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Targeted Restriction of Viral Gene Expression and Replication by the ZAP Antiviral System

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Keywords

ZAP, zinc finger antiviral protein, ZC3HAV1, PARP13, CpG dinucleotide, virus, interferon-stimulated gene

Abstract

The zinc finger antiviral protein (ZAP) restricts the replication of a broad range of RNA and DNA viruses. ZAP directly binds viral RNA, targeting it for degradation and inhibiting its translation. While the full scope of RNA determinants involved in mediating selective ZAP activity is unclear, ZAP binds CpG dinucleotides, dictating at least part of its target specificity. ZAP interacts with many cellular proteins, although only a few have been demonstrated to be essential for its antiviral activity, including the 3′–5′ exoribonuclease exosome complex, TRIM25, and KHNYN. In addition to inhibiting viral gene expression, ZAP also directly and indirectly targets a subset of cellular messenger RNAs to regulate the innate immune response. Overall, ZAP protects a cell from viral infection by restricting viral replication and regulating cellular gene expression. Further understanding of the ZAP antiviral system may allow for novel viral vaccine and anticancer therapy development.

INTRODUCTION

Vertebrate cells contain several pattern recognition receptors (PRRs) that discriminate between viral and cellular RNA to elicit an antiviral response while avoiding chronic autoimmune activation. The best-characterized PRRs that detect viral RNA in the cytosol bind double-stranded RNA (dsRNA) as the pathogen-associated molecular pattern (PAMP) (1). For example, the RIG-I-like receptors RIG-I and MDA5 bind dsRNA and induce a signaling cascade that activates nuclear factor-κB (NF-κB), interferon regulatory factor 3 (IRF3), and IRF7 to stimulate type I interferon (IFN) as well as proinflammatory cytokine and chemokine gene expression. PKR, OAS1, OAS2, and OAS3 also bind dsRNA in the cytosol. PKR inhibits cap-dependent translation by phosphorylating eIF2α and stimulating stress granule formation. OAS1–3 activate RNase L, leading to global RNA degradation. Even though most cellular RNA does not contain substantial stretches of dsRNA, these PRRs are highly regulated to ensure that they are activated only when necessary. Mutations in some of the dsRNA PRRs, such as MDA5, lead to dysregulated activation and can cause autoimmune diseases such as type I interferonopathies (2).

In contrast to PRRs that detect dsRNA, the zinc finger antiviral protein (ZAP) is a PRR that binds single-stranded RNA (ssRNA) and directly inhibits viral replication through multiple mechanisms, including targeting RNA for degradation and inhibiting its translation. As ssRNA is the predominant form of cellular RNA, ZAP must be selective in its activity or risk altering global cellular gene expression. In recent years, substantial progress has been made in elucidating the mechanisms of ZAP antiviral activity. However, the full range of ZAP-sensitive viruses, how ZAP targets specific transcripts, the role of ZAP cofactors for inhibiting viral replication, and how ZAP regulates cellular gene expression remain to be fully characterized.

ZAP DOMAIN STRUCTURE AND SUBCELLULAR LOCALIZATION

ZAP, also known as zinc finger CCCH-type containing, antiviral 1 (ZC3HAV1) or poly (ADP-ribose) polymerase (PARP) family member 13 (PARP13), evolved from a duplication of the PARP12 gene in tetrapods (3). Both proteins inhibit a range of viruses, although their antiviral mechanisms of action may differ. There are at least four ZAP isoforms produced by alternative splicing and polyadenylation: ZAP-extra long (XL), ZAP-long (L), ZAP-medium (M), and ZAP-short (S) (4–8). All ZAP isoforms have an N-terminal RNA binding domain (RBD) with four CCCH-type zinc finger motifs (ZnF1–4) as well as an integrated central domain structure that contains a fifth CCCH zinc finger motif (ZnF5) and two WWE modules (4–6, 9–11) (**Figure 1**). Part of the central domain has homology to TIPARP, and this region is sometimes referred to as a TIPARP homology region (5, 6). In addition, ZAP-XL and ZAP-L contain a C-terminal PARP-like domain that does not have ADP-ribosyltransferase activity because it lacks the histidine, tyrosine, and glutamate (H-Y-E) catalytic triad (5, 6, 12). These domains are highly conserved between mammalian, bird, and reptile ZAP orthologs (3, 13). Because ZAP-L and ZAP-S have the highest expression levels and their antiviral function is best characterized, this review focuses on these isoforms (5, 6). Another ZAP-like gene, *ZC3HAV1L*, consisting of only the region paralogous to the ZAP RBD, also exists in mammals (3, 5). It is not known whether this protein has antiviral activity.

