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CHAPTER THREE

Negative Regulation of Cytosolic Sensing of DNA

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Contents

Abstract

In mammals, cytosolic detection of nucleic acids is critical in initiating innate antiviral responses against invading pathogens (like bacteria, viruses, fungi and parasites). These programs are mediated by multiple cytosolic and endosomal sensors and adaptor molecules (c-GAS/STING axis and TLR9/MyD88 axis, respectively) and lead to the production of type I interferons (IFNs), pro-inflammatory cytokines, and chemokines. While the identity and role of multiple pattern recognition receptors (PRRs) have been elucidated, such immune surveillance systems must be tightly regulated to limit collateral damage and prevent aberrant responses to self- and non-self-nucleic acids. In this review, we discuss recent advances in our understanding of how cytosolic sensing of DNA is controlled during inflammatory immune responses.

1. INTRODUCTION

The innate immune system, which is conserved across virtually all multi-cellular organisms on the planet (from sponges to insects, plants, and vertebrates), includes a diverse set of receptors capable of detecting

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molecular patterns like sugars, lipids, polymers, and nucleic acids that are principal in prompting protective responses (Broz and Monack, 2013; Kieser and Kagan, 2017; Kumar et al., 2011a). In vertebrates, cytosolic detection of nucleic acids (derived from microbes like viruses, intracellular bacteria, fungi and parasites) is critical in initiating innate (characterized by production of type I IFNs) and adaptive (characterized by T and B cell responses) immune responses (Wu and Chen, 2014). Recent efforts have focused on identifying relevant immune surveillance sensors and components of downstream signaling—Toll-like receptors (TLRs) and their cognate ligands, cytosolic sensing of RNA (primary mediated by the RIG-I/IPS-1 axis), cytosolic sensing of DNA (primary mediated by the cGAS/STING axis), and the inflammasome pathway (primary mediated by NOD-like receptors; NLRs) (Broz and Monack, 2013; Kieser and Kagan, 2017; Kumar et al., 2011a). Yet, while the identities of immune surveillance systems have been revealed, inflammatory programs mediated by these sensors must be tightly regulated to prevent aberrant and inappropriate responses to self-derived ligands (like self RNA and DNA) that may be released from damaged cells, senescent cells, apoptotic cells, or during fertilization (Barber, 2017; de Oliveira Mann and Kranzusch, 2017). Indeed, RIG-I, the cytosolic receptor for RNA distinguishes self from non-self RNA through interaction with a 5′-triphosphate that is unique to viral RNA (Wu and Chen, 2014). Though exceptions to this requirement have been reported, the ability of RIG-I to distinguish self from non-self in this way ensures that anomalous immune responses to cellular RNA do not occur. Responses to DNA are far more agnostic. cGAS, in collaboration with STING, does not distinguish between cellular and foreign DNA (Barber, 2015; Crowl et al., 2017). Indeed, HT-DNA (Herring Testes-DNA), as well as ISD (Interferon-Stimulated DNA) oligonucleotides, is known to act as potent stimulators of cGAS/STING in multiple mammalian cell types. It has also been suggested that chronic cGAS/STING activation induced by self DNA may be responsible for induction of aberrant inflammatory diseases like systemic lupus erythematosus (SLE), Aicardi-Goutières syndrome (AGS), and polyarthritis (Barber, 2015; Crowl et al., 2017). While apoptotic cells represent a possible source of DNA, the existence of DNases in cytoplasmic (e.g., DNase-III, also known as TREX1) and lysosomal compartments (e.g., DNase-II) can clear potential ligands and ensure that inappropriate responses are not initiated (Barber, 2015; Crowl et al., 2017). In the event of mitochondrial DNA (mtDNA) release into the cytoplasm (which occurs following mitochondrial damage), intracellular caspase activation controls

the aberrant immune response (McArthur et al., 2018; Rongvaux et al., 2014; White et al., 2014). Similarly, cGAS/STING and the necessary cofactors, and cellular DNA are compartmentalized such that sensing of self-DNA is avoided; the receptor in the cytosol and the ligand (DNA) in the nucleus (Barber, 2015). However, recent publications have illustrated that cell cycle progression in the context of DNA-damage may lead to the formation of micronuclei which elicit cGAS/STING-mediated DNA sensing (Harding et al., 2017; Mackenzie et al., 2017). In addition, cellular DNA can serve as a cGAS/STING ligand following cellular senescence (defined as the senescence-associated secretory phenotype; SASP) (Gluck et al., 2017; Yang et al., 2017). Induction of SASP factors, like inflammatory cytokines and chemokines, may then reinforce senescent cells via autocrine and paracrine routes. Similarly, during fertilization, sperm cell-derived DNA can be found in oocyte cytoplasm may serve to activate cytoplasmic nucleic acid sensing (NAS) pathway to induce inflammatory responses. Thus, there are at least two contexts unrelated to infection where cytosolic responses must be repressed: during mitosis when chromosomal DNA naturally exists in cytoplasm, and during fertilization when sperm-derived DNA enters oocyte cytoplasm. Such control can be achieved through the downregulation of molecules necessary for prompting responses to nucleic acids, and/or the upregulation of a negative regulators that act as molecular safeguards. In this review, we provide an update on cellular machinery that negative controls cytosolic sensing of DNA and discuss therapeutic opportunities for the germ-cell specific NLR family member, NLRP14, a recently identified inhibitor of DNA-sensing during fertilization.

2. CANDIDATE SENSORS OF INTRACELLULAR DNA

In contrast to the identification of molecular machinery responsible for TLR and RNA-sensing RIG-like receptor (RLR) pathways, identification of a universally accepted cytosolic DNA sensor and its related signaling pathway involved a more untidy trajectory (Unterholzner, 2013). Since the existence of a cytosolic sensor for DNA was first postulated (one that could elicit TLR-independent production of IFN to transfected double-stranded DNA; dsDNA) (Ishii et al., 2006; Stetson and Medzhitov, 2006), a great deal of effort has gone into revealing its identity. First, DNA-dependent activator of IRF3 (DAI; also referred to as DLM-1/ZBP1) was shown to participate in the activation of IRF3 downstream of dsDNA

sensing (Takaoka et al., 2007). Evidence suggests that following cellular exposure to synthetic dsDNA or DNA virus infection, DAI can form a signaling complex together with TBK1 and IRF3 to initiate production of type I IFNs. However, reports also suggested that cells from DAI-deficient mice did not exhibit impaired responses to synthetic B-form dsDNA and DNA genomes derived from bacteria (Ishii et al., 2008).

