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A Proteomics Perspective on Viral DNA Sensors in Host Defense and Viral Immune Evasion Mechanisms

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Abstract

The sensing of viral DNA is an essential step of cellular immune response to infections with DNA viruses. These human pathogens are spread worldwide, triggering a wide range of virus-induced diseases, and are associated with high levels of morbidity and mortality. Despite similarities between DNA molecules, mammalian cells have the remarkable ability to distinguish viral DNA from their own DNA. This detection is carried out by specialized antiviral proteins, called DNA sensors. These sensors bind to foreign DNA to activate downstream immune signaling pathways and alert neighboring cells by eliciting the expression of antiviral cytokines. The sensing of viral DNA was shown to occur both in the cytoplasm and nucleus of infected cells, disproving the notion that sensing occurred by simple spatial separation of viral and host DNA. A number of omic approaches, in particular mass spectrometry-based proteomic methods, have significantly contributed to the constantly evolving field of viral DNA sensing. Here, we review the impact of omic methods on the identification of viral DNA sensors, as well as on the characterization of mechanisms involved in host defense or viral immune evasion.

Introduction

The ability of mammalian cells to recognize pathogenic nucleic acids has long been established as an essential step in the initiation of intrinsic and innate immune responses. Viral pathogens can be diverse in terms of their DNA or RNA genomes and subcellular sites of viral replication. During infection, the viral nucleic acids, which are deposited in the nucleus or cytoplasm of infected cells, act as potent stimulators of immune response pathways that induce the expression of a variety of interferons and inflammatory cytokines (Figure 1). Detection of nucleic acids is part of the inbuilt cellular patrol achieved by proteins known as pattern recognition receptors (PRRs). These receptors can detect pathogen-associated molecular patterns (PAMPs), which, in addition to nucleic acids, can include proteins, lipids, glycans, and glycolipids^{1; 2; 3}. Remarkably, mammalian cells can distinguish self from non-self despite similarities between these PAMPs and equivalent cellular molecules.

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Given their essential roles in cellular defense, significant effort has been placed on the discovery of PRRs and the characterization of the mechanisms involved in nucleic acid detection and subsequent pathway-specific induction of immune response. The discovery of Toll-like receptors was the initial driving force for studying PRRs. A wealth of important information was accumulated with regard to mechanisms of viral RNA detection, in part made possible by the identification of differences between viral and cellular RNA. For example, the Rig-I-like receptors (RLR) sense pathogenic RNA by recognizing several PAMPs, which include 5'triphosphate RNA, processing signatures by RNaseL, and properties related to RNA length and structure (reviewed in ^{4; 5}). In contrast to the growing understanding of RNA sensors, the knowledge regarding DNA sensors is not yet as mature.

In order for a protein to be classified as a viral DNA sensor, the protein must satisfy two requirements: 1) It must physically interact with viral DNA, and 2) It must subsequently stimulate an innate immune program, such as the expression of interferons (IFN α , β , γ , λ) and/or chemokines and proinflammatory cytokines (e.g. IL-1 β , IL-18, IL-6, TNF, CXCL10, and CCL5). The endosomal, membrane-bound Toll-like receptor 9 (TLR9) was the first identified DNA sensor⁶. TLR9 recognizes unmethylated cytosine-phosphate-guanine (CpG) nucleotide sequences, which are abundant in many DNA viruses and bacterial pathogens, but occur infrequently in the human genome^{7; 8; 9; 10}. However, immune responses to a variety of DNA stimuli were induced in the absence of TLR9, predicting the presence of other DNA sensors^{11; 12; 13; 14}. Additionally, cells were able to respond to adenine-thymine (AT)-rich DNA even following chemical inhibition of TLR9¹⁵. As foreign and mammalian DNA have similar features, including AT-rich regions, these findings suggest that distinguishing self from non-self may be more challenging for DNA sensors than for their RNA sensing counterparts. How then can cells recognize foreign DNA? For most viral DNA sensors, this still remains an open question. Given the lack of a clear difference between host and viral DNA, the recognition of foreign DNA was thought for many years to rely primarily on compartmentalization, i.e. the spatial separation between virus and host DNA. The first cytoplasmic viral DNA sensor, the DNA-dependent activator of IFN-regulatory factors (DAI), was only discovered within the last decade¹⁶, and since then, the characterization of cytoplasmic sensors has been the focus of most DNA sensing studies. These studies have been the main contributors to expanding the field of DNA sensing, leading to the discovery of several cytoplasmic viral DNA sensors, including Absent in melanoma 2 (AIM2), cyclic-GMP-AMP synthase (cGAS), DNA-protein kinase (DNA-PK), DEAD box polypeptide- 41 and -60 (DDX41 and DDX60), DEAH box helicase -9 and -36 (DHX9 and DHX36), Interferon inducible protein 16 (IFI16), and RNA polymerase III (RNA Pol III) (Table 1). Two other proteins, Mre11¹⁷ and Lrrfip1¹⁸, have also been described as cytosolic sensors of exogenous DNA, but the roles of these proteins in sensing in response to DNA virus infections have not yet been characterized. However, this sole understanding of cytoplasmic sensing brought a conceptual challenge. The majority of DNA viruses are known to replicate their genomes in host cell nuclei as a part of their natural viral life cycle (Figure 1A). These viruses include wide-spread human pathogens, such as human cytomegalovirus (HCMV), herpes simplex virus 1 (HSV-1), adenovirus (AdV), hepatitis B (HBV), and human papilloma virus (HPV). In certain immune cells, such as macrophages and plasmacytoid dendritic cells (pDCs), the cellular cytoplasmic environment can cause the

leakage of viral DNA, for example by proteasomal-mediated degradation or proteolysis of viral capsid proteins. As a result, the viral DNA is partly exposed in the cytoplasm¹⁹ or in endosomes^{20; 21}, allowing for sensing in these subcellular compartments (Figure 1B). However, in many natural cellular hosts of these viruses (non-immune cells), the viral DNA is protected by capsid proteins during its transit through the cytoplasm, prior to its deposition in the nucleus (Figure 1A). Therefore, it became evident that mammalian cells must have some means to also recognize the presence of viral DNA in the nucleus, and that proteins capable of nuclear sensing should exist. Indeed, in recent years, this emerging field of DNA sensing was further expanded by the identification of a nuclear DNA sensor, the interferon inducible protein IFI16^{22; 23; 24; 25}. More recently, another interferon inducible protein, IFIX, was also shown to act as a sensor and bind nuclear HSV-1 DNA in infected cells²⁶. Overall, the development and application of omic technologies have had a significant impact on this field²⁷, aiding the discovery and characterization of both cytoplasmic and nuclear DNA sensors. Here, we provide an overview of the field of DNA sensing, with an emphasis on the contribution of proteomic approaches to the discovery and characterization of viral DNA sensors. For a broader view of microbial immune sensing, we also point the readers to additional valuable reviews^{28; 29; 30}. In this review, we discuss omic workflows that have been used for studying virus-host interactions in the context of DNA sensing, including both DNA-protein and protein-protein interactions. We next elaborate on the mechanisms by which these interactions induce immune responses. We go on to review the current knowledge regarding the mechanisms that viruses have evolved to block immune response. We also emphasize the role of post-translational modifications (PTMs) of either host or viral proteins that contribute to host defense or viral immune evasion. Lastly, we provide a perspective for both the future of proteomics in studying viral DNA sensors and important standing questions regarding the intracellular recognition of viral nucleic acids.

