we first investigated the dimer forming ability of K224R. Immunoblot assay using disuccinimidyl suberate (DSS)-treated reconstituted Sting−/− MEF cells or CRL-5800 cells indicated that K224R could form dimer normally (Figure 3.12A and 3.12B). Further analysis using native-PAGE confirmed that K224R substitution did not affect its ability to form dimers (Figure 3.12C and 3.12D). We also observed that K224R was co-precipitated with biotin-labeled cGAMP or diGMP, indicating that K224R substitution did not affect the ability of

**Figure 3.12 K224R mutation does not affect STING dimer formation**

(A, B) Primary Sting−/− MEF cells (A) or CRL-5800 cells (B) reconstituted with hSTING or its mutants were transfected with dsDNA (4 µg/ml) for 6 hr and then treated with DSS (2 mM) for 30 min. Cell lysates were then immunoblotted with indicated antibodies.

(C, D) Primary Sting−/− MEF cells (C) or CRL-5800 cells (D) reconstituted with hSTING or its mutants were transfected with dsDNA (4 µg/ml) for 6 hr, and cell lysates were analyzed by native PAGE with anti-STING antibody.
Figure 3.13 K224R mutation does not affect interactions between STING and CDNs

HEK293T cells were transfected with STING or its mutants for 24 hr, and cell lysates were incubated with biotin-labeled cGAMP or diGMP (10 µM) followed with UV crosslinking (1J/cm²). Crosslinked lysates were precipitated with streptavidin agarose and then immunoblotted with indicated antibodies.

STING to bind CDNs (Figure 3.13). Taken together, these data suggest that substitution of lysine 224 with arginine does not disrupt STING structure.

3.2.4. **Hyperactivity of STING K289R mutant is caused by increased ubiquitination on K224**

Mutagenesis analysis demonstrated K224 to be the predominant ubiquitination site of STING, as the K224R mutation abolished STING ubiquitination (Figure 3.3). Interestingly, we also noted that K289R mutation significantly increased dsDNA-induced STING ubiquitination as well as type I IFN production (Figure 3.3, 3.8). K289R mutant was also more quickly phosphorylated and degraded than wild-type STING in response to dsDNA (Figure 3.11). To investigate the mechanism of this hyper-activity, we further substituted K224 or the previously reported K150 with arginine within the K289R mutant. Ubiquitination assay demonstrated that additional substitution of K224, but not K150, abolished the increased ubiquitination on K289R mutant (Figure 3.14A). Accordingly,
dsDNA-induced IFNβ production was reduced in cells reconstituted with K224/289R compared to K289R (Figure 3.14B). Additional K224R mutation also inhibited dsDNA-induced phosphorylation and degradation of K289R (Figure 3.14C). These data suggest that the hyper-activity of K289R is due to increased ubiquitination on K224.

**Figure 3.14 Hyperactivity of STING K289R mutant is caused by increased ubiquitination on K224**

(A) Reconstituted Sting−/− MEF cells were transfected with or without dsDNA (4 µg/ml) for 6 hr. Cell lysates were immunoprecipitated with anti-STING antibody and then immunoblotted with the indicated antibodies.

(B) Reconstituted Sting−/− MEF cells were transfected with dsDNA (4 µg/ml) or poly I:C (4 µg/ml) for 16 hr, and IFNβ production was measured by ELISA.

(C) Reconstituted Sting−/− MEF cells were transfected with dsDNA (4 µg/ml) for the indicated time periods, and cell lysates were immunoblotted with indicated antibodies.

**3.2.5. Ubiquitination on K224 is essential for STING activity in human cells**

We have demonstrated that ubiquitination on K224 is essential to activate hSTING and downstream signaling pathway in reconstituted Sting−/− MEFs. In order to confirm that ubiquitination on K224 plays the same role in STING function in human cells, we
Figure 3.15 Ubiquitination on K224 is essential for STING activity in human cells

(A) CRL-5800 cells were reconstituted with empty vector, hSTING or its mutants using retroviruses. Cells were transfected with dsDNA (4 µg/ml) for 3 hr. Cell lysates were immunoprecipitated with anti-STING antibody and then analyzed by immunoblot with the indicated antibodies.

(B) Reconstituted CRL-5800 cells were transfected with dsDNA (4 µg/ml) for the indicated time periods, and cell lysates were immunoblotted with the indicated antibodies.

Figure 3.16 Ubiquitination on STING K224 is required for dsDNA-induced IRF3 and STING translocation in human cells

(A) Reconstituted CRL-5800 cells were transfected with dsDNA (4 µg/ml) for 6 hr, and then stained with anti-IRF3 antibody and imaged by confocal microscopy.

(B) Reconstituted CRL-5800 cells were transfected with dsDNA (4 µg/ml) for 3 hr, and then stained with anti-STING antibody and imaged by confocal microscopy.

retrovirally reconstituted wild-type hSTING, K150R, K224R and K289R into a lung cancer cell line CRL-5800, which does not express endogenous STING. Similarly as in
reconstituted *Sting*−/− MEFs, we observed that ubiquitination of K224R in CRL-5800 was significantly reduced in the presence of cytosolic DNA (Figure 3.15A). Immunoblot assay indicated markedly reduced phosphorylation of TBK1 as well as kinetically delayed phosphorylation of IRF3 in cells reconstituted with K224R when treated with dsDNA (Figure 3.15B). In addition, dsDNA-induced phosphorylation and degradation of K224R were greatly inhibited as compared with wild type hSTING (Figure 3.15B).

Further analysis using immunofluorescence microscopy verified that dsDNA-induced nuclear translocation of IRF3 as well as STING trafficking were greatly inhibited in cells reconstituted with K224R (Figure 3.16A and 3.16B). Accordingly, CRL-5800 cells reconstituted with K224R exhibited significantly decreased induction of *Cxc110* mRNA in response to dsDNA (Figure 3.17A and 3.17B). These data confirm that ubiquitination on K224 is essential for STING trafficking as well as TBK1-IRF3 axis activation in the presence of cytosolic DNA in human cells.