IFI16 (IFN-γ-inducible protein 16), a PYHIN family member protein, was also shown to be involved in the recognition of synthetic dsDNA and DNA genomes of nuclearly replicating viruses (e.g., HSV-1, KSHV, HCMV, and EBV) (Unterholzner et al., 2010). In addition, IFI16 was also shown to participate in the DNA damage response through promoting apoptosis and senescence, suggesting that such processes may mediate inflammatory diseases (Unterholzner, 2013). Related to IFI16 is yet another putative cytosolic DNA sensor, absent in melanoma 2 (AIM2), which, through an inflammasome dependent pathway, induces production and secretion of IL-1β and IL-18 rather than type-I IFNs after DNA exposure (Burckstummer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009). More recently, AIM2-like receptors (ALRs), consisting of 13 members possessing a Pyrin signaling domain and a DNA-binding HIN domain, have been shown to not contribute to type I IFN production downstream of DNA sensing, nor to autoimmune diseases like as AGS which are associated with responses to self-DNA (Gray et al., 2016). The establishment of human IFI16-deficient cells further illustrated competent type-I IFN responses to HCMV infection. Nevertheless, IFI16 may participate in the recognition of cytosolic DNA in a cell type and/or species-specific manner.

An additional putative DNA sensor, the DDX41 helicase, was identified through RNAi screening and shown to be involved in DNA recognition in immunocompetent cells rather than epithelial cells (Zhang et al., 2011). Shortly after its discovery, DDX41 mediated signaling was shown to go through the bacterial-derived second messenger cyclic dinucleotide (CDN) molecules cyclic di-AMP and cyclic di-GMP (Parvatiyar et al., 2012). Solved crystal structures confirmed that the binding regions for these ligands overlapped, suggesting that DDX41 can recognize multiple ligands via the DEAD domain (Omura et al., 2016). Of note, both IFI16 and DDX41 have been proposed to act upstream of STING (see below) through physical interactions. Yet, their collaborative role in modulating cytosolic sensing of DNA requires further investigation using genetically engineered mice. Additionally, several DNA damage inducible host factors like the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), and its binding co-factors Ku70/80, or Mre11 (Meiotic recombination 11) have

also been implicated in cytosolic sensing through direct interactions with DNA ligands (Ferguson et al., 2012; Kondo et al., 2013). Importantly, while DNA-PKcs appear to have critical roles in DNA-mediated immune responses, DNA-PKcs deficient cells exhibit normal expression of many ISGs upon stimulation with DNA and DNA virus infection. Nonetheless, these observations reinforce the notion that DNA damage response induces type I IFN production (Brzostek-Racine et al., 2011). Use of DNA damage induced agents like 7,12-dimethylbenz-α-anthracene (DMBA) has helped shed light on the underlying events initiate the DNA damage-induced immune response via cytosolic DNA sensing pathway and implicates nucleosome leakage in eliciting cGAS/STING-dependent signal activation via recognition of self-DNA (Barber, 2015). Finally, LRRFIP1, cytosolic protein, has been shown to recognize both dsDNA and dsRNA derived from pathogens, and may control the production of type-I IFNs through the transcriptional co-activator β-catenin rather than IRF3—potentially acting as an amplifier of cytosolic NAS (Yang et al., 2010). Collectively, evidence indicates that while the long list of candidate cytosolic DNA sensors may have redundant functions, they vary in ligand specificity as well as cell type and tissue distribution.

3. DNA SENSING VIA THE cGAS/STING PATHWAY

Between 2008 and 2009, prior to the identification of several candidate DNA sensors described the above, Barber and colleagues reported that STING (Stimulator of IFN genes, also referred as MITA, MPYS, or ERIS, and encoded by TMEM173), an endoplasmic reticulum (ER) localized protein consisting of multiple transmembrane regions, acts as an essential molecule for cytosolic sensing of DNA (Barber, 2014; Ishikawa and Barber, 2008; Ishikawa et al., 2009). Several DNA species appear to trigger STING-dependent signaling via TBK1/IRF3 and IKK/NF-κB axis in a length dependent manner and STING has also been attributed with a role in responses to plasmid-based vaccines and induction of long-term immunity. And though evidence suggests that STING may interact directly with dsDNA, the physiological relevance of these observations remains to be explored (Abe et al., 2013). Nevertheless, upon ligand stimulation, STING translocates from the ER to the perinuclear-Golgi region, forms a signaling complex with TBK1 and leads to phosphorylation and activation of IRF3. In addition, STING signaling appears to self-regulate by inducing protein degradation through a ubiquitin-mediated proteasome pathway and termination of signal transduction. A subsequent study indicated that STING dimers

bind cyclic dinucleotides (CDNs), suggesting that STING acts as the direct innate immune sensor for CDNs (Burdette et al., 2011). Other reports indicated that the type-I IFN and pro-inflammatory cytokine inducing flavonoid compounds, 5,6-dimethylxanthenone-4-acetic acid (DMXAA, also known as Vadimezan or ASA404 and initially identified as a potent tumor vasculature disrupting agent in mice), and 10-carboxymethyl-9-acridanone (CMA) may trigger STING dependent signal activation through direct interactions (Prantner et al., 2012; Roberts et al., 2007). However, it was also known that these compounds displayed such STING activating properties in mice but not human cells. Consistent with the unfortunate failure of these drugs in human clinical trials, these observations may be explained by structural differences in the DMXAA-binding sites across the human and mouse proteins (Gao et al., 2013).