A. DNA-protein interactions during viral DNA sensing

Proteomic methods employed for discovering viral DNA sensors

Technological advances in methods that integrate mass spectrometry-based proteomics within studies of DNA-protein interactions have contributed significantly to the field of viral DNA sensing (Table 1). One commonly used approach involves affinity purification (AP) in conjunction with analysis by mass spectrometry (MS). The isolation of proteins that interact with DNA can be performed using beads conjugated to dsDNA (or ssDNA) or by spiking the sample with biotinylated DNA and performing the AP with streptavidin beads. The isolated proteins are usually subjected to proteolytic digestion (in-solution or in-gel following SDS-PAGE separation) and identified by MS (Figure 2). In fact, five of the twelve viral DNA sensors (Table 1) were identified using this type of proteomic approach, including AIM2³¹, IFI16³², DNA-PK^{33; 34}, and the DEAH/RNA Helicases DHX9 and DHX36³⁵. Additionally, two other DNA sensors, RNA Pol III³⁶ and cGAS³⁷, were characterized using a modified proteomic approach, by coupling biochemical fractionation with MS. One example of an elegant integration of OMIC technologies came from the laboratory of Yong-Jun Liu, where the RNA helicases DHX9 and DHX36 were discovered as DNA sensors³⁵, and an RNA sensing complex consisting of 3 DEXD/Hbox helicases was identified by AP-MS/MS³⁸. These findings led them to expand these studies using a

bioinformatic approach to find additional DExD/Hbox helicases that may have nucleic acid sensing capability³⁹. By coupling MS data with bioinformatic tools, they identified DDX41, which was further experimentally validated as a new viral DNA sensor³⁹. Most recently, the interferon inducible protein X (IFIX) was discovered as a viral DNA sensor by using an integrative proteomic, virology, and molecular biology approach²⁶. Thus, nine out of 12 viral DNA sensors were identified through approaches integrating mass spectrometry. Collectively, these studies emphasize the value of omic approaches for identifying previously unknown viral DNA sensors. One technical challenge to consider when performing AP-MS experiments is distinguishing DNA-protein from protein-protein interactions. Comparisons done in the presence or absence of Dnase I or Benzonase treatment can help prioritize the proteins identified from AP-MS for follow-up studies. Several techniques, such as *in vitro* electromobility shift assay (EMSA)⁴⁰ and the α -screen⁴¹, described below, can help determine if the protein binds directly to DNA or if it interacts with DNA indirectly through another protein.

AIM2 was reported as a DNA sensor by multiple groups around the same time^{31; 42; 43}. In addition to the AP-MS approach described above³¹, AIM2 was also identified by other groups using an α -screen⁴³. The α -screen allows DNA-protein or protein-protein interactions to be studied using basic donor-acceptor principles, where microbeads are coated with the molecules of interest and placed in the cell lysate. For example, if photosensitive donor beads come in close proximity to the chemiluminescent acceptor beads, the interaction is detected spectrophotometrically in a microplate reader (as reviewed in ⁴¹). Both MS-based and α -screen approaches were also valuable in identifying IFI16, the other AIM2-like receptor (ALR), as a sensor and to validate its interaction with DNA³².

Only three viral DNA sensors were found independently of the use of mass spectrometry, TLR9, DAI, and DDX60. Of note, two of these (TLR9 and DAI) were the first two viral DNA sensors to be reported, suggesting that advances in MS-based proteomics and the resulting increase in the popularity of these methods have helped to expand this field with the discovery of additional viral DNA sensors. TLR9 was initially shown to recognize CpG motifs in bacterial DNA⁶. Later, surface plasmon resonance (SPR) was used to demonstrate a direct interaction between TLR9 and CpG DNA⁴⁴, and, more recently, a bioinformatics approach was employed to model the 3D structure of the TLR9-CpG complex⁴⁵. It should be noted that current technology now allows bridging SPR with protein identification by MS⁴⁶. Several years after the discovery of TLR9, DAI, the first cytosolic DNA sensor, was described¹⁶. DAI, previously known as DLM-1 or ZBP-1, was predicted to act as a sensor given its identification in a DNA chip screen and its upregulation upon IFN β or B-DNA stimulation¹⁶. Indeed, its requirement for IFN response to transfected vaccinia virus (VACV) DNA was observed in L929 fibroblasts¹⁶. Furthermore, the co-localization of DAI with B-DNA was demonstrated by Fluorescence Resonance Energy Transfer (FRET), and their interaction was confirmed by isolation assays using biotin-labeled B-DNA and streptavidin beads, followed by western blotting (AP-WB)¹⁶. DDX60 was identified using a similar approach. A DNA microarray was used to identify potential antiviral genes, followed by a gel shift assay to confirm DDX60 binding to nucleic acids and IFN β induction following herpes simplex 1 (HSV-1) infection⁴⁷. Altogether, these findings portray the

evolution of DNA-protein interaction technology. The characterization of the first viral DNA sensors (TLR9, DAI, and DDX60) was predicated on knowing the identity of the protein itself. Conversely, the identities of the other eight viral DNA sensors were found without a priori knowledge of their identity, which underscores the value of mass spectrometry-based approaches in the growing field of DNA sensing.

Structural insights into DNA-protein interactions

While several DNA sensors, TLR9, DHX9, DHX36 and RNA Pol III, were shown to have preference for binding to GC or AT content^{6; 9; 15; 35; 44}, other sensors, such as AIM2⁴⁸, IFI16⁴⁸, IFIX²⁶ and cGAS⁴⁹, are able to effectively bind DNA in a sequence-independent manner. This is an interesting difference, exemplifying distinct host defense strategies to recognize viral invasion. The ability to bind viral DNA in a sequence-independent manner can point to broad-spectrum antiviral factors that are efficient against numerous pathogens. Structural studies have contributed not only to elucidating how these DNA sensors bind to DNA, but also to how signaling events are mediated through changes in protein conformation. Here, we highlight the significance of these structural studies for characterizing AIM2, IFI16, and cGAS, and the downstream IFN β effector protein Interferon regulatory factor 3 (IRF3). The AIM2-like receptors AIM2 and IFI16 are members of the PYHIN family. This family is termed PYHIN due to the two domains that characterize these proteins (Figure 3); the pyrin (PY) domain that mediates homotypic interactions with other pyrin domain-containing proteins, and one or two HIN domains that bind DNA. The HIN domains of AIM2 and IFI16 have been shown to bind DNA in a sequence independent manner (⁴⁸ and reviewed in⁵⁰). The positively charged residues of the HIN200 domain bind DNA via electrostatic attraction to the negative charge of the sugar-phosphate backbone of DNA, as determined by x-ray crystallography and molecular replacement⁴⁸. For both AIM2 and IFI16, the DNA binding interface is formed by two oligonucleotide/oligosaccharide binding (OB) folds within each HIN domain⁴⁸. In agreement with this, the recently reported DNA sensor, IFIX, which also contains a HIN domain, was shown to bind DNA in a sequence-independent manner²⁶. Due to the sequence similarities between these PYHIN proteins, it is likely that IFIX also binds DNA via an OB fold electrostatic mechanism. The studies by Jin et al. also predicted the likely mechanism for AIM2 in sequestering inflammasome components, whereby the pyrin domain is autoinhibited by the HIN domain until its release through HIN domain binding to DNA^{48; 51}. This may be a common auto-inhibition mechanism for other PYHIN proteins.