**Figure 3.17** Ubiquitination on STING K224 is required for dsDNA-induced cytokine production in human cells

**(A)** Reconstituted CRL-5800 cells were transfected with dsDNA (4 µg/ml) for 3 hr, and induction of *Cxc110* mRNAs was measured by real-time PCR. *, p<0.05.

**(B)** Reconstituted CRL-5800 cells were transfected with dsDNA (4 µg/ml) for the indicated time periods, and induction of *Cxc110* mRNAs was measured by quantitative PCR. *, p<0.05.
Chapter 4: MUL1 Modulates dsDNA-mediated STING Ubiquitination

4.1 Identification of MUL1 as a potential activator of dsDNA-mediated STING-dependent pathway

We have demonstrated that ubiquitination of STING is required for dsDNA-induced STING trafficking and downstream signaling activation. Although several ubiquitin ligases have been reported to regulate STING ubiquitination, the precise mechanism is yet to be established. To identify the ubiquitin ligase which catalyzes STING ubiquitination in response to cytosolic DNA, we performed a siRNA-based screening in Sting<sup>−/−</sup> MEFs reconstituted with hSTING (Figure 4.1A). Briefly, siRNA of 369 ubiquitin E3 ligase genes were individually transfected into cells for 72 hr prior to treatment with dsDNA or poly I:C. ELISA was then carried out to identify approximately 35 E3 ligases, the silencing of which was seen to significantly inhibit dsDNA-induced, but not poly I:C-mediated IFNβ production. Among the 35 genes, immunoblot assay further demonstrated that 18 of them were required for activating dsDNA-induced IRF3 phosphorylation. A subsequent ubiquitination assay further identified 6 genes that might be involved in regulating STING ubiquitination, because silencing of them greatly reduced STING ubiquitination (Figure 4.1B). We repeatedly confirmed that these 6 genes could influence dsDNA-induced IFNβ production in Sting<sup>−/−</sup> MEFs reconstituted with hSTING (Figure 4.1C). However, only suppression of Mul1 notably reduced dsDNA-induced IFNβ production in wild-type MEFs (Figure 4.1D). Further analysis indicated that only silencing of Mul1 markedly inhibited dsDNA-induced phosphorylation of IRF3 in wild-type MEFs (Figure 4.1E). Taken together, these data indicate that MUL1 might play a role in regulating STING ubiquitination and downstream signaling activation.
Figure 4.1 Identification of MUL1 as a potential ubiquitin E3 ligase of STING

(A) Schematic chart flow of screening procedure for identifying STING ubiquitin ligase. (B) Reconstituted Sting\textsuperscript{–/–} MEF cells were transfected with the indicated siRNAs for 72 hr followed with dsDNA (4 µg/ml) treatment for 6 hr. Cell lysates were precipitated with anti-STING antibody and then immunoblotted with the indicated antibodies. (C) Reconstituted Sting\textsuperscript{–/–} MEF cells were transfected with the indicated siRNAs for 72 hr followed with dsDNA (4 µg/ml) treatment for 16 hr, and IFN\textbeta production was measured by ELISA. (D) Wild-type MEF cells were transfected with the indicated siRNAs for 72 hr followed with dsDNA (4 µg/ml) treatment for 16 hr, and IFN\textbeta production was measured by ELISA. (E) Wild-type MEF cells were transfected with the indicated siRNAs for 72 hr followed with dsDNA (4 µg/ml) treatment for the indicated time periods. Cell lysates were immunoblotted with the indicated antibodies.

4.2 MUL1 interacts with STING and ubiquitinates STING

4.2.1 MUL1 interacts with STING

To further confirm the roles of MUL1 in STING ubiquitination, we transfected FLAG-tagged MUL1 and STING individually or together into HEK293T cells. Immunoprecipitation assays indicated that STING could interact with FLAG-tagged MUL1 (Figure 4.2A and 4.2B). By using HA-tagged STING truncation mutants (Figure 4.2C, upper panel), we determined that the N-terminal region of STING mediated its association with MUL1 (Figure 4.2C, lower panel). Likewise, the transmembrane region of MUL1 was mapped to facilitate this interaction (Figure 4.2D). We further confirmed that MUL1 interacted with STING endogenously by similar immunoprecipitation assays (Figure 4.2E and 4.2F). Notably, the endogenous association between MUL1 and STING was gradually reduced after dsDNA stimulation (Figure 4.2E and 4.2F). Immunofluorescence microscopy analysis further demonstrated that MUL1 co-localized with STING in resting reconstituted MEF cells (Figure 4.3). However, MUL1 did not co-translocate with STING to perinuclear region after dsDNA stimulation (Figure 4.3). Thus,
MUL1 may associate with STING in the vicinity of the ER in resting cells and then disassociate with STING after it has been ubiquitinated in response to cytosolic DNA.

**Figure 4.2 MUL1 interacts with STING**

(A, B) STING and FLAG-tagged MUL1 were transfected individually or together into HEK293T cells for 30 hr. Cell lysates were immunoprecipitated with anti-STING (A) or anti-FLAG (B) antibodies and then immunoblotted with the indicated antibodies.

(C) Schematic diagram of STING and its truncation mutants (upper panel). HA-tagged STING or its mutants were individually transfected into HEK293T cells along with FLAG-tagged MUL1. Cell lysates were immunoprecipitated with an anti-HA antibody and then immunoblotted with the indicated antibodies (lower panel).

(D) Schematic diagram of MUL1 and its truncation mutants (upper panel). FLAG-tagged MUL1 or its mutants were individually transfected into HEK293T cells along with HA-tagged STING. Cell lysates were immunoprecipitated with an anti-FLAG antibody and then immunoblotted with the indicated antibodies (lower panel).
(E, F) hTERT-BJ1 cells were transfected with dsDNA (4 µg/ml) for the indicated time periods. Cell lysates were immunoprecipitated with rabbit IgG or anti-STING (E) or anti-MUL1 (F) antibodies and then immunoblotted with the indicated antibodies.

Figure 4.3 MUL1 co-localizes with STING
Primary Sting^−/− MEF cells were reconstituted with hSTING and FLAG-tagged hMUL1 using retrovirus. Cells were transfected with dsDNA (4 µg/ml) for 9 hr, and then stained with anti-STING and anti-FLAG antibodies.