While the identification of STING undoubtedly contributed to our understanding of the underlying molecular events that control cytosolic DNA-mediated immune responses, it was universally accepted that a sensor upstream of STING remained to be discovered. Indeed, the breakthrough came in 2013 when Chen and colleagues uncovered a role for cyclic GMP-AMP (cGAMP), and the catalytic enzyme (cyclic GMP-AMP synthase (cGAS), encoded by MB21D1 and C6orf150) in response to several cytosolic DNA ligands (Sun et al., 2013; Wu et al., 2013). cGAMP is a CDNs that consist of varying phosphodiester linkages (a 2′5′-phosphodiester linkage and a canonical 3′5′-phosphodiester linkage; the cGAMP isomer is known as 2′3′- cGAMP) and cGAS undergoes conformational rearrangement following direct binding to DNA which leads to the synthesis of cGAMP from cellular stores of ATP and GTP. cGAMP produced by cGAS upon DNA stimulation in turn acts as a cognate STING ligand to activate signaling and downstream production of type I IFN (Barber, 2014)—it is interesting to note that similarly to other PRRs (TLR, RLR and the inflammasome pathway) cGAS activation of the STING pathway does not require direct interaction the sensor and adaptor that mediates signal transduction. Importantly, the cGAS/STING pathway plays a crucial role in the induction of autoimmune and inflammatory diseases (see Section 4), highlighting the need to properly control the DNA sensing pathway for the maintenance of cellular homeostasis and immune response. Taken together, while the functional and physiological relevance of several candidate DNA sensors (e.g., DDX41, IFI16, and DNA-PKcs) needs to be fully investigated, cGAS and STING have emerged as bona fide players in cytosolic sensing of DNA (Fig. 1).

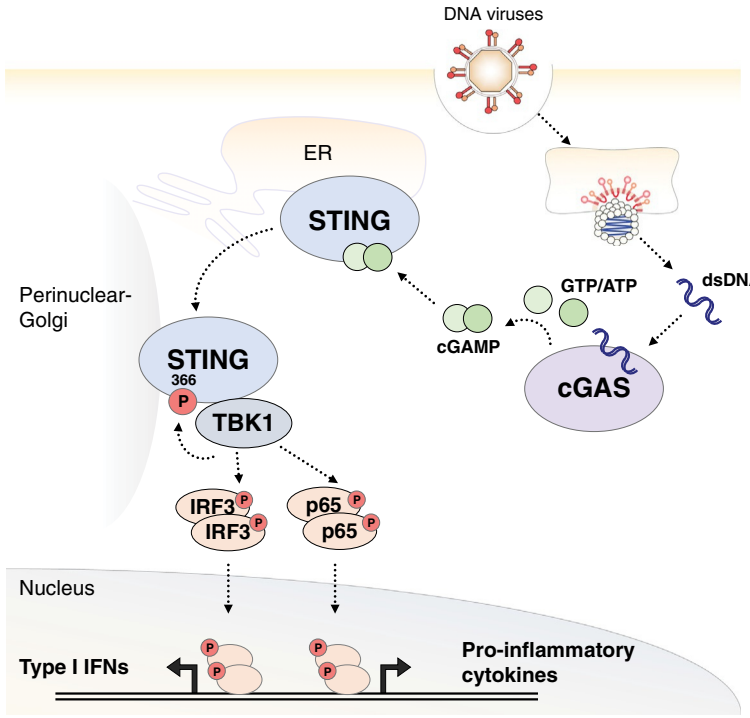

Fig. 1 Cytosolic sensing of DNA via cGAS/STING. Upon viral infection, cGAS recognizes viral dsDNA and utilizes GTP/ATP to catalyze cGAMP and trigger activation of STING. In addition to foreign DNA, cGAS promotes STING-dependent signal activation in response to nucleosome and micronuclear DNA that results from DNA damage. Following cGAMP binding, STING translocates from the ER to perinuclear-Golgi, and forms a signaling complex with TBK1 (phosphorylation of STING at S-366 occurs after translocation) leading to IRF3-mediated type I IFN production as well as NF-κB (p65)-mediated signaling. *Abbreviations: cGAS*, cyclic GMP-AMP Synthase; *STING*, Stimulator of IFN Genes; *cGAMP*, cyclic GMP-AMP; *dsDNA*, double-stranded DNA; *ER*, Endoplasmic Reticulum; *TBK1*, TANK-Binding Kinase 1; *IRF3*, Interferon Regulatory Factor 3; *P*, Phosphorylation.

4. REGULATION OF cGAS/STING DEPENDENT SIGNALING

Intracellular Post-Translational Modifications (PTM), including phosphorylation, ubiquitination, and ubiquitin-like modifications like sumoylation and ISGylation, glutamylation, acetylation or methylation, are critical in controlling cellular responses. These modulators of pathway activity participate in signal transduction by tuning enzymatic states, subcellular localization,

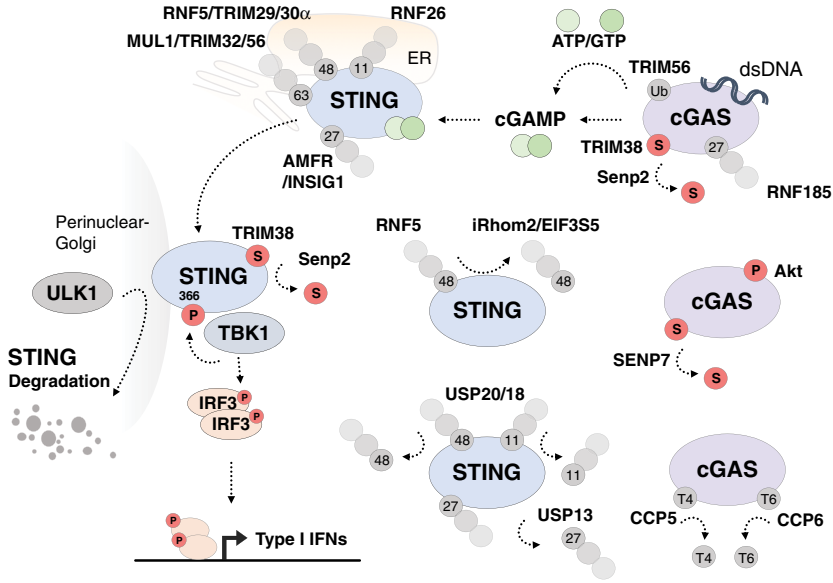

Fig. 2 Regulation of cGAS/STING-signaling through Post-Translational Modifications (PTMs). Ubiquitin E3 ligases as well as ubiquitin specific proteases (USPs) regulate STING function and localization. In addition, ubiquitin-mediated proteasome pathway can lead to degradation of STING and termination of signaling activation—ULK1 controls STING degradation. Detailed description and identity of targeted residues in cGAS and STING are described in the text. *Ub*, Ubiquitin; *S*, SUMOylation; *P*, Phosphorylation; *11*, K11-linked Ub; *27*, K27-linked Ub; *33*, K33-linked Ub; *48*, K48-linked Ub; *63*, K63-linked Ub; *T4*, TTLL (tubulin tyrosine ligase-like enzymes) 4; *T6*, TTLL6; *ULK1*, UNC-51-like kinase.