The crystal structure of cGAS also revealed why this sensor can bind DNA in a sequence independent manner, as cGAS associates with a span of 7 nucleotides by binding the sugar-phosphate strands in the minor groove⁴⁹. Two arginine residues further stabilize the cGAS-DNA interaction by their insertion into the minor groove⁴⁹. Additionally, another study showed the requirement of a 2:2 stoichiometry of cGAS-DNA⁵², demonstrating the requirement of a key lysine residue (mouse K335 ; human K347) for dimer formation and interaction with DNA, both of which are important for IFN β induction via IRF3 activation⁵². cGAS was also observed in an autoinhibited state until it bound to its ligand, which caused a conformational change in its activation loop⁵². Intriguingly, this conformational change only occurred upon cGAS binding to B-form DNA, and was not observed upon binding to A-

form RNA. The authors reasoned that the lack of conformational change in cGAS in complex with dsRNA could explain why cGAS-dependent RNA sensing had not been observed⁵². However, in another study, cGAS was observed to play an antiviral role during infection with RNA viruses⁵³, suggesting a possible interplay between the DNA and RNA sensing pathways.

A common mechanistic feature of DNA sensing activity described above is protein autoinhibition prior to the conformational change induced by the protein-DNA interaction. These conformation changes induce dimer or oligomer formation, which is required for downstream IFN signaling. IRF3, a crucial component in Stimulator of interferon genes (STING)-dependent IFN activation (Figure 3 and section below), has also been shown to be autoinhibited, though the mechanism of release is different. The N- and C-terminal domains of IRF3 interact to hide a hydrophobic surface implicated in IRF3 oligomerization and subsequent immune response⁵⁴, with phosphorylation likely serving to release the autoinhibited state⁵⁴. It remains to be determined how many DNA sensors and pathway-associated proteins are self-controlled in a similar manner. Structural studies are expected to continue to significantly contribute to understanding the mechanisms involved in DNA sensing.

B. Protein-protein interactions involved in host immune response

Upon binding to viral DNA, the sensors rely on the formation of cascades of protein-protein interactions that mediate downstream immune signaling. In this section, we review the main pathways through which DNA sensors are known to signal (Figure 3).

Protein interactions within the STING-dependent DNA sensing pathway

Currently, several identified viral DNA sensors (IFI16, cGAS, DDX41, DNAPK, and likely DAI and IFIX) have been shown to stimulate an immune response that signals through the stimulator of interferon genes (STING) (Table 1 and Figure 3). Although the signaling events upstream of STING are still being elucidated, the downstream signaling cascade starts to be well understood. The adaptor protein STING is an endoplasmic reticulum resident protein⁵⁵. Upon stimulation, STING forms a dimer and recruits TANK binding kinase 1 (TBK1) and IRF3^{55; 56; 57}, triggering a signaling cascade mediated by several phosphorylation events (Figure 3). Specifically, STING binding stimulates the autophosphorylation of TBK1 at Ser172^{58; 59}. TBK1 then phosphorylates IRF3 on a serine/threonine cluster, which enables IRF3 homodimer formation and interaction with CREB-binding protein and p300^{60; 61}. Two different potential sites for IRF3 phosphorylation, site 1 (Ser385 or Ser386) and site 2 (localized between Ser396 and Ser405), have been reported, yet the necessity of phosphorylation at both sites and the order of the phosphorylation events have been debated^{62; 63; 64; 65; 66}. Panne and colleagues used constitutively activated TBK1 *in vitro* to demonstrate that phosphorylation at site 2 relieves IRF3 autoinhibition, which allows phosphorylation at site 1 and IRF3 dimerization and activation⁶⁷. However, Bergstroem et al. performed MS/MS studies on IRF3 in human embryonic kidney (HEK)293 cells infected with Sendai virus, and *in vitro* studies with TBK1, which indicated that phosphorylation at site 1 precedes that of site 2⁶⁸. In addition these studies identified constitutive phosphorylations at Ser173 and Ser175, as well as a new site of phosphorylation

by TBK1 during viral infection at Thr390⁶⁸. While more studies are needed to fully characterize the temporality of these events, the importance of phosphorylation is well established for the nuclear localization of the IRF3 complex, where it binds to type I IFN promoters to activate transcription^{62; 69}. Type I IFNs then work in an autocrine and paracrine manner to elicit innate immune response and the expression of antiviral cytokines.

Several studies have indicated that IFI16 triggers immune signaling through the STING-dependent pathway. Interestingly, IFI16 is the one sensor shown to sense foreign DNA in both the cytoplasm and the nucleus^{23; 24; 25; 32}, and published reports have associated both of these signaling events with the STING pathway. The dual localization of IFI16 seems to be cell type-dependent, expanding its range of sensing. Indeed, IFI16 has been reported to induce the expression of antiviral cytokines in response to nuclear-localized DNA during HSV-1, HCMV, and KSHV infections, and to cytoplasmic-localized VACV DNA and proviral DNA from human immunodeficiency virus (HIV) (Table 1).

IFI16 sensing ability was first identified in the cytoplasm, in cells transfected with VACV DNA³². A co-IP assay demonstrated an interaction between IFI16 and the adaptor protein STING, suggesting that IFI16 cytoplasmic sensing signals through this pathway³². Later, a study integrating AP-MS with mutagenesis, microscopy, and cytokine assays, demonstrated that the localization of IFI16 can be modulated by its acetylation²³ (Figure 3). Targeted mass spectrometry determined that, in several different cell types, endogenous IFI16 is acetylated at residues Lys99 and Lys128 within its multipartite nuclear localization signal (NLS). The acetylation was shown to prevent the nuclear import of IFI16, leading to its accumulation in the cytoplasm and allowing for sensing in response to transfected VACV²³. Furthermore, cytoplasmic sensing by IFI16 has been observed following HSV-1 infection in macrophages, where the viral capsid is broken down prior to the docking of the viral capsid at the nuclear pore¹⁹. This process was shown to involve the ubiquitination and proteasomal degradation of the HSV-1 capsid protein Vp5¹⁹. More recently, similar IFI16 cytoplasmic sensing in primary macrophages has been reported in response to ssDNA from HIV proviral DNA⁷⁰.