4.2.2 MUL1 ubiquitinates STING in vitro
To evaluate whether MUL1 directly ubiquitinates STING, we purified glutathione S-transferases (GST)-tagged MUL1 protein as well as a previously-reported MUL1 mutant H319A, which reportedly eliminates its ubiquitin ligase activity (191). A screening assay using 29 purified ubiquitin E2 ligases indicated that members from the UBE2D family collaborated with MUL1 to ubiquitinate STING in vitro (Figure 4.4A). A more sophisticated in vitro ubiquitination assay further confirmed that MUL1 catalyzed the formation of polyubiquitin chains on STING, whereas MUL1 H319A did not (Figure 4.4B). To determine which type of polyubiquitination was catalyzed on STING by MUL1, we performed in vitro ubiquitination assay using pre-linked K48-Ub₂ or K63-Ub₂ proteins and observed that MUL1 preferably catalyzed K63-linked, but not K48-linked
Figure 4.4 MUL1 ubiquitinates STING in vitro

(A) Purified E1, ubiquitin, STING (152-379 aa), GST-MUL1 proteins were mixed together with the indicated E2 proteins and incubated at 37°C for 2 hr. The mixture was then analyzed by immunoblot with indicated antibodies.
(B) Purified E1, E2, ubiquitin, STING (152-379 aa), GST-MUL1 or GST-MUL1 H319A proteins were mixed together as indicated and incubated at 37°C for 2 hr. The mixture was then analyzed by immunoblot with indicated antibodies.

(C) Purified E1, E2, STING (152-379 aa) and GST-MUL1 proteins were mixed together with ubiquitin, K48-Ub2 or K63-Ub2 proteins and incubated at 37°C for 2 hr. The mixture was then analyzed by immunoblot with indicated antibodies.

(D) In vitro ubiquitinated STING by GST-MUL1 as described above was analyzed by mass spectrometry. Highlighted lysine residues were identified as the ubiquitination sites.

polyubiquitin chains on STING (Figure 4.4C). Importantly, mass spectrometry analysis of ubiquitinated STING proteins generated from in vitro ubiquitination assay indicated that MUL1 could catalyze ubiquitination on four lysine residues (K224, K236, K289 and K338) (Figure 4.4D). Taken together, our data demonstrate that MUL1 catalyzes K63-linked polyubiquitination of STING on lysine 224.

4.3 MUL1 regulates STING-dependent innate immune responses in murine cells

4.3.1 MUL1 regulates dsDNA-induced type I IFN production by modulating STING ubiquitination

To further explore the function of MUL1 in STING-dependent innate immunity, we suppressed MUL1 expression using Mul1 siRNA in Sting−/− MEFs reconstituted with hSTING and wild-type MEFs. It was observed that silencing of MUL1 significantly reduced dsDNA-induced, but not poly I:C-mediated IFNβ production in both reconstituted Sting−/− MEFs (Figure 4.5A) and wild-type MEFs (Figure 4.5B). Subsequent time-coursed analysis confirmed the inhibition of dsDNA-induced IFNβ production by MUL1 suppression, especially at early time point (Figure 4.5C and 4.5D). Ubiquitination assay demonstrated that dsDNA-induced STING ubiquitination was greatly inhibited in MUL1-suppressed reconstituted Sting−/− MEFs (Figure 4.6A), indicating that reduced induction of type I IFN was due to impaired STING ubiquitination. Previously we have
shown that loss of ubiquitination on STING K224 prevented dsDNA-triggered type I IFN production by inhibiting the activation of TBK1-IRF3 but not NF-κB or AP-1 (Figure 3.7 and 3.8). Since suppression of MUL1 prevented STING ubiquitination, a similar inhibition of the TBK1-IRF3 activity should be observed. As expected, suppression of MUL1 was seen to significantly inhibit dsDNA-induced phosphorylation of TBK1 and IRF3 in reconstituted Sting−/− MEFs (Figure 4.6B). However, phosphorylation of p65 or p38 was largely not affected (Figure 4.6B). Notably, STING phosphorylation and

Figure 4.5 Suppression of MUL1 impairs dsDNA-induced type I IFN production

(A, B) siRNA-treated reconstituted Sting−/− MEF cells (A) or wild-type MEF cells (B) were transfected with dsDNA (4 µg/ml) or poly I:C (4 µg/ml) for 16 hr, and IFNβ production was measured by ELISA. NS, nonspecific siRNA. *, p<0.05. ns, not significant.

(C, D) siRNA-treated reconstituted Sting−/− MEF cells (C) or wild-type MEF cells (D) were transfected with dsDNA (4 µg/ml) for the indicated time periods, and IFNβ production was measured by ELISA. *, p<0.05.
Figure 4.6 Suppression of MUL1 inhibits dsDNA-induced TBK1 and IRF3 activity due to impaired STING ubiquitination

(A) siRNA-treated reconstituted Sting<sup>−/−</sup> MEF cells were transfected with dsDNA (4 µg/ml) for the indicated time periods. Cell lysates were immunoprecipitated with anti-STING antibody and then analyzed by immunoblot with the indicated antibodies.

(B, C) siRNA-treated reconstituted Sting<sup>−/−</sup> MEF cells (B) or wild-type MEF cells (C) were transfected with dsDNA (4 µg/ml) for the indicated time periods, and cell lysates were immunoblotted with indicated antibodies.

Degradation were also significantly inhibited in the presence of cytosolic DNA in MUL1-suppressed cells (Figure 4.6B). Likewise, same effects of MUL1 suppression were observed in wild-type MEFs (Figure 4.6C). Accordingly, confocal microscopy analysis
indicated the translocation of p65, but not of IRF3, into the nucleus in MUL1-silenced cells in response to cytosolic DNA (Figure 4.7A and 4.7B). However, silencing of MUL1 did not affect poly I:C-induced translocation of IRF3 or p65 into the nucleus (Figure 4.7A and 4.7B), confirming that MUL1 specifically regulates STING-dependent pathway. Previous data indicate that STING K224R failed to translocate in the presence of cytosolic DNA due to loss of ubiquitination (Figure 3.10). Similarly, dsDNA-triggered STING trafficking was seen to be inhibited, although not completely blocked, in MUL1-suppressed cells (Figure 4.7C), likely due to impaired STING ubiquitination. Collectively, our data suggest that MUL1 regulates dsDNA-mediated STING-dependent signaling pathway by modulating STING ubiquitination.