protein stability and degradation, as well as protein-protein interactions. Not surprisingly, recent evidence implicates several PTMs in the cGAS/STING pathway (summarized in Fig. 2). TRIM56 and TRIM32, both E3 ubiquitin ligases, can positively regulate STING-dependent signal activation through conjugation of K63-linked poly-ubiquitination of STING (TRIM56 at position K150, and TRIM32 at K20, K224, and K236 (Tsuchida et al., 2010; Zhang et al., 2012). Similarly, K27-linked poly-ubiquitination of STING at four Lysine residues (K137, K150, K224, and K236), mediated by E3 ubiquitin ligase complexes of AMFR (Autocrine motility factor receptor)-GP78/INSIG1 (Insulin-induced gene 1), also leads to positive regulation of STING function (Wang et al., 2014).

More recently, MUL1 (Mitochondrial E3 ubiquitin protein ligase 1) was also shown to be responsible for K63-linked poly-ubiquitination of STING at K224, leading to specific enhancement of IRF3-dependent signaling but

not NF-κB (Ni et al., 2017). Conversely, two distinct ubiquitin ligases, RNF5 (Ring finger protein 5) and TRIM30α (Tripartite motif containing 30α), lead to termination of signal activation through K48-linked poly-ubiquitination of STING at K150 and K275 which target it to subsequent degradation (Wang et al., 2015a; Zhong et al., 2009). Epstein-Barr virus (EBV), a DNA virus, coopts this regulatory property of the DNA sensing pathway to evade host innate immune response. EBV induces expression of TRIM29 which in turn induces K48-linked poly-ubiquitination of STING, and attenuation of STING-mediated antiviral response (Xing et al., 2017).

Proper regulation of signal transduction requires the poly-ubiquitin conjugation system to be reversible. Indeed, inactive rhomboid protein 2 (iRhom2) contributes to stabilize STING protein by recruiting the de-ubiquitinating enzyme EIF3S5 (Eukaryotic translation initiation factor 3 subunit 5) and de-conjugating RNF5-mediated K48-linked poly-ubiquitination (Luo et al., 2016). In addition, it appears that iRhom2 may regulate the translocation of STING in response to DNA through recruitment of ER translocon-associated protein TRAPβ, which was previously identified as a STING-interacting protein (Ishikawa and Barber, 2008). Conversely, the ubiquitin specific protease 20 (USP20) can counteract K33-linked poly-ubiquitination and RNF5-mediated K48-linked poly-ubiquitination of STING, thereby facilitating STING-mediated signaling (Zhang et al., 2016). Similarly, RNF26 catalyzes K11-linked poly-ubiquitination of STING at the same conjugating site as RNF5 (K150), thereby preventing RNF5-mediated poly-ubiquitination of STING, which positively regulates STING function (Qin et al., 2014). Another ubiquitin specific protease, USP18, also participates in this process, suggesting that collaboration between two distinct USPs may catalyze de-ubiquitination of STING for signal activation. Moreover, USP13 has also been shown to act as a de-conjugating enzyme of K27-linked and K33-linked poly-ubiquitination of STING, though the role of K33-linked poly-ubiquitination of STING remains to be defined (Sun et al., 2017). Nevertheless, de-ubiquitinated STING fails to form signaling complexes with TBK1, thereby suppressing signal transduction, supporting a repressive role for USP13.

While multiple ubiquitin ligases have been implicated in directly regulating STING protein, much less is known about their modulatory role on cGAS function. A recent report illustrated that the ubiquitin ligase RNF185 acts as a positive regulator of cGAS via K27-linked poly-ubiquitination at both K173 and K384 and may contribute to the enzymatic activity of cGAS

and promoting synthesis of cGAMP (Wang et al., 2017a). It was also reported that RNF185 expression is elevated in PBMCs of SLE patients, suggesting that it could play a role in auto-inflammatory disorders. Though an initial report described a role for TRIM56 in targeting STING for ubiquitination, it may also conjugate mono-ubiquitin to cGAS at K335 (Seo et al., 2018). Thus, negative regulation of cGAS function by a ubiquitin ligase remains to be identified. In short, while the role of multiple lysine (K)-residues in STING protein has been implicated in regulating its activity, it remains unclear how ubiquitination of STING and cGAS is regulated to orchestrate productive and appropriate immune responses. Additionally, the biological significance and machinery regulating non-degradative poly-ubiquitin linkage (like K27 and K11) of STING remains to be fully resolved.

Recent studies have also implicated SUMOylation in modulating signaling through the cGAS/STING axis. The first report indicated that TRIM38, an E3 ubiquitin ligase, may promote stabilization of cGAS and STING by targeting them for SUMOylation (Hu et al., 2016). In contrast, Senp2, a SUMO-specific protease, was shown to mark these proteins for degradation via proteasomal and chaperone-mediated autophagy pathways. Though cGAS K217 and K464 (which are conserved between mouse and human), and STING K338 (corresponding to K337 in murine STING), were shown to regulate this process, a conflicting report suggested that SUMOylation at K335, K372, and K382 residues of cGAS suppressed functions including DNA-binding, conformational rearrangement, and enzymatic activity—and that SENP7 may reverse this suppression through catalyzing de-SUMOylation of cGAS (Cui et al., 2017).