While a subset of IFI16 is localized to the cytoplasm in certain cell types, its predominant localization is nuclear. Indeed, IFI16 was identified as the first known protein able to detect viral DNA in the nucleus. Importantly, using NLS deletion mutants, IFI16 nuclear localization was demonstrated to be required for the efficient binding to viral DNA and for eliciting IFN- β response following HSV-1 infection²³. In agreement, another study showed that IFI16 was retained in the nucleus during the early stages of HSV-1 infection²⁴. This was also the case for the infection with another nuclear-replicating virus, HCMV, as IFI16 was observed to remain nuclear early in infection and to be required for triggering antiviral cytokine expression following its binding to HCMV DNA²⁵. Interestingly, although IFI16 binds viral DNA in the nucleus, these studies have shown that following both HSV-1 and HCMV infections, the immune signaling occurs through the STING-dependent pathway^{24; 25}. Therefore, a question that remains to be addressed is how the signals are transmitted between IFI16 and STING. A yet-to-be identified protein or a small molecule may interact with IFI16 to facilitate signaling.

Another protein in the same PYHIN family as IFI16, the interferon inducible protein IFIX, was also recently found to act as a DNA sensor²⁶. Similar to IFI16, IFIX may also function both in the cytoplasm and nucleus. In response to transfection with VACV 70mers, IFIX was observed to co-localize with cytoplasmic viral DNA and to aid the induction of IFN β , thereby satisfying both requirements for a DNA sensor²⁶. Interestingly, IFIX retained its predominant nuclear localization during early HSV-1 infection and was shown by chromatin immunoprecipitation to bind HSV-1 DNA in infected cells²⁶. Its antiviral function was further strengthened by the demonstration that IFIX knockdown triggers an increase in HSV-1 titer. As HSV-1 is a nuclear replicating virus in non-immune cells, this study points to IFIX as the second DNA sensor that associates with nuclear viral DNA. Due to the similarities in the domain structures, localization, and functions of IFIX and IFI16, it is possible that the induction of IFN β by IFIX involves the STING-dependent pathway, although this remains to be investigated.

Using a multi-disciplinary approach that worked backwards from STING activation, cGAS was also recently identified as a potent cytoplasmic DNA sensor⁷¹. After establishing that the activator of STING was not protein, DNA or RNA, a mass spectrometry approach was utilized to discover that it was a small molecule, the cyclin guanosine monophosphate-adenosine monophosphate (cGAMP). The cyclic GMP-AMP synthase cGAS was then shown to bind DNA, to catalyze the synthesis of cGAMP in a DNA-dependent manner, and to be required for IFN induction^{37; 71; 72}. Additionally, cGAS knockdown led to reduced levels of IRF3 dimers in response to HSV-1. Collectively, these studies established cGAS as a STING-dependent viral DNA sensor where the interactions of cGAS in this immune program are cGAS with DNA, cGAS with its product cGAMP, and cGAMP with STING. Whether cGAS and IFI16 have interdependent sensing functions remains to be established. However, a recent study demonstrated that IFI16, cGAS, and STING are all necessary for eliciting cytoplasmic sensing in macrophages in response to *Listeria monocytogenes* infection, and that the resulting IFN β expression was independent of the presence of cyclic-di-AMP, but rather was dependent on *Listeria* DNA⁷³. Interestingly, this study indicated that IFI16 was necessary for sustained IFN induction and that, although acting through the same STING-dependent pathway in response to *Listeria* DNA, IFI16 and cGAS do not seem to be redundant⁷³. Therefore, interconnected functions between different DNA sensors, such as IFI16 and cGAS may exist. Indeed, another study indicated that following HIV-1 infection IFI16 signals through a pathway dependent on cGAS and STING⁷⁰. In terms of viral DNA sensing, it is not yet understood whether IFI16 and cGAS function through parallel or within the same pathway(s), and whether the coordination of their functions is also expanded to the nucleus.

The DNA sensor DDX41 was found to bind directly to STING and to induce IFN β in response to either transfected DNA or HSV-1 infection³⁹. The Asp-Glu-Ala-Asp (DEAD) domain of DDX41 was shown to act as the DNA binding domain, and the shRNA-mediated knockdown of either DDX41 or STING led to severe reductions in IFN expression³⁹. DDX41 has also been shown to play a role in sensing AdV in the mouse macrophage-like cell line RAW264.7⁷⁴. In this study, the immune response was tested by detecting levels of activated IRF3 (phosphorylation level at pSer388), which were reduced when DDX41,

STING, or TBK1 were knocked down⁷⁴. Although direct interactions were not tested, this study indicated a correlation between DDX41 and AdV DNA sensing.

DNA-dependent protein kinase (DNA-PK) is a complex made up of the Ku70/Ku80 heterodimer and the catalytic subunit of the kinase-DNA-PKcs and is best known for its role in DNA repair⁷⁵. Recently, this complex was identified as a cytoplasmic viral DNA sensor that activates IFN λ 1 in response to HIV³³. Although the transcriptional regulation of type III IFNs has not been elucidated in response to many viruses, IFN λ 1 was shown to be regulated by IRF7, NF- κ B, and IRF3 in HepC infected hepatocytes⁷⁶, which suggests the possible involvement of STING-TBK1. Additionally, Ferguson et. al. showed that the induction of IFN β in response to mouse VACV (mAV) or HSV-1 was reduced following knockdown of *prkdc* (gene for DNA-PKcs)³⁴. The immune response to mAV was abolished in the absence of IRF3, which implicated the STING pathway³⁴.

There is also evidence that DAI-mediated IFN induction may also occur via the STING-dependent pathway, whereby an interaction between DAI, IRF3, and TBK1 has been observed¹⁶; the latter two proteins are a requirement for STING-dependent IFN activation (Figure 3). The induction of IFN expression in response to viral DNA is known to be dependent on STING in the cells in which DAI was shown to function (i.e., mouse fibroblasts)⁷⁷, further supporting its likely signaling through a STING pathway. However, after the identification of DAI, several other viral DNA sensors (described above) have been discovered and shown to be more potent activators of IFN response. Therefore, it remains to be determined whether the function of DAI in this response is redundant. Recent evidence suggests the involvement of DAI in another antiviral response, through pathway the receptor interacting protein 3 (RIP-3)-dependent necrosis pathway⁷⁸ (see section below).