**Figure 4.7** Suppression of MUL1 inhibits dsDNA-induced IRF3 translocation and STING trafficking
(A, B) siRNA-treated reconstituted Sting−/− MEF cells were transfected with dsDNA (4 µg/ml) or poly I:C (4 µg/ml) for 6 hr, and then stained with anti-IRF3 (A) or anti-p65 (B) antibodies.

(C) siRNA-treated reconstituted Sting−/− MEF cells were transfected with dsDNA (4 µg/ml) for the indicated time periods, and then stained with the indicated antibodies.

### 4.3.2 MUL1 regulates antimicrobial defense against HSV-1 invasion

We have been using synthetic dsDNA, which mimics the genomic DNA of bacteria or DNA virus, to facilitate our study on cytosolic DNA-mediated innate immunity.

![Figure 4.8](image)

**Figure 4.8 MUL1 modulates innate immune defense against HSV-1 invasion**

(A, B) siRNA-treated reconstituted Sting−/− MEF cells (A) or wild-type MEF cells (B) were infected with HSV-1 γ34.5 (MOI=10) for the indicated time periods, and cell lysates were immunoblotted with indicated antibodies.

(C) siRNA-treated reconstituted Sting−/− MEF cells were infected with HSV-1 γ34.5 (MOI=10) for 6 hr, and induction of IFNβ mRNAs was measured by real-time PCR.

(D) siRNA-treated reconstituted Sting−/− MEF cells were infected with HSV-1 γ34.5 (MOI=2 or 10) for 24 hr, and viral titer was measured by plaque assay.
Although synthetic dsDNA can potently activate STING-dependent signaling pathway, it does not present the whole picture of how cells response to real microbial evasion. To further validate the role of MUL1 in STING-dependent innate immunity, we infected Mull siRNA-treated cells or control cells with γ34.5-deleted HSV-1 (in short as HSV-1 γ34.5), an attenuated HSV-1 strain. Immunoblot analysis indicated that viral infection-triggered phosphorylation of IRF3 was inhibited in both MUL1-silenced reconstituted Sting\(^{-/}\) MEFs (Figure 4.8A) and wild-type MEFs (Figure 4.8B). Consistently, knockdown of MUL1 greatly reduced the induction of IFN\(\beta\) mRNA in response to HSV-1 γ34.5 infection (Figure 4.8C). Plaque assay indicated that viral titer in Mull siRNA-treated reconstituted Sting\(^{-/}\) MEFs was significantly higher than that in control cells, suggesting that suppression of MUL1 facilitated HSV-1 γ34.5 replication (Figure 4.8D). Taken together, our data suggest that MUL1 plays a critical role in facilitating STING-dependent antimicrobial innate immune response.

### 4.3.3 MUL1 deficiency attenuates dsDNA-induced innate immune response

Although knockdown of MUL1 significantly inhibited dsDNA-induced STING ubiquitination and type I IFNs production, suppression of MUL1 did not completely eliminate cytosolic DNA triggered STING activity, perhaps due to inefficiency of knockdown or more likely due to the existence of alternate ubiquitin ligases. To clarify this issue, we generated Mull deficient mice and isolated Mull\(^{-/}\) MEFs. Ubiquitination assay demonstrated that dsDNA-induced STING ubiquitination was attenuated in Mull knockout MEFs compared to wild type MEFs (Figure 4.9A). Cytosolic DNA-mediated phosphorylation of IRF3 was seen to be reduced in Mull\(^{-/}\) cells, while phosphorylation of p65 and p38 were less affected (Figure 4.9B). Accordingly, Mull\(^{-/}\) MEFs exhibited
impaired IFNβ production in response to dsDNA or cGAMP stimulation (Figure 4.9C).

However, poly I:C transfection induced similar level of IFNβ production in both wild type and Mul⁻/⁻ MEFs, indicating that MUL1 specifically regulates STING- dependent

Figure 4.9 MUL1 deficiency attenuates dsDNA-induced innate immune response

(A) Wild type (WT) and Mul1 knockout (KO) MEFs were transfected with dsDNA (4 µg/ml) for the indicated time periods. Cell lysates were immunoprecipitated with anti-STING antibody and then analyzed by immunoblot with the indicated antibodies.

(B) WT and Mul1 KO MEFs were transfected with dsDNA (4 µg/ml) for the indicated time periods, and cell lysates were immunoblotted with indicated antibodies.

(C) WT and Mul1 KO MEFs were transfected with poly I:C (4 µg/ml), dsDNA (4 µg/ml) or cGAMP (8 µg/ml) for 16 hr, and IFNβ production was measured by ELISA. *, p<0.05.

(D) WT and Mul1 KO MEFs were infected with HSV-1 (MOI=0.1 or 1) for 24 hr, and viral titer was measure by plaque assay. *, p<0.05.
pathway. Consistently, MUL1 deficient MEFs were seen to facilitate HSV-1 replication (Figure 4.9D), likely due to impaired production of type I IFNs and cytokines. Taken together, these data confirm that MUL1 regulates cytosolic DNA-induced innate immune response by modulating STING ubiquitination. However, MUL1 deficiency did not completely abrogate STING activity, indicating that other STING E3 ligases also contribute to this process.