Other PTMs, including glutamylation, mediated by tubulin tyrosine ligase-like enzymes (TTLLs), have also been shown to be involved in modulating cGAS-mediated immune responses (Xia et al., 2016). Poly-glutamylation and mono-glutamylation of cGAS, mediated by TTLL6 and TTLL4, respectively, negatively regulate cGAS-mediated DNA binding and enzymatic activity. Conversely, the intracellular carboxypeptidases CCP5 and CCP6 counteract mono-glutamylation and poly-glutamylation of cGAS, suggesting that cGAS function is tightly regulated through cellular glutamylation and de-glutamylation. In addition to glutamylation, recent work also implicates palmitoylation, the covalent attachment of fatty acids (like palmitic acid) to cysteine (C) residues of a substrate proteins, in modulating STING (specifically, at positions C88 and C91) mediated signaling and type I IFN production downstream of DNA sensing (Mukai et al., 2016).

Perhaps most recognized and best studied PTM is the phosphorylation of substrate proteins, and STING is no exception. Recent studies have shown that STING possesses a number of serine (S) residues in the C-terminal region that can act as potential phosphorylation sites, though the primary phosphorylation site is Serine-366 (S366) (Konno et al., 2013; Tanaka and Chen, 2012). While TBK1, whose primary target is IRF3, was suggested to be involved in the phosphorylation of STING, in vitro experiments hinted otherwise (Konno et al., 2013). In addition, STING-phosphorylation and downstream degradation appear normal in TBK1-deficient mouse embryonic fibroblasts stimulated with STING agonists, suggesting that if TBK1 does indeed target STING, its role is redundant (Abe and Barber, 2014). Subsequent studies reported that the autophagy-related serine/threonine protein kinases ULK1 and ULK2 as well as the ribosomal protein S6 kinase 1 (S6K1) are involved the phosphorylation of STING (Konno et al., 2013; Wang et al., 2016). While multiple candidate STING kinases are being explored, only one has been implicated in directly controlling cGAS function, and its identity came out of studies on HSV-host interactions. Induced by HSV-1, Akt, also known as protein kinase B (PKB), phosphorylates cGAS at S305 (S291 in murine cGAS), thereby suppressing the synthesis of cGAMP and attenuating cGAS/STING-mediated antiviral responses (Seo et al., 2015). Thus, HSV infection has coopted cellular machinery to negatively regulate cGAS function and evade immune responses. Taken together, while the PTMs that control cGAS/STING function remain to be fully defined, understanding the underlying network that controls these processes may contribute to the establishment of potent therapeutic approaches for tuning cGAS/STING-mediated signaling (see Section 4).

5. AUTO-INFLAMMATORY RESPONSES MEDIATED BY cGAS/STING

While cytosolic sensing of nucleic acids and induction of innate immune responses is critical for eliminating invading pathogens, inappropriate responses to DNA from necrotic or apoptotic cells can result in the development of autoimmune diseases like SLE, AGS (characterized by high levels of anti-nuclear antibodies (ANA) and high levels of circulating pro-inflammatory cytokines). To limit such aberrant inflammatory responses, vertebrates have evolved mechanisms to tightly control the availability of self DNA and regulate inflammatory responses through cGAS/STING dependent signaling (Fig. 3). For example, mice lacking DNase II, which acts in

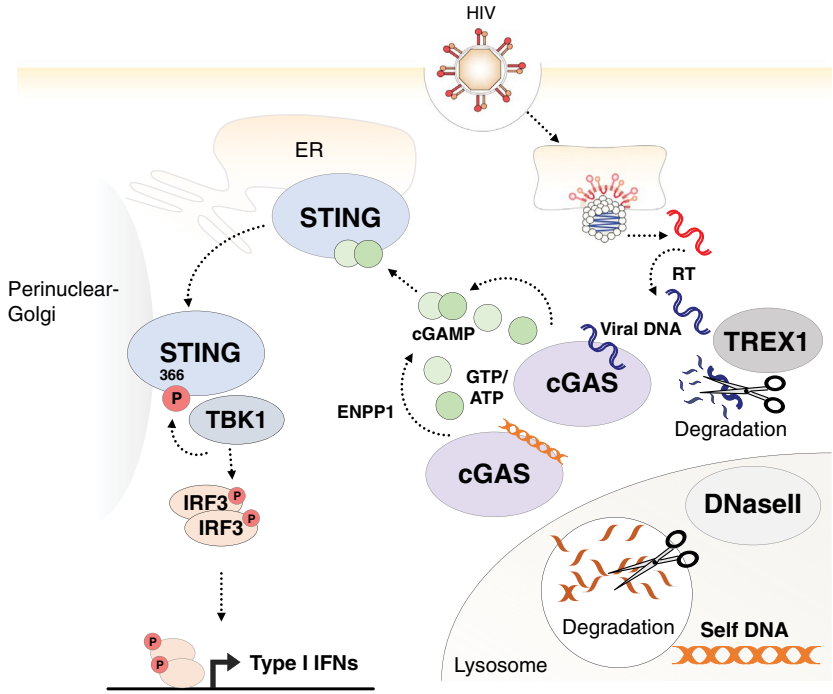

Fig. 3 Intracellular nucleases that modulate cGAS/STING signaling responses: Recognition of self and non-self DNA can augment production of pro-inflammatory genes in virtually all nucleated cells. To prevent an aberrant inflammatory, the three-prime repair exonuclease 1 (TREX1) targets self and non-self DNA for degradation in the cytoplasm, while DNase II plays a similar role in lysosomes. Hydrolysis of cGAMP by ENPP1 also prevents inappropriate activation of STING. *Abbreviations: RT*, Reverse transcription; *HIV-1*, Human Immunodeficiency virus-1.

macrophages to degrade chromosomal DNA derived from apoptotic cells, exhibit embryonic lethality due to anemia induced by the accumulation of undigested DNA (Kawane et al., 2001). Rescue of these mice through deletion of the type I IFN receptor, IFNAR highlights the pathogenic potential of IFN, though aged mice develop TNFα-mediated severe polyarthritis (Kawane et al., 2006). Additionally, mice lacking the three-prime repair exonuclease 1, TREX1 (encoded by DNase III), which degrades nicked dsDNA as well as single-stranded DNA, exhibit a significantly shortened lifespan due to constitutive activation of pro-inflammatory genes across multiple organs (Yang et al., 2007). Similarly, using genetically engineered mice, DNase I has also been implicated in the development of SLE (Napirei et al., 2000), though its role in nucleic acid sensing remains to be elucidated.