Interactions that lead to the formation of the inflammasome

In addition to the STING-dependent pathway, selected DNA sensors have been shown to trigger immune response through the formation of an inflammasome. The inflammasome (reviewed in ^{79; 80}) is a complex with important roles in defense against foreign pathogens, triggering immune response through the maturation and subsequent release of proinflammatory cytokines such as IL-1 β , and IL-18. Cytokine maturation is carried out through the proteolytic activity of caspase 1, which is recruited to the inflammasome in its immature form (procaspase 1) via its C-terminal caspase-recruitment domain (CARD). Currently, in the context of DNA sensing, only the ALRs (i.e., AIM2 and IFI16) have been shown to elicit the formation of inflammasome macromolecular assemblies^{22; 31; 42; 43}. These inflammasomes are composed of the DNA sensor (AIM2 or IFI16), caspase 1, and the apoptosis-associated speck-like protein containing a CARD (ASC). The N-terminal pyrin domain of ASC mediates homotypic pyrin domain interactions with AIM2 or IFI16, while its CARD binds procaspase 1 to trigger the formation of the heterotetrameric caspase 1. Furthermore, the PTM status of ASC modulates inflammasome formation, either by inhibiting or promoting protein interactions. Hara et al. reported that phosphorylation of ASC Tyr146 (Tyr144 in mouse) is needed for speck aggregate formation and subsequent cytokine signaling of IL-18⁸¹. Indeed, small molecule inhibition or knockdown of Syk and Jnk kinases reduced inflammasome formation upon exposure of cells to foreign DNA (i.e.,

poly (dA:dT))⁸¹. Martin et al. found that I κ B kinase α (IKK α) downregulates inflammasome formation through its interaction with ASC⁸². ASC is phosphorylated by IKK α at residues Ser193 and Ser16, which triggers the segregation of ASC-IKK α to the perinucleus. Another phosphorylation event, the IKKi phosphorylation of Ser58 on ASC, leads to the disassembly of the ASC-IKK α association, reestablishing the formation of the inflammasome⁸². Protein kinase R (PKR) was also shown to regulate inflammasome formation in response to poly (dA:dT)⁸³. PKR was found to interact with several inflammasome components, including AIM2, NLRP3 and ASC⁸³. PKR knockdown in macrophages or the chemical inhibition of PKR autophosphorylation showed diminished AIM2 inflammasome formation, although no phosphorylations were detected on inflammasome components⁸³. Interestingly, the requirement of PKR in inflammasome activation has recently been challenged, as He et al. found PKR to be dispensable for inflammasome activity⁸⁴. As these studies were done in a similar system (i.e., macrophages from Pkr $^{-/-}$ mice) and both used induction with poly(dA:dT), it remains to be determined whether other differences in the experimental workflows or inflammasome formation readouts triggered this discrepancy in results. Further studies will help resolve this issue. In addition to the role of phosphorylation in inflammasome regulation, ubiquitination was also shown to regulate ASC aggregation. Inhibition of deubiquitinase (DUB) activity was reported to impede ASC aggregation, although this occurs to a lesser degree in AIM2 inflammasomes responding to cytoplasmic DNA than NLRP3 inflammasomes responding to lipopolysaccharide, a component of the outer membrane of bacteria⁸⁵.

Interesting is the seemingly cell-type and, in some cases, virus-type specific inflammasome responses. These differences may reflect the differential expression of inflammasome components in different cell types, the subcellular localization of the viral dsDNA, the ability of host cells to induce immune response through diverse mechanisms, and the ability of certain viruses to suppress immune responses by specifically blocking inflammasome formation. For example, to date, AIM2 inflammasome formation has been demonstrated only in macrophages, dendritic cells, and keratinocytes, whereas IFI16 inflammasomes have been shown in fibroblasts and endothelial cells. In macrophages and dendritic cells, AIM2 inflammasomes formed in response to VACV and mouse cytomegalovirus (MCMV), but not in response to HSV-1⁸⁶. AIM2 was also shown to play a role in sensing adenovirus (AdV) in RAW264.7 mouse macrophages; however, AIM2 inflammasome formation was not evaluated. These results showed an AIM2- and DDX41-dependent decrease in phosphorylated IRF3⁷⁴, suggesting the involvement of the STING-dependent pathway. In a clinical study, AIM2 was found to sense hepatitis B (HBV) DNA in human glomerular mesangial cells, where IL-18 levels were decreased in response to transfected Hep-B DNA when AIM2 was knocked down⁸⁷. Finally, in keratinocytes, AIM2 inflammasome formation was seen in response to human papilloma virus (HPV-16) DNA⁸⁸. The selection of the cell type in which the studies are performed is critical, as the ability of different cells to trigger immune responses can vary substantially. The studies mentioned above used cells that are permissive to infection. It remains unknown if AIM2 senses these viruses in cell types other than those listed above. Remarkably, AIM2 senses MCMV but not HSV-1 even though both are herpesviruses with similar modes of infection and replication. Because the capsids of both CMV and HSV-1 are broken down in the cytoplasm of macrophages¹⁹, triggering the

release of viral DNA in the cytosol, the selectivity of AIM2 in sensing these viruses seems remarkable. Since cytoplasmic IFI16 was shown to sense cytoplasmic HSV-1 DNA in macrophages independent of inflammasome assembly, this differential specificity of AIM2 and IFI16 may represent a tactic through which a detrimental overactive immune response is avoided. This hypothesis is in agreement with the study by Reinholz et al that showed that, although the AIM2 inflammasome is triggered in response to HPV-16 in keratinocytes (as evidenced by IL-1 β secretion without increase in IFN β), AIM2 knockdown triggers a decrease in IL-1 β levels, while IFN β levels are still observed to increase⁸⁸. It will be interesting to see how this mystery unfolds in forthcoming studies.

Interestingly, while several groups have shown that IFI16 signals through a STING-dependent pathway following infections of fibroblasts with HSV-1²⁴ and HCMV²⁵, one group observed the formation of IFI16 inflammasomes in endothelial cells and human fibroblasts in response to the herpesviruses Kaposi Sarcoma-associated herpesvirus (KSHV) and HSV-1, respectively^{22; 89; 90}. By fluorescent in situ hybridization and immunofluorescence microscopy, IFI16 was shown to co-localize with KSHV DNA and ASC in nuclei of infected cells²². In addition, this study from the Chandran laboratory reported interactions between IFI16, ASC, and mature caspase 1 by immunoaffinity purification, and showed an IFI16-dependent increase in IL-1 β in response to KSHV infection²². Sensing via IFI16 inflammasomes was also observed during KSHV and EBV latent infection^{90; 91}, a persistent form of infection in which viral particles are not being produced, yet the viral genome is still present. In addition, a study from the same lab reported that IFI16 can form inflammasomes in human foreskin fibroblasts (HFF) early during HSV-1 infection; however, due to the proteasomal degradation of IFI16 dependent on the viral protein ICP0 and the entrapment of caspase 1 in actin clusters, HSV-1 infection inhibits IFI16 inflammasomes by 8 hours post infection⁸⁹. Interestingly, in contrast to these observations following HSV-1 infection in HFFs, inflammasome formation was not detected following HCMV infection in these cells⁹². These distinct responses of IFI16 to different herpesviruses remain to be further characterized.

Alternative immune response pathways employed in viral DNA sensing

In addition to the STING-dependent and the inflammasome pathways described above, viral DNA sensors have been observed to signal through several alternative immune pathways. For example, DAI was shown to function both through the activation of IRF3 and NF κ B^{16; 93}. Each of these pathways uses a set of kinases to signal and activate the relevant transcription factors. DAI has been demonstrated to have two receptor-interacting protein (RIP) homotypic interaction motifs (RHIMs), which mediate recruitment of RIP-1 and -3^{94; 95; 96}. In addition RIP-3 phosphorylation activity has been shown to be essential for NF κ B signaling via the activation of the IKK complex, suggesting that RIP-3 is an effector of DAI signaling^{94; 97; 98}.