4.4 MUL1 partially regulates STING activity in human cells

We have demonstrated that suppression of MUL1 expression inhibited STING ubiquitination as well as the activation of STING-dependent signaling pathway in murine cells. To further evaluate the function of MUL1 in human system, we RNAi suppressed MUL1 expression in a human endothelial cell line EA.hy926. Knockdown of MUL1 significantly inhibited dsDNA-induced IFNβ production, but poly I:C-mediated IFNβ production was largely unaffected (Figure 4.10A). Time-course assay confirmed that MUL1-suppressed cells exhibited impaired production of dsDNA-induced IFNβ (Figure 4.10B). Accordingly, STING ubiquitination was seen to be reduced in MUL1-silenced cells, although to a moderate extent (Figure 4.10C). Similar as in murine cells, suppression of MUL1 in EA.hy926 cells inhibited dsDNA-mediated phosphorylation of IRF3, but did not affect the phosphorylation of p65 or p38 (Figure 4.10D). Confocal microscopy analysis further confirmed that suppression of MUL1 inhibited translocation of IRF3 into the nucleus when in the presence of cytosolic dsDNA, but not poly I:C (Figure 4.10E). To extend the analysis further, we infected control or Mul1 siRNA-transfected EA.hy926 cells with HSV-1 γ34.5. It was observed that suppression of MUL1 significantly inhibited viral infection-induced IRF3 activation (Figure 4.11A) as well as
Figure 4.10 MUL1 partially regulates STING activity in human cells

(A) siRNA-treated EA.hy926 cells were transfected with dsDNA (4 µg/ml) or poly I:C (4 µg/ml) for 16 hr, and IFNβ production was measured by ELISA.
(B) siRNA-treated EA.hy926 cells were transfected with dsDNA (4 µg) for the indicated time periods, and IFNβ production was measured by ELISA.
(C) siRNA-treated EA.hy926 cells were transfected with dsDNA (4 µg/ml) for the indicated time periods. Cell lysates were immunoprecipitated with anti-STING antibody and then analyzed by immunoblotting with the indicated antibodies.

![Graphs and Images]
siRNA-treated EA.hy926 cells were transfected with dsDNA (4 µg/ml) for the indicated time periods, and cell lysates were immunoblotted with indicated antibodies. siRNA-treated EA.hy926 cells were transfected with dsDNA (4 µg/ml) or poly I:C (4 µg/ml) for 3 hr, and then stained with anti-IRF3 antibody. Cells with nuclear IRF3 staining were counted as a percentage of total cells (5 fields of cells counted per sample).

IFNβ production (Figure 4.11B). Consistently, suppression of MUL1 was seen to facilitate HSV-1 γ34.5 replication, due to reduced type I IFN production (Figure 4.11C). Collectively, these data demonstrate an important role of MUL1 in regulating STING ubiquitination and activity in human cells, although other STING E3 ligases may also contribute to this process, as in the murine cells.

**Figure 4.11 MUL1 modulates innate immune defense against HSV-1 invasion**

(A) siRNA-treated EA.hy926 cells were infected with HSV-1 γ34.5 (MOI=10) for the indicated time periods, and cell lysates were immunoblotted with indicated antibodies. (B) siRNA-treated EA.hy926 cells were infected with HSV-1 γ34.5 (MOI=10) for the indicated time periods, and IFNβ production was measured by ELISA. (C) siRNA-treated EA.hy926 cells were infected with HSV-1 γ34.5 (MOI=1 or 10) for 24 hr, and viral titer was measured by plaque assay.
Chapter 5: Discussion

The presence of cytosolic dsDNA species trigger the production of host defense proteins including type I IFNs and pro-inflammatory cytokines, a signaling event that is controlled by the ER-resident protein STING (63). Activators of STING include genomic DNA or cyclic dinucleotides from invading microbes or leaked DNA from the nucleus or mitochondria of damaged cells (48, 63). Cytosolic dsDNA associates with the synthase cGAS to produce cGAMP that binds STING, which leads to the translocation of STING from the ER, through the Golgi apparatus to the perinuclear lysosomal compartments, where it is degraded (51, 74, 75, 94, 211). STING trafficking is critical for the activation of transcription factor IRF3 and NF-κB, which then induce the robust production of type I IFNs and pro-inflammatory cytokines (51, 78). However, the regulatory mechanisms of how cytosolic DNA initiates STING trafficking were not well understood. Several post-translational events, including palmitoylation, phosphorylation and ubiquitination, have been reported to modulate STING activity (94, 97-103). However, none of these studies established any role of STING posttranslational modification in regulating STING trafficking. In this study, we demonstrated that K63-linked polyubiquitination on lysine 224 of STING is essential to initiate its translocation triggered by cytosolic dsDNA. By using a siRNA-based screening, we further identified an E3 ligase MUL1 which can catalyze the K63-linked ubiquitination of STING on lysine 224.

5.1 STING ubiquitination: DNA pathway or RNA pathway

Numerous studies have well established the essential role of STING in the innate immune response to cytosolic DNA. STING can be activated by DNA from a variety of origins including viral DNA (such as adenovirus and herpes simplex virus), purified E.
coli DNA, calf thymus DNA, plasmid DNA, and synthetic dsDNA (such as ISD, poly(dA:dT) and poly(dGC:dGC)) (51, 55). Invading pathogens such as adenovirus, HSV-1, KSHV, *Listeria monocytogenes* and *M. tuberculosis* also potently activate STING-dependent pathway (51, 96, 107, 212). Activated STING undergoes post translational modifications such as ubiquitination and phosphorylation, and then translocates to perinuclear lysosomal compartments, where it is quickly degraded (63, 94). On the other hand, although STING seems to involve in immune response to RNA virus infection, the exact role of STING in RNA-sensing pathway remains to be fully elucidated. STING has been shown to interact with key components of the RNA-sensing pathway, such as RIG-I and MAVS (48-50). In addition, STING deficiency facilitates replication of certain RNA virus such as VSV and sendai virus (51, 92). However, STING seems to be dispensable for the induction of IFNβ in response to RNA stimulation or RNA virus infection (92, 93). In addition, poly I:C transfection or SeV infection is not able to trigger STING trafficking or phosphorylation (92, 94). Therefore, the role of STING in immune response to RNA virus seems to be quite different from that in the DNA-sensing pathway.