Importantly, the dramatic phenotypes observed in both DNase II and TREX1 deficient mice can be reversed through abrogation of cGAS or STING gene expression, suggesting that the cGAS/STING axis can initiate auto-inflammatory diseases (Ahn et al., 2012; Gall et al., 2012; Gao et al., 2015; Gray et al., 2015). Adding credibility to these studies, mutations in TREX1 have also been implicated in AGS and SLE patients. Furthermore, a recent report suggests that mutations in STING (specifically, N154S, V155M, and V147L) may cause vascular and pulmonary syndrome (VAPS), an auto-inflammatory disease characterized by abnormal inflammation across multiple tissues, including skin, vasculature, and lung (Liu et al., 2014)—in vitro interrogation of these mutations revealed that they result in a gain-of-function phenotype and likely contribute to constitutive production type I IFN (Jeremiah et al., 2014). Furthermore, mutations in RNaseH2, which degrades RNA/DNA hybrids, have also been implicated in the development of AGS (Crow et al., 2006), and mutant mice possessing mutated human allele (G37S) die perinatally (Pokatayev et al., 2016). Collectively, these studies and observations highlight the importance of tightly regulating DNA sensing and point to intracellular DNases like DNase II and TREX1 critical gatekeepers in controlling cGAS/STING access to self-DNA.

6. VIRAL STRATEGIES TO MODULATE cGAS/STING FUNCTION

Strong selection pressure, coupled with high mutation rates and short generation times, has led to intricate strategies employed by viruses to counter host immune surveillance and establishment of infection. While the subject is more comprehensively covered by others, here we focus on a few examples from recent reports that highlight the roles of PTMs and protein stability in regulating cytosolic sensing of DNA (Fig. 4). For example, the hepatitis B virus (HBV) polymerase suppresses cytosolic sensing of DNA by interfering with K63-linked poly-ubiquitination of STING (Liu et al., 2015). Though the clinical significance of this observation remains to be resolved—given that hepatocytes lack functional STING dependent signaling (Thomsen et al., 2016)—the data highlight the role of poly-ubiquitination in regulating STING function. Indeed, other viruses have evolved machinery to modulate K63-linked poly-ubiquitination of STING. The human coronavirus (HCoV)-NL63, severe acute respiratory syndrome (SARS) CoV, porcine epidemic diarrhea virus (PEDV) papain-like protease,

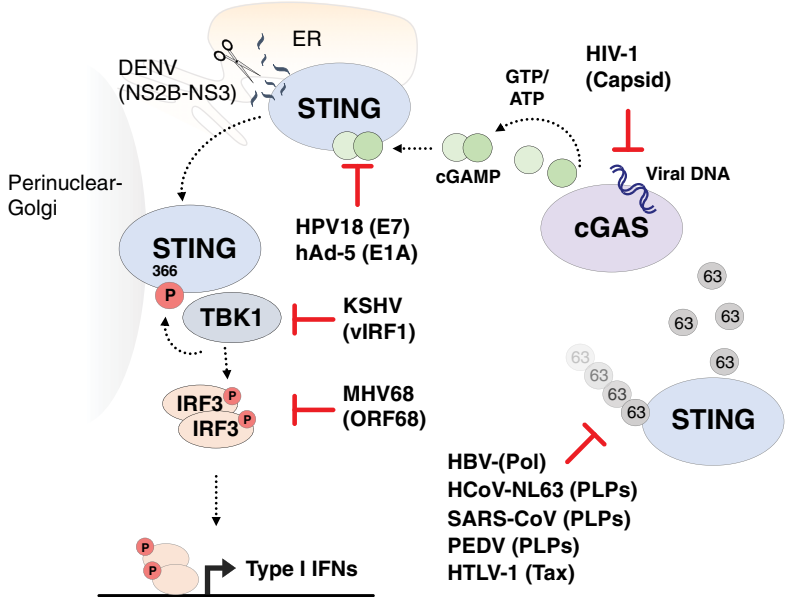

Fig. 4 Viral strategies to evade cytosolic DNA sensing: To escape cytosolic sensing, HIV-1 capsid protein suppresses cGAS-mediated sensing of DNA by recruiting cyclophilin-A (Cyp-A). Other viral proteins, including HTLV-1 Tax protein, HBV polymerase (HBV-Pol), papain-like proteases (PLPs) derived from human coronavirus (HCoV)-NL63, severe acute respiratory syndrome (SARS), and porcine epidemic diarrhea virus (PEDV), interfere K63-linked poly-ubiquitination of STING. E7 and E1A of human papillomavirus 18 (HPV18) and human adenovirus type-5 (hAd5) suppress DNA sensing through direct association with STING. Kaposi sarcoma-associated herpesvirus (KHSV) encodes a viral interferon regulatory factor 1 (vIRF1) to suppress IRF3-mediated production of IFN. The NS2B/NS3 protease of dengue virus (DENV) cleaves human STING at the N-terminal of TM3 region. Finally, murine gammaherpesvirus 68 (MHV68) encodes a de-ubiquitination (DUB) enzyme that contributes to the establishment of latent infection. *Abbreviation*: *HTLV-1*, Human T Lymphotropic Virus type-1; *HIV-1*, Human Immunodeficiency Virus-1; *HBV*, Hepatitis B Virus; *ORF*, Open Reading Frame.

hepatitis C virus non-structural protein 4B (NS4B), as well as the Tax protein of human T lymphotropic virus type-1 (HTLV-1), all suppress STING through either direct physical interactions or regulation of the poly-ubiquitination process (Chen et al., 2014; Ding et al., 2013; Nitta et al., 2013; Sun et al., 2012; Wang et al., 2017b; Yang et al., 2014).