Two DNA sensors, RNA Pol III and DDX60, were reported to signal through the RNA-sensing RIG-I-like receptors (Figure 3). RIG-I is a PRR that recognizes short 5' triphosphate uncapped RNA and signals through MAVS to induce type I IFN. RNA Pol III was shown to synthesize 5' triphosphate from AT-rich viral DNA, which is subsequently sensed by RIG-I

in HEK293 cells transfected with poly dA-dT or during AdV, HSV-1 or EBV infection³⁶. DDX60 was demonstrated to associate with RIG-I, MDA5 and LGP2 in HEK293FTs in the absence of RNA, and to directly bind dsDNA⁴⁷. A reduction in IFN β expression in HSV-1 infected HeLa cells was seen upon DDX60 knockdown⁴⁷. However, the DDX60-dependent IFN induction following HSV-1 infection was observed as moderate at 19 hours post infection (hpi) and only significant at 26 hpi⁴⁷. As the initial IFN response to HSV-1 is usually documented at ~6hpi, these observations suggest that DDX60, while not critical for the initial induction, it may be important for amplifying the IFN response. The exact mechanisms behind this RLR-dependent DNA sensing by DDX60 remain to be understood, as does the apparent redundancy of RNA pol III sensing⁹⁹; however these findings provide further evidence for the divergence of DNA sensing pathways¹⁰⁰.

Three DNA sensors, DHX9, DHX36, and TLR9, signal through MyD88 (Figure 3). MyD88 leads to the activation of IRF7 and NF- κ B¹⁰¹. In plasmacytoid dendritic cells (pDCs) DHX-9 and -36 were shown to bind class B and A CpG, respectively, and to associate with the TIR domain of MyD88³⁵. Additionally, siRNA-mediated knockdown of DHX-9 and -36 in HSV-1 infected pDCs led to a decrease in TNF α and IFN α ³⁵. TLR9 signaling through MyD88 during viral infection is well understood and relies on downstream IRAK1-dependent IRF7 activation (reviewed in²⁸). Interestingly, pDC sensing of HSV-1 DNA was observed to occur in both TLR9-dependent and -independent manners¹². Viral DNA sensing by TLR9 has been shown only in pDCs, probably because TLR9 expression is limited to immune cells.

Altogether, the immune signaling pathways described above highlight the relevance of protein-protein interactions in antiviral response against DNA viruses. The use of mass spectrometry-based proteomic approaches within these studies promises to continue to lead to insights into the dynamic nature of these interactions and their regulation by PTMs. The observations that the expression patterns of DNA sensors and their subsequent signaling pathways are both diverse and, in some cases, redundant, underscore the strength of this branch of innate immunity. This is especially important in light of the fact that most viruses have evolved effective means to evade cellular immune detection.

C. Protein-protein interactions involved in viral immune evasion

The efficacy of immune response is influenced by the dynamic balance between host defense and the ability of viruses to block these host defense mechanisms. In fact, viral immune evasion mechanisms are not only detrimental from a clinical perspective, but the lack of understanding of these evasion mechanisms can also impede basic research. Without the knowledge of how viruses block host immune responses, the mechanisms for how DNA sensors trigger immune signaling are challenging to decipher. In this section we review viral proteins that impede viral DNA sensing pathways and proteins.

One mechanism by which viruses block immune signaling is through direct sequestration of anti-viral host factors. AP-MS has proven invaluable for identifying unknown host factors that are targeted by viral proteins²⁷. These studies were instrumental in elucidating mechanisms of viral evasion during DNA sensing and promotion of a pro-viral replicative

environment (Figure 4). For example, an AP-MS study determined that IFI16 is targeted by the viral tegument protein pUL83 during infection with HCMV¹⁰². Later on, a multidisciplinary study, integrating mass spectrometry and domain mapping, demonstrated that pUL83 targets specifically the pyrin domain of IFI16 to block its ability to oligomerize⁹² (Figure 4). This inhibition by pUL83 was shown to impede the ability of IFI16 to stimulate STING/TBK1/IRF3-dependent IFN and proinflammatory cytokine production in human fibroblasts⁹². Interestingly, pUL83 was shown to also inhibit the oligomerization of the IFIX pyrin domain, suggesting that IFIX may also have antiviral functions inhibited by this viral protein during HCMV infection; more studies are needed to investigate this possibility. Additionally, this AP-MS study also identified numerous phosphorylation sites on pUL83. Interestingly, phosphorylation at Ser364 by a host kinase was shown to lead to a partial rescue of IFI16 oligomerization⁹². This may reflect a host response to release a subset of IFI16 for signaling. Therefore, omic approaches can provide valuable insights into the dynamic virus-host interplay, whereby both have evolved strategies to foster their survival.

In addition to the role of HCMV pUL83 in inhibiting pyrin domain oligomerization and IFI16 immune signaling, the recruitment of IFI16 by pUL83 was also shown to play a role in regulating viral transcription¹⁰². Specifically, pUL83 targets IFI16 to the HCMV major immediate-early promoter, and this recruitment seems to be necessary for the stimulation of viral transcription¹⁰². More recently, the ability of IFI16 to modulate viral transcription has been expanded. One study demonstrated that, during HCMV infection, IFI16 bound to Sp1 binding regions to inhibit sp1-like transcription factors, leading to a decrease in virus replication¹⁰³. Another study showed that, during HSV-1 infection, IFI16 is connected to heterochromatin marks on the viral genome, and associated with the transcriptional repression of HSV-1 genomes¹⁰⁴. Furthermore, IFI16 knockdown led to an increase in transcriptionally active histone marks on HSV-1 DNA¹⁰⁴. Altogether, these studies indicate that IFI16 can act as an antiviral factor both through stimulation of an immune response upon sensing of viral DNA and through its transcriptional regulation functions. Both of these antiviral functions seem to be targeted by viral proteins for inhibition of host defense.

Another mechanism by which viruses block immune signaling is through degradation of anti-viral host factors. Orzalli et. al. has demonstrated that the HSV-1 immediate early protein ICP0 can abrogate nuclear IFI16 expression and sensing²⁴ (Figure 4). During HSV-1 infection, IFI16 was observed to be rapidly targeted for proteasomal degradation, which was shown to be in part dependent on the E3 ubiquitin ligase activity of ICP0²⁴ provided by its RING finger domain¹⁰⁵. While these studies imply the involvement of IFI16 ubiquitination, the sites of modification remain to be determined. Furthermore, the sole involvement of ICP0 or the requirement of other viral factors for IFI16 degradation is still being debated¹⁰⁶. The ectopic expression of ICP0 alone, in the absence of infection, was not sufficient to degrade IFI16; however, infection with an ICP0 null virus in conjunction with the expression of the HCMV immediate-early protein IE1 was able to largely recapitulate IFI16 degradation¹⁰⁶. Indeed, another study used an ICP0 mutant virus that lacks E3 ligase activity to show that ICP0 is necessary, but not sufficient, for the degradation of IFI16¹⁰⁷. The mechanism involved in IFI16 degradation requires further investigation, but, collectively, these studies suggest that IFI16 degradation during infection is dependent on the function of