Previous study reported that SeV infection triggered RNF5-mediated K48-linked polyubiquitination on STING, which promoted its proteasomal degradation in HEK293 cells (101). The same group further reported that SeV or HSV-1 infection also induced TRIM32-mediated K63-linked polyubiquitination on STING, which was required for its activation in HEK293 cells (100). Knock down of RNF5 or TRIM32 by siRNA reduced SeV-induced STING ubiquitination which resulted in enhanced or impaired IFNβ mRNA induction respectively (100, 101). However, in our study we found that neither poly I:C
transfection nor VSV infection could induce STING ubiquitination in hTERT cells or 
Sting\(^{-/-}\) MEF cells reconstituted with hSTING (Figure 3.1C - E), indicating that cytosolic 
RNA is not able to active STING ubiquitination. Our data are in consistent with previous 
findings that cytosolic RNA does not activate STING (92, 94). In addition, as mentioned 
above, STING is dispensable for RNA virus-induced IFN\(\beta\) production, including SeV (92, 
93), which is also conflicting with the RNF5 and TRIM32 story. Furthermore, the 
HEK293 cells used in these two studies have been shown to be defective in response to 
cytosolic DNA except poly (dA:dT), which is recognized through RNA polymerase III-
RIG-I pathway (46, 79). Therefore, it is very likely that RNF5 and TRIM32 might 
involve in regulating ubiquitination of components in the RNA-sensing pathway, rather 
than modulating STING ubiquitination. Actually, the same group has reported that viral 
infection triggered RNF5 to target MAVS, the key adaptor protein of RNA-sensing 
pathway, for K48-linked ubiquitination and proteasomal degradation (213). In addition, 
another group demonstrated that TRIM32 was employed by the intrinsic immunity to 
limit viral replication upon influenza A virus infection (214). However, SeV infection-
induced IFN\(\beta\) production and SeV replication were not affected in TRIM32 deficient 
cells (214). In general, our data indicate that STING ubiquitination is a cytosolic DNA-
induced, but not RNA-mediated event.

5.2 STING ubiquitination: sites and patterns

A number of studies about STING ubiquitination have been published since 
STING was identified, with most of the studies focusing on ubiquitination of human 
STING and one report investigating mouse STING ubiquitination (99-103, 187). When 
referred to ubiquitination of hSTING, five ubiquitin E3 ligases have been reported to
catalyze four different types of polyubiquitin chains on STING. However, data from these studies frequently appear conflicting, especially on the ubiquitination sites and patterns. Three of these studies reported that STING was ubiquitinated exclusively on one lysine residue 150, but with three different ubiquitination types (K11, K48 and K63), which was catalyzed by ubiquitin E3 ligase RNF26, RNF5 and TRIM56 respectively (99, 101, 102). However, another group reported that Sting⁻/⁻ MEFs reconstituted with hSTING K150R could produce IFNβ normally compared with cells reconstituted with wild-type hSTING in response to DNA stimulation (69). In our study, we found that although Sting⁻/⁻ MEFs reconstituted with hSTING-K150R exhibited partially impaired IFNβ production in response to DNA (Figure 3.6A), the K150R substitution did not significantly reduce STING ubiquitination (Figure 3.3C), indicating that K150 is not the ubiquitination site, at least not the predominant one. In the previously mentioned TRIM32 study, the authors demonstrated that TRIM32 could ubiquitinate STING on multiple lysine residues (K20, K150, K224, and K236) including K150, with K63-linked polyubiquitin chains (100). However, another group recently reported that STING was ubiquitinated by the E3 ligase AMFR on similar lysine residues (K137, K150, K224, and K236), but with K27-linked polyubiquitin chains (103). In addition, the authors reported that TRIM56 and TRIM32 were not able to ubiquitinate STING when overexpressed in HEK293T cells.

The reason that multiple lysine residues were mapped as the ubiquitination sites is probably because both studies only used mutagenic approach with an overexpression system in HEK293T cells to identify the ubiquitination sites. However, results of mutagenesis should be interpreted with caution, because other lysine residues could be
used for chain formation when the preferred site is substituted, especially when the substrate and E3 ligase are overexpressed (215). To clarify this issue, we first attempted to purify endogenous ubiquitinated STING protein from dsDNA-transfected hTERT cells for mass spectrometry analysis. However, due to extremely low abundance of endogenous ubiquitinated STING, we were not able to achieve this goal (data not shown). Instead, we next employed a tandem affinity purification method to purify ubiquitinated STING from HEK293T cells expressing hSTING and HA-tagged ubiquitin. We repeatedly identified three ubiquitination sites (K224, K236 and K338) on STING using mass spectrometry (Figure 3.2C). We next employed a more physiologically relevant approach to characterize plausible key sites of STING ubiquitination by using reconstituted primary Sting<sup>−/−</sup> MEFs. Our data indicate that only substitution of K224, but not other lysine residues, almost completely abolished STING ubiquitination in the presence of cytosolic DNA, suggesting that K224 is the predominant ubiquitination site of STING (Figure 3.3B, C). Although substitution of other lysines, such as K150 and K236, partially reduced dsDNA-induced IFNβ production (Figure 3.6A), they did not appear to significantly affect STING ubiquitination in our model (Figure 3.3C). Interestingly, we noted that substitution of K289 to K289R greatly increased dsDNA-induced STING ubiquitination, for reasons that remain to be determined (Figure 3.3B, C). Nevertheless, additional substitution of K224 to arginine abolished the increased ubiquitination on K289R, further demonstrating that K224 is the major site that polyubiquitin chains are anchored.

Previous studies have identified four different ubiquitination patterns of STING, including K11, K27, K48 and K63-linked polyubiquitination (99-103). In our study, mass
spectrometry analysis identified K48- and K63-linked polyubiquitination in the purified protein sample (Figure 3.2D). Mutagenesis analysis using ubiquitin variants further confirmed that STING was predominantly ubiquitinated with K63-linked polyubiquitin chains (Figure 3.5 B, C). In addition, proteasome inhibitors MG132 or lactacystin did not significantly enrich ubiquitinated STING, suggesting that STING might not be modified with K48-linked polyubiquitination (Figure 3.4). Therefore, the K48-linked polyubiquitination detected by the mass spectrometry in our sample probably came from contamination of other ubiquitinated proteins which were co-precipitated with ubiquitinated STING.