Other examples include the human papillomavirus 18 (HPV18) derived E7 protein and human adenovirus type-5 (hAd5) derived E1A protein, both of which suppress cytosolic sensing of DNA through direct interactions with STING (Lau et al., 2015). An LxCxE motif encoded in both proteins, and

conserved among many DNA tumor viruses, mediates disruption of STING-dependent signaling, suggesting a possible functional link between oncogenesis and antagonization of STING function. Another oncogenic DNA virus, Kaposi sarcoma-associated herpesvirus (KHSV) encodes a viral interferon regulatory factor 1 (vIRF1) gene capable of preventing DNA sensing via direct association with STING (Ma et al., 2015). Additionally, though details remain to be resolved, the murine gammaherpesvirus 68 (MHV68) encoded de-ubiquitination (DUB) enzyme ORF68 was shown to antagonize cytosolic sensing of DNA (Sun et al., 2015). In addition to targeting STING (described above), the HCV employs the NS3/4A protease to target the mitochondrial adaptor IPS-1 for cleavage, thereby short-circuiting the signaling cascade (Chan and Gack, 2016). In a rather exquisite example of host restriction, dengue virus (DENV), a mosquito-borne flavivirus which infects hundreds of millions of people annually, encodes the NS2B/NS3 protease which cleaves human but not murine STING (Aguirre et al., 2012). Importantly, as TBK1 mediated signaling is not suppressed by NS2B/NS3 in murine cells, mice produce higher levels of type I IFNs and effectively restrict DENV replication. Though far fewer examples exist of viral targeting of cGAS, recent reports suggest that capsid proteins of HIV-1 and HIV-2 may dampen cGAS function by recruiting cellular CPSF6 and cyclophilin-A (Lahaye et al., 2013; Rasaiyaah et al., 2013). What is clear is that understanding virus-mediated immune evasion strategies can provide critical insights into regulatory functions that control cellular responses to DNA in the cytosol.

7. MODULATION OF INNATE IMMUNE RESPONSE BY NLRs

The nucleotide-binding domain leucine-rich repeat-containing receptor (NLR) family contains five subfamilies that are subclassified according to the composition of domains in their N-termini—the NLRC family contains a caspase activation and recruitment domain (CARD), and the NLRP family contains a pyrin domain (PYD). NLRs are known for their role in triggering caspases to cleave pro-IL-1β and IL-18 into mature proinflammatory cytokines in response to various pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs) (Saxena and Yeretssian, 2014; Zhong et al., 2013). However, recent advances have revealed that some members of the NLR family

may also regulate tissue homeostasis and modulate innate immune signaling pathways (Kufer and Sansonetti, 2011; Saxena and Yeretssian, 2014; Zhong et al., 2013).

Several NLRPs (including NLRP2, 4, 5, 7, 9, and 14) are expressed primarily in mammalian oocytes, and NLRP5, NLRP7, and NLRP14 have been shown to have important roles in reproduction and development (McDaniel and Wu, 2009; Murdoch et al., 2006; Tong et al., 2000; Westerveld et al., 2006; Zhang et al., 2008). Additionally, NLRC3 and NLRC5, as well as several NLRPs, have been demonstrated to function as negative regulators of PRRs (Table 1). For example, NLRP2 suppresses

Table 1 Summary of Inhibitory Functions Mediated by Inflammasome Family Members

NLRP subfamily	Function	Reference
NLRP2	Negative regulator of TLR/TNFR pathway	Bruey et al. (2004), Fontalba et al. (2007)
NLRP4	Negative regulator of RIG-I/IPS-1 pathway	Cui et al. (2012)
NLRP6/12	Negative regulator of NF-κB (The role of anti-viral in intestinal)	Zaki et al. (2011), Allen et al. (2012), Lupfer and Kanneganti (2013), Chen et al. (2014), Anand et al. (2012), Wang et al. (2015b)
NLRP11	Negative regulator of TLR pathway Negative regulator of RIG-I/IPS-1 pathway	Wu et al. (2017), Qin et al. (2017b), Ellwanger et al. (2018)
NLRP14	Negative regulator of cGAS/STING pathway Negative regulator of RIG-I/IPS-1 pathway	Abe et al. (2017)
NLR		
NLRX1	Negative regulator of RIG-I/IPS-1 pathway Negative regulator of cGAS/STING pathway	Moore et al. (2008), Guo et al. (2016), Qin et al. (2017a), Ma et al. (2017)
NLRC3	Negative regulator of cGAS/STING pathway	Zhang et al. (2014), Tocker et al. (2017)
NLRC5	Negative regulator of TLR pathway	Cui et al. (2010)

both TLR and TNFα-mediated NF-κB activation at the level of IKK complex formation (Bruey et al., 2004; Fontalba et al., 2007). Similarly, NLRP4 inhibits RIG-I signaling by recruiting DTX4, an E3 ubiquitin ligase which marks TBK1 for degradation (Cui et al., 2012). NLRP6 and NLRP12 suppress NF-κB signaling and are involved in the maintenance of intestinal homeostasis and tumorigenesis (Allen et al., 2012; Anand et al., 2012; Chen, 2014; Lupfer and Kanneganti, 2013; Zaki et al., 2011)—more recent reports also point to a role for NLRP6 in anti-viral immunity in mouse intestines (Wang et al., 2015b). NLRX1, the only mitochondrially localized member of this family, modulates sensing of dsRNA and virus-induced ROS production (Guo et al., 2016; Ma et al., 2017; Moore et al., 2008; Qin et al., 2017a; Tattoli et al., 2008). In collaboration with IQGAP1, NLRC3 suppresses DNA sensing lymphoid cells, myeloid cells, and epithelial cells through interactions with STING (Tocker et al., 2017; Zhang et al., 2014). In addition, NLRC5 can suppress TLR signaling by modulating IKK activation (Cui et al., 2010), though NLRC5 deficiency does not affect this signaling pathway (Kumar et al., 2011b). Finally, the primate specific NLRP11 suppresses TLR-mediated activation of NF-κB by targeting TRAF6 for degradation in myeloid cells and B-cells (Ellwanger et al., 2018; Qin et al., 2017b; Wu et al., 2017). Together, these emerging properties call into question whether the established role for this family in activating proinflammatory responses may in fact represent an exception rather than a rule.