herpesvirus immediate-early proteins. Nevertheless, it is interesting that related herpesviruses, such as HSV-1 and HCMV, have evolved different means for blocking IFI16 antiviral functions. While HSV-1 adopts a destructive approach, HCMV recruits IFI16, which possibly better suits its longer infection life cycle. Altogether, this knowledge regarding viral immune evasion mechanism is critical for gaining a better understanding of the mechanisms involved in sensing of viral DNA. As IFI16 is able to trigger an immune response following infection with the ICP0 mutant virus, a proteomic study took advantage of this knowledge to characterize IFI16 protein interactions in the context of HSV-1 infection¹⁰⁷. Interestingly, by using AP-MS followed by validation studies, IFI16 was shown to interact with components of cellular promyelocytic leukemia (PML) nuclear bodies during infection with the HSV-1 ICP0 mutant virus. By similar means, another study demonstrated that IFIX also associates with PML nuclear bodies; however, this association was already detected in uninfected cells²⁶. It remains to be determined whether this interaction also occurs during infection. The interaction of these sensors with PML nuclear bodies is intriguing, given that PML bodies are known to localize to herpesvirus replication compartments¹⁰⁸. It is tempting to speculate that these associations are playing a role in recruiting these sensors to viral DNA to facilitate immune response. Another possibility is that these interactions have antiviral functions through other means, such as transcriptional repression of the viral genome or initiation of apoptosis, as PML bodies have been shown to possess both functions¹⁰⁹.

Viral proteins have been shown to inhibit other viral DNA sensors following diverse infections. For example, the C16 protein of VACV binds the Ku70/80 complex, thereby impeding the ability of the Ku complex to bind DNA¹¹⁰. This disruption in DNA binding leads to a decrease in antiviral cytokines¹¹⁰. Other viral proteins counter immune response by directly inhibiting the downstream signaling factors, IRF3 and/or TBK1 (Figure 4). These viral proteins include the Varicella-zoster herpesvirus (VZV) immediate early proteins encoded by ORFs 61 and 62, and the HSV-1 proteins ICP0 and ICP34.5. In VZV-infected HEK293 cells, the E3 ligase ORF61 triggers the specific ubiquitination and degradation of active, phosphorylated IRF-3¹¹¹. In human embryonic lung fibroblasts, ORF62 was shown to inhibit phosphorylation of IRF3 by TBK1¹¹². While the specific mechanism remains to be fully understood, it was demonstrated that ORF62 did not bind to or cause the degradation of either IRF3 or TBK1¹¹². The HSV-1 protein ICP34.5 was also reported to interact with TBK1, which inhibited its interaction and phosphorylation of IRF3¹¹³. An alternative HSV-1 mechanism for inhibiting IRF3 involves ICP0. In this case ICP0 does not disrupt IRF3 phosphorylation or dimerization, but instead sequesters activated IRF3 in complex with its cofactors CBP/p300 to nuclear foci away from host chromatin¹¹⁴. Other HSV-1 proteins have been shown to interfere with IRF3 and/or IFN responses (as reviewed in ¹¹⁵), highlighting the elaborate mechanisms used by viruses to suppress host immune responses. Interestingly, the ability of viruses to block immune responses was also recently connected to the establishment of a latent infection. During infection with the murine gammaherpesvirus 68 (MHV68), the inhibition of the STING-dependent pathway by the viral tegument ORF64 deubiquitinase was shown to be important for the ability of the virus to establish a latent reservoir¹¹⁶.

Closing Remarks

The exciting and relatively young field of DNA sensing has significantly expanded the understanding of mammalian immune response to virus infections. Here, we reviewed the current knowledge of viral DNA sensors, including the viruses these sensors detect and the activated cellular pathways associated with host defenses. A number of viral DNA sensors have been identified, and, importantly, recent studies have demonstrated that these defense factors can recognize viral DNA in both the cytoplasm and nuclei of infected cells. As expected for an emerging field in which many mechanisms are still to be fully elucidated, some interesting, but seemingly, paradoxical findings have been described. For example, IFI16 and cGAS have both been shown to act as cytoplasmic viral DNA sensors and to be required for IFN expression in macrophages infected with HIV. However, an involvement for cGAS in nuclear sensing has not been observed. It is also not yet known if IFI16 and cGAS work together in the same signaling cascade or if their activities are independent of one another within parallel pathways. Given the knowledge of a number of viral DNA sensors, and the possible future identification of additional defense factors, it becomes increasingly important to understand their possible coordinated or redundant functions. Also remaining to be further examined is how these immune signaling pathways are regulated since, in certain cell types, multiple pathways have been shown to be active. For example, AIM2 inflammasomes have been observed in response to MCMV in macrophages, but not in response to another herpesvirus, HSV-1⁸⁶. Conversely, IFI16 has been shown to mediate IFN expression in response to HSV-1 in the same cell type¹⁹. The opposite regulation of PYHIN proteins has also been observed in keratinocytes infected with HPV-16, where AIM2 inflammasomes were formed, while IFI16 was downregulated; however, in the absence of AIM2, IFI16-dependent IFN response was upregulated⁸⁸. Thus, there may be a coordinated regulation of these sensing pathways that prevents the cell from producing a detrimental overactivation of immune responses. This is relevant, as overactivation of the immune system is known to contribute to the development of autoimmune diseases¹¹⁷. The mechanisms involved in regulating nucleic acid sensors in this context have been the focus of recent work^{118; 119; 120}. In this review, we have also summarized the contribution of omic technologies to the identification of viral DNA sensors and to characterizing the mechanisms involved in host defense or in viral immune evasion. The implementation of modern omic technologies is expected to continue to enhance the knowledge of viral DNA sensing. Specifically, the use of quantitative mass spectrometry-based proteomics promises to provide a better understanding of the dynamic nature of protein interactions during infection. Furthermore, there is still limited information regarding the impact of posttranslational modification on the activation or inhibition of protein interactions and downstream signaling pathways. While phosphorylation, acetylation, and ubiquitination have been reported to modulate DNA sensing pathways, other PTMs are likely to play critical roles in immune response. Therefore, future omic studies can continue to significantly contribute to this important and still emerging field of DNA sensing.

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Figure 1. Localization-dependent viral DNA sensing

A. Model of nuclear viral DNA sensing in response to infection with nuclear-replicating viruses (e.g., HSV-1) in non-immune cells (e.g., fibroblasts or natural host dermal cells). The virus particle fuses to the host membrane (1). The capsid is assisted by microtubules to move through the cytoplasm and then dock at the nuclear pore (2). The viral genome is then released into the nucleus (3), where it is sensed by a nuclear viral DNA sensor (4). This culminates in the expression of Type I IFN (5). B. Model of cytoplasmic viral DNA sensing as it occurs in immune cells, such as macrophages (1-4a) or dendritic cells (4b). In macrophages, the HSV-1 capsid is broken down in the cytoplasm before reaching the nucleus (2), triggering leakage of viral DNA in the cytoplasm (3), and leading to the detection of viral DNA by cytoplasmic DNA sensors (4a) and induction of Type I IFN (5). In dendritic cells, the DNA of viruses that are endocytosed are sensed by TLR9 in the endosomal compartment (4b). This sensing event results in the expression of Type I IFN (5). Continuous arrows represent signaling; dashed arrows represent movement.