5.3 STING ubiquitination, trafficking and function

5.3.1 Role of STING ubiquitination in IRF3 and NF-κB activation

Cytosolic DNA is recognized mainly through cGAS, which generates cGAMP to bind and activate STING (54, 55). Following binding with cGAMP, STING undergoes a dramatic translocation from the ER, through the Golgi apparatus, to perinuclear vesicles (51). It is believed that transcription factors IRF3 and NF-κB are recruited to STING complex and get activated during this process. Indeed, a recent study showed that IRF3 is recruited to STING/TBK1 complex after their trafficking from the ER to the ER-Golgi intermediate compartments (ERGIC) (74). However, how STING activates NF-κB signaling is largely unclear. A previous study in our lab indicates that dsDNA-mediated NF-κB pathway activation is through a STING-TRAF6-TBK1 axis (77). However, whether this activation occurs before or after STING trafficking is still unknown. In this study, we found that the ubiquitination deficient mutant K224 was not able to efficiently activate TBK1/IRF3 phosphorylation in the presence of cytosolic DNA. However, the
activation of NF-κB and AP-1 pathway were largely not affected (Figure 3.7, 3.8). Thus, ubiquitination of STING on K224 appears to principally affect TBK1/IRF3 but not NF-κB or AP-1 signaling. These results may help explain why the transcriptional activation of some genes such as Cxcl2 and Csf2 were unaffected by loss of K224 ubiquitination, since they only require NF-κB signaling for transcriptional activity, unlike type I IFNs which require NF-κB, AP-1 as well as IRF3/7. Notably, a previous study in our lab demonstrated that phosphorylation on S366 of STING by ULK1 also inhibited IRF3 activity, but not NF-κB activity (94). Thus, results from both studies suggest that the STING-dependent activation of TBK1/IRF3 axis may share different regulatory mechanisms with that of NF-κB and AP-1 pathways. We further found that the K224R variant failed to translocate from the ER in response to DNA stimulation (Figure 3.9, 3.10), which explained why IRF3 was not activated in cells reconstituted with this variant, as IRF3 activation requires translocation of STING/TBK1 complex to the ERGIC. Considering that activation of NF-κB and AP-1 pathways is not affected by inhibiting of STING trafficking, this indicate that activation of these two pathways may occur immediately after STING binding with CDNs in the vicinity of the ER or occur on route from the ER to the Golgi apparatus. Further investigations are needed to fully understand the mechanism of this process.

5.3.2 Role of STING ubiquitination in regulating STING trafficking, phosphorylation and degradation

Previous studies reported that K63-linked polyubiquitination on K150 of STING catalyzed by TRIM56 or TRIM32, or the K27-linked polyubiquitination on multiple lysine residues of STING catalyzed by AMFR, was required for STING/TBK1
interaction (99, 100, 103). However, another group demonstrated that STING C-terminal region (a.a 341-379) alone was sufficient to interact with TBK1 and promote IRF3 phosphorylation (97), suggesting that STING could interact with TBK1 directly without additional posttranslational modifications. In our study, we found that several STING variants, including K150R and K224R, interacted with TBK1 normally (Figure 3.10B), further confirming that STING may not need additional ubiquitination to recruit TBK1. On the other hand, our data indicate that the ubiquitination deficient variant K224R failed to initiate trafficking in response to cytosolic DNA (Figure 3.9, 3.10), suggesting that ubiquitination of STING might be a prerequisite of DNA-induced STING trafficking.

Several studies have demonstrated that cytosolic DNA-evoked STING trafficking is an autophagy-like process, and it is dependent on autophagy-related proteins VPS34 and ATG9a (75, 94). In addition, STING-positive vesicles were seen to co-localize with essential autophagosome component LC3 and p62, an important receptor for selective autophagosomal degradation of ubiquitinated targets (75). However, STING-positive vesicles do not have morphological characteristics of the double-membrane structures as canonical autophagosomes and STING trafficking is independent of ATG7 and ATG16L1 (75). Thus, the exact mechanism of autophagy-related STING trafficking and degradation remains to be elucidated. Ubiquitination has been known to play important roles in regulating degradation of various cellular structures, including protein aggregates, mitochondria, and microbes, through proteasome or lysosomes (210, 216). While K48-linked polyubiquitination usually leads substrates for proteasomal degradation, K63-linked polyubiquitin chain-marked cargoes are preferentially targeted to the autophagic/lysosomal degradation pathway (210, 216). In this study, we demonstrated
that STING was predominantly ubiquitinated with K63-linked polyubiquitin chains. It is known that STING is mainly degraded in lysosomal compartments, because lysosome inhibitor chloroquine (94), but not proteasome inhibitor MG132 or lactacystin (Figure 3.4), prevents STING degradation. In addition, we found that deprivation of STING ubiquitination (via substitution of K224 or suppression of MUL1) greatly prevented STING trafficking and degradation (Figure 3.10 and 3.11E). Taken together, these data indicate that the K63-linked polyubiquitination on K224 of STING is a key event to initiate cytosolic DNA-induced noncanonical autophagy-dependent STING trafficking, which is required to activate downstream signaling pathway as well as subsequent STING degradation in lysosomes.

We further investigated the spatial and temporal nature of STING ubiquitination event. Our previous studies demonstrate that BFA, which inhibits protein transport from the ER to the Golgi apparatus, prevents STING trafficking events (51, 94). In this study, we additionally noted that BFA also prevented STING ubiquitination (Figure 3.11A), suggesting that this modification occurs immediately after STING associates with CDNs, perhaps following a conformational rearrangement of STING. Ubiquitination could thus occur when STING is still in the ER, or during early stage of the ER to the Golgi transportation process since we observed that the K224 variant retained strong co-localization with the ER resident protein calreticulin.