8. NLRP14: A NEW RHEOSTAT OF CYTOSOLIC NUCLEIC ACID SENSING

As highlighted above, inappropriate activation of the NAS pathway can result in highly destructive immune responses. Yet, molecular dampeners, structural determinants and topological barriers described above as well as novel checks and balances yet to be discovered tightly regulate the STING/cGAS axis and defend against such circumstances. One instance when distinguishing between self and non-self DNA is particularly important is fertilization, when sperm cell derived DNA can be found in oocyte cytoplasm. Yet, while STING/cGAS and rest of the NAS pathway (which are all expressed in oocytes) are afforded access to this foreign DNA, a nucleic acid sensing response is not triggered. In a recent publication, Abe and colleagues reasoned that germ cells must therefore possess a robust mechanism to negatively regulate cytosolic nucleic acid sensing

(Abe et al., 2017). Through a combination of data-mining and experimental approaches, the group identified 20 candidate genes that may serve as putative regulators of NAS in oocytes. Among these, four (TRIM42, TGIF2LX, C4orf42, ZPLD1) significantly suppressed cGAS-mediated signaling, and one, NLRP14 (NACHT, LRR and PYD Domains-Containing 14; a component of the inflammasome family of proteins), shut off NAS virtually all together. What followed were a series of observations that delineate a role for NLRP14 as a rheostat for nucleic acid sensing, highlight the importance of controlling innate immune responses to foreign and endogenous ligands, and suggest that tight regulation of these processes is critical in maintaining proper immunologic homeostasis in germline. Specifically, the authors demonstrate that NLRP14 associates with both STING and MAVS and prevents downstream signal transduction (Fig. 5). In turn, these adaptor

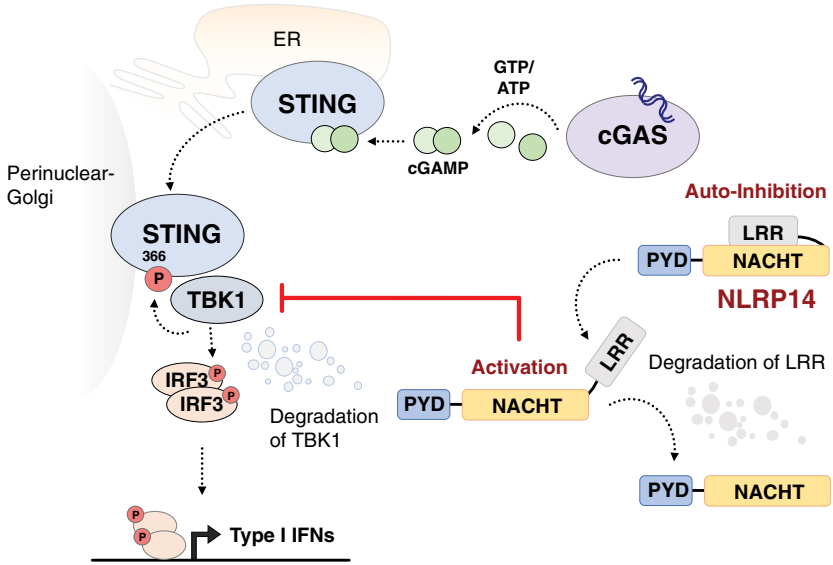

Fig. 5 NLRP14-mediated inhibition of cytosolic nucleic acid sensing. Upon activation of STING dependent signaling, conformational rearrangement from inhibitory-form to an active-form of NLRP14 is induced. Targeting of TBK1 for K48-linked poly-ubiquitination is then initiated and results in proteasome dependent degradation of TBK1 and termination of STING dependent as well as RIG-I dependent signaling. Similarly, NLRP14 is targeted for degradation to avoid persistent immunosuppression. *Abbreviations: NLRP14*, Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing protein 14; *PYD*, Pyrin domain; *NACHT*, NAIP (neuronal apoptosis inhibitory protein), CIITA (MHC class II transcription activator), HET-E (incompatibility locus protein from Podospora anserine) and TP1 (telomerase-associated protein), *LRR*, Leucine-rich repeat; *Ub*, Ubiquitin; *P*, Phosphorylation.

molecules induce the proteasomal degradation of NLRP14, a feedback loop that may be critical in preventing persistent immunosuppression and proper induction of innate immune responses under appropriate conditions (for example, post fertilization of oocytes). Importantly, several human diseases, including Crohn's disease, Celiac disease, Blau syndrome, rheumatoid arthritis, Type-1 and -2 diabetes, SLE, Cryopyrin-associated periodic syndromes (CAPS), inflammatory bowel diseases, colitis as well as colon cancer, have been shown to be associated with mutations in NLRPs (Saxena and Yeretssian, 2014; Zhong et al., 2013). Other NLRPs (like NLRP2, NLRP5, and NLRP7) have been associated with miscarriage and infertility (Docherty et al., 2015; Huang et al., 2013). In line with these observations ectopic expression of a K108X NLRP14 allele (rs76274604; coding for a nonsense mutation that introduces an early STOP codon in NLRP14) results in reduced suppression of TBK1-mediated signaling (Abe et al., 2017). With an allele frequency of 1.7% in the human population, and a minor allele frequency of 3% in East Asian and Ad Mixed American populations, infertility associated with homozygosity of this gene may affect 3 in 10,000 individuals. As an immunological rheostat, NLRP14 safeguards against inappropriate cytosolic responses to nucleic acids and the acquisition of such a function may have been a prerequisite to sexual reproduction. Indeed, homologs of the inflammasome family of proteins have been found widely across life—arguing in favor of their involvement in immunity and broader cellular processes, as is observed with other expanded gene families.

9. CONCLUDING REMARKS

The past decade has seen rapid advancement in our understanding of how cells sense cytosolic DNA, including the identification of receptors and molecular machinery that participate in this process as well as recognition of multiple diseases associated with this pathway. Recent findings highlight the importance of controlling innate immune responses to DNA and suggest that tight regulation of these processes is critical in maintaining proper immunologic homeostasis across multiple organs. Thus, understanding the underlying network of regulators that control signaling through the cGAS/STING axis will undoubtedly contribute to the development of potent therapeutic agents against auto-immune and inflammatory diseases, as well as certain cancers.

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