Structures for all of the major ZAP domains (the RBD, central domain, and PARP-like domain) have been solved (10–12, 14, 15). Three reports have elucidated the ZAP RBD structure, with two showing ZAP bound to RNA containing CpG dinucleotides (10, 14, 15). ZAP forms a stable complex with ssRNA and shows no binding affinity for dsRNA (15). The RBD binds ssRNA through a high-affinity interaction with a CpG dinucleotide mediated by a hydrophobic pocket formed by ZnF2 and less specific electrostatic interactions (14, 15). While mammalian ZAP orthologs

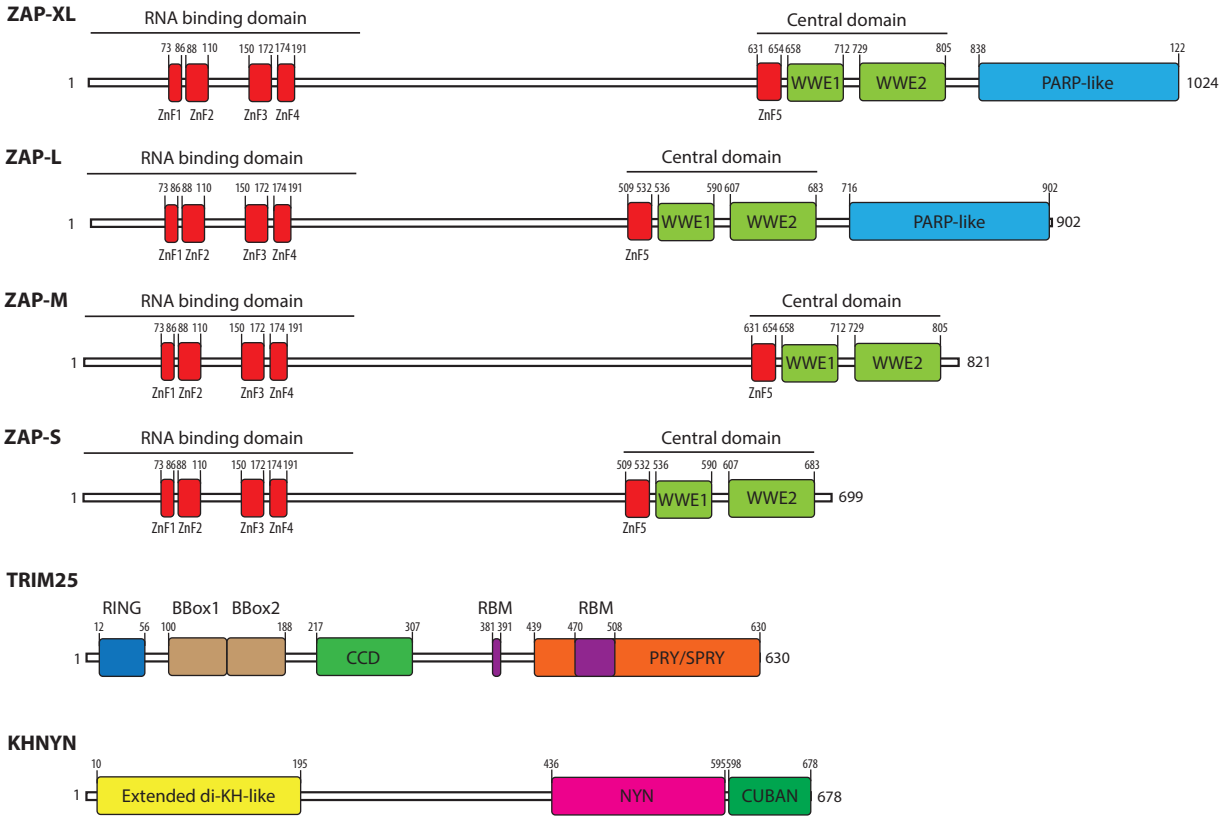

Figure 1

Schematic of ZAP, TRIM25, and KHNYN. All four ZAP isoforms contain an N-terminal RNA binding domain spanning residues 1–225 with four CCCH-type zinc finger (ZnF1–4) motifs. ZnF2 specifically binds CpG dinucleotides in RNA. All isoforms also contain a central domain structure with a fifth CCCH zinc finger motif (ZnF5) and two WWE modules, with the second WWE module binding poly(ADP-ribose). ZAP-XL and ZAP-L contain a catalytically inactive C-terminal PARP-like domain. TRIM25 is an E3 ubiquitin ligase with an N-terminal catalytic RING domain that mediates ubiquitylation, a coiled-coil domain that enables dimerization, and a C-terminal PRY/SPRY domain required for the interaction with ZAP. TRIM25 binds to RNA through two potential RBMs. KHNYN contains an N-terminal extended di-hnRNP KH-like domain, an NYN domain with putative endonuclease activity, and a C-terminal CUBAN domain. Abbreviations: CCD, coiled-coil domain, CUBAN, cullin binding domain associating with NEDD8; KH, K-homology; PARP, poly(ADP-ribose) polymerase; RBM, RNA binding motif; ZAP, zinc finger antiviral protein; ZAP-L, ZAP-long; ZAP-M, ZAP-medium; ZAP-S, ZAP-short; ZAP-XL, ZAP-extra long.