Figure 2. Proteomic workflows for identifying DNA-protein and protein-protein interactions

DNA-coated beads or antibody-coated beads are placed in the cell lysate from uninfected or virally infected cells to capture interactions. Affinity purification is usually followed by enzymatic digestion, and the resulting peptides are analyzed by mass spectrometry. These analyses lead to the identification of proteins that interact with either DNA or protein. Protein interaction networks can be constructed to gain insights into complexes and modulated cellular pathways. These studies can be expanded by mapping PTMs that can contribute to the formation of interactions. Affinity purification using antibody-coupled beads can also be used to detect the DNA that associates to the proteins of interest, e.g. by PCR.

Figure 3. Viral DNA sensing pathways and associated PTMs

The main signaling pathways of viral DNA sensors are depicted. The nuclear DNA sensor IFI16 and the cytoplasmic DNA sensors cGAS, DDX41, IFI16 and DNA-PK signal through STING. ^aDAI was also shown to activate TBK1 and IRF3 prior to the discovery of STING. ^bIFI16 was shown to induce IFN following VACV, but the downstream signaling pathway has not been yet investigated. IFIX was also reported to bind HSV-1 DNA, a nuclear replicating virus. RNA Pol III and DDX60 signal through RIG-I, which further signals through MAVS. DHX9 and DHX36 signal through MyD88. AIM2 and IFI16 interact with ASC and Caspase 1 to form inflammasomes, which lead to the proteolytic cleavage of proinflammatory cytokines IL-1β and IL-18. Continuous arrows represent signaling; dashed arrows represent movement.

Figure 4. Mechanisms involved in viral immune evasion

Several viral proteins known to inhibit DNA sensing are depicted. The HCMV pUL83 binds to the IFI16 pyrin (PY) domain, inhibiting IFI16 oligomerization and the transmission of immune signals through the STING-dependent pathway. Phosphorylation of pUL83 seems to rescue IFI16 oligomerization. The HSV-1 protein ICP0 is thought to be partly involved in the degradation of IFI16 following infection, thereby preventing IFN induction. Other viral proteins inhibit the STING/IRF IFN induction pathway directly. HSV-1 ICP34.5 prevents TBK-1 phosphorylation and activation. The VZV proteins ORF62 and ORF61 prevent IRF3 from being phosphorylated by TBK1 and targeted for degradation, respectively. Finally, ICP0 causes IRF3 to be segregated in the nucleus and prevents it from up-regulating IFN production.

Table 1**Overview of viral DNA sensors**

Viral DNA sensors (PRR) are listed, together with the viruses shown to activate their sensing functions. Indicated are the viral substrates recognized by the different DNA sensors, the subcellular compartment in which sensing occurs, and the pathways through which the sensors have been shown to signal immune responses. The methods used to first identify the protein and/or show evidence of DNA binding are included. Note: additional non DNA pathogenic substrates are not included in this table.

PRR	Viral Substrates	Substrate	Sensing Location	Pathway	Immune response	Methods	Refs
AIM2	CMV, AdV, VACV, HBV, HPV16	dsDNA	Cytoplasm	Inflammasome	IL-1 β , IL-18	α -Screen; DNA beads AP-MS	31; 42; 43; 86; 87; 88
cGAS	HSV-1, MAV/VACV ^a , AdV	dsDNA	Cytoplasm	STING	IFN α , IFN β	Biochemical fractionation-MS	37; 121; 122
DAI	HSV-1, CMV	dsDNA	Cytoplasm	STING? RIP-3	IFN β , necrosis ^b	FRET; IP-WB	16; 78; 123
DDX41	HSV-1, AdV	dsDNA	Cytoplasm	STING	IFN α , IFN β	MS; bioinformatics screen	39; 74
DDX60	HSV-1	dsDNA	Cytoplasm	RIG-I	IFN β	DNA microarray, gel shift assay	47
DHX9	HSV	Class B CpG	Cytoplasm	MyD88	TNF α , IL-6	CpG beads AP-MS	35
DHX36	HSV	Class A CpG	Cytoplasm	MyD88	IFN α	CpG beads AP-MS	35
DNA-PK	HSV-1, HSV-2G, VACV,	dsDNA	Cytoplasm	STING	IFN λ 1, IFN β , IL-6	DNA beads AP-MS	33; 34; 110
IFI16	HSV-1, CMV, KSHV, VACV70mer, HIV	dsDNA, ssDNA	Nucleus Cytoplasm	STING Inflammasome	IFN β , CXCL10, CCL5, CCL20, IL-6, IL-1 β , IL-18	DNA beads AP-MS; α -Screen	22; 23; 24; 32; 70; 92
IFIX	VACV70mer, HSV-1	dsDNA	Cytoplasm Nucleus*	n.d.	IFN β	AP-MS; dsDNA array	26
RNA Pol III	HSV-1, EBV, AdV	AT-rich dsDNA	Cytoplasm	RIG-I	IFN β	chromatography fractionation; MS	15; 36
TLR9	HSV-1+2, CMV, AdV, EBV	Unmethylated CpG motif	Endosome	MyD88	IFN α , IFN β	Luciferase reporter; SPR; bioinformatic modeling	9; 12; 44; 45; 124; 125; 126; 127

^a modified vaccinia virus (MAV/VACV);

^b programmed necrosis through the RIP-3 pathway.

* IFIX was shown to bind HSV-1 DNA, a nuclear replicating virus, suggestive of another possible nuclear sensor, which remains to be demonstrated.

Table 2

PTMs involved in regulating DNA sensing pathways

Overview of the types and sites of posttranslational modifications shown to have roles in regulating DNA sensors and/or downstream pathway components. The function of each of the modifications, as well as the protein responsible for the modification, are indicated when known.

Protein	Modification	Site	Function	Modifying protein	Refs
TBK1	Phosphorylation	Ser172	Activation	TBK-1	58; 59
	Ubiquitination	Lys48	Degradation	DTX4	119
IRF3	Ser385 or Ser386 Ser396 – Ser405 Removal of autoinhibition, Dimerization and activation				
	Phosphorylation	Ser173/Ser175	Constitutive	TBK-1	60; 61; 62; 63; 64; 65; 66; 67; 68
	Ubiquitination	Thr39	n/a (occurs during infection)	ORF61 (VZV)	68
IFI16	Acetylation	Lys99 Lys128	Cytoplasmic localization	p300	111
ASC	Phosphorylation	Tyr146	Aggregation	-	23
	Ubiquitination	-	Inhibits aggregation	-	81
RIP3	Phosphorylation	-	Promotes IKK complex activation	RIP3 (autophosphorylation upon DAI binding)	85
Vp5 (HSV-1)	Ubiquitination	-	Capsid degradation	-	94
pUL83 (HCMV)	Phosphorylation	Ser364	Prevents pul83 mediated IFI16 signaling inhibition	-	19
DDX41	Ubiquitination	Lys9 Lys115	Degradation	Trim21	92
					120