Crosstalk between phosphorylation and ubiquitination, which acts either positively or negatively in both directions to regulate these processes, have been widely observed (217). Previous study in our lab demonstrated that STING was phosphorylated predominantly on four serine residues including S366 after trafficking through the Golgi
Phosphorylation of S366 inhibits STING function after STING/TBK1 has successfully trafficked to trigger IRF3 activity (94). Since we have shown that STING ubiquitination is required for trafficking, it is not surprising to observe that STING K224R was not able to be phosphorylated in the presence of cytosolic DNA (Figure 3.11D). On the other hand, phosphorylation-deficient STING variants were readily able to be ubiquitinated (Figure 3.11B), confirming that phosphorylation of STING is not required for STING ubiquitination, but rather the opposite.

5.4 MUL1 and STING ubiquitination

Studies from several groups have reported a number of ubiquitin E3 ligases which were proposed to catalyze STING ubiquitination (99-103). However, data from these studies appear conflicting. To clarify this issue, we employed an ubiquitin E3 ligase siRNA library-based screening and identified mitochondrial E3 ubiquitin protein ligase 1 (MUL1) as a novel STING ubiquitin E3 ligase (Figure 4.1). MUL1 is a transmembrane protein locates in mitochondrial outer membrane as well as ER/mitochondria contact region (189, 193). MUL1 has been reported to play extensive roles in various processes including: mitochondrial dynamics, cell growth, apoptosis, and mitophagy, through its ubiquitin E3 ligase activity or SUMO E3 ligase activity (189, 191, 193, 196, 199). Our data here indicate that MUL1 directly interacts and ubiquitinates STING, preferentially with K63-linked polyubiquitin chains (Figure 4.2, 4.4). Suppression of MUL1 expression by siRNA inhibited dsDNA-induced STING ubiquitination, further confirming the essential role of MUL1 in mediating STING ubiquitination (Figure 4.6A). MUL1 may interact with STING in resting cells (Figure 4.2), although STING is not ubiquitinated until association with CDN’s. How MUL1 ubiquitinates STING in the presence of
cytosolic DNA has yet to be determined, presumably a conformational rearrangement of 
STING following association with CDN’s may facilitate STING ubiquitination by MUL1. 
MUL1 might dissociate with STING after STING is ubiquitinated, because it was not 
seen to co-translocate with STING to the perinuclear regions (Figure 4.3). Ubiquitination 
of STING by MUL1 is essential for the activation of STING-dependent innate immunity, 
because suppression of MUL1 greatly impaired cytosolic DNA-mediated type I IFNs 
production in murine cells (Figure 4.5). In consistent with data described above, this 
inhibition was dependent on TBK1/IRF3 activity, but not NF-κB or AP-1 (Figure 4.6B 
and C). As expected, suppression of MUL1 in murine cells also inhibited STING 
trafficking, thus preventing its phosphorylation and degradation (Figure 4.6B, 4.6C and 
4.7C). Notably, MUL1 has been reported to interact with GABARAP (GABA receptor 
associated protein) (201), a member of the ATG8 family that plays a major role in 
autophagy. This could supports our findings that MUL1-mediated STING ubiquitination 
is essential for DNA-induced autophagy-related STING trafficking, although how this 
ubiquitination event initiates STING-dependent autophagy machinery still awaits further 
investigations.

In order to further clarify the role of MUL1 in STING-dependent pathway, we 
attempted to generate Mull knockout mice. Although one research group previously 
reported that they were not able to obtain heterozygous (Mull+/−) progeny from chimeric 
mice (191), we successfully generated Mull deficient mice by targeted homologous 
recombination in embryonic stem (ES) cells. We noted that Mull deficiency indeed 
inhibited cytosolic DNA-evoked STING ubiquitination, IRF3 phosphorylation as well as 
type I IFN production in MEFs, confirming its role in regulating STING activity.
However, MUL1 deficiency did not completely abolish DNA-induced innate immune response, indicating that other E3 ligases also participate in modulating ubiquitination-dependent STING activation and thus can compensate MUL1’s role when it is absent.

We also investigated the role of MUL1 in regulating STING ubiquitination and activity in human cells. Although suppression of MUL1 did inhibit cytosolic DNA-induced IRF3 activation and type I IFNs production in human cells, the effects of MUL1 knockdown on STING ubiquitination, phosphorylation and degradation seemed to be moderate (Figure 4.10). This might due to inefficiency of knockdown of MUL1 or more likely due to the existence of alternate STING E3 ligases which are able to compensate MUL1 function, as data from Mull deficient cells indicate.

5.5 Conclusions and future directions

Based upon my work on STING ubiquitination, there are several conclusions which are listed below: 1) Human STING protein is predominantly ubiquitinated on lysine 224 with K63-linked polyubiquitination chains in the presence of cytosolic DNA. 2) Deprivation of K224 ubiquitination prevents cytosolic DNA-induced type I IFNs production by inhibiting IRF3 but not NF-κB or AP-1 signaling. 3) Ubiquitination on K224 is required for initiating STING trafficking, phosphorylation and degradation via non-canonical autophagy. 4) MUL1 is an E3 ligase able to ubiquitinate STING on K224 and modulate STING-dependent signaling pathway.

Additional future work may help to better understand the role of STING ubiquitination in innate immunity. First, we noticed that the K224R variant still exhibited weak ability to activate IRF3 and IFNβ, suggesting that ubiquitination on other lysine residues might also contributes. Whether this is a compensation of K224R substitution or
it occurs endogenously is unknown. Purification of endogenous ubiquitinated STING for mass spectrometry analysis would be helpful to answer this question. Second, as being discussed above, how ubiquitination of STING initiate STING trafficking via non-canonical autophagy is unclear. Ubiquitin-binding receptors p62 and NBR1 are well known to couple certain ubiquitinated proteins for selective autophagic degradation in lysosomes (218-220). Considering that p62 has been shown to co-localized with STING in response to cytosolic DNA, it could be a good target for studying the initiation of STING-dependent non-canonical autophagy machinery. Third, we noted that suppression of MUL1 using siRNA or MUL1 deficiency did not completely abrogate STING activity in both mice and human cells, indicating the existence of redundant E3 ligases. Knockdown or knockout of previously reported STING E3 ligases such as TRIM32 and TRIM56 in Mul1−/− cells may clarify the roles of these ligases. A new screening using different strategy might also be necessary to identify the redundant E3 ligase if it is an unreported one.
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