selectively bind to the CpG dinucleotide, bird orthologs have a lower preference for this motif (3). The specificity for CpG dinucleotides in mammalian ZAP orthologs results from residues in the ZnF2 binding pocket accommodating only a CpG, with any other nucleotide combination leading to steric clashes (14, 15). Therefore, CpG dinucleotides in ssRNA are a PAMP in ZAP's role as a PRR. The other three CCCH-type zinc finger motifs (ZnF1, ZnF3, and ZnF4) can bind RNA, albeit through lower affinity electrostatic interactions and undetermined sequence specificity (14, 15).

The ZAP central domain binds to poly(ADP-ribose), a post-translational modification deposited by active PARP proteins, through its second WWE module (11). While this interaction is required for optimal antiviral activity, how poly(ADP-ribose) regulates ZAP is unclear. The PARP-like domain is under positive evolutionary selection and is speculated to have been shaped by

repeated episodes of host-pathogen interactions (5, 16). At its C terminus, the PARP-like domain contains a cysteine (CaaX) motif (17), which mediates S-farnesylation. This covalent isoprenoid modification can enhance protein affinity for cellular membranes by increasing hydrophobicity. S-farnesylation of Cys-899 in ZAP-L localizes it to endolysosomes or the endoplasmic reticulum (ER) (7, 17, 18). Loss of S-farnesylation results in ZAP-L showing a diffuse distribution pattern in the cytosol, similar to ZAP-S, which lacks the PARP-like domain (7, 17). Importantly, C-terminal epitope tags or other modifications should not be used to study ZAP-L antiviral activity because they prevent the S-farnesylation modification and lead to protein mislocalization.

Rat ZAP has been reported to be a CRM1-dependent nucleocytoplasmic shuttling protein and is localized in the cytoplasm at steady state (19). Under conditions of cellular stress, both ZAP-L and ZAP-S localize to stress granules, which may be important for their antiviral activity (18, 20–25). ZAP targeting to stress granules is regulated by the RBD and the interaction between the WWE2 module and poly(ADP-ribose), which also localizes to stress granules (11, 18, 20, 21). ZAP is an IFN-stimulated gene (ISG) that is also induced by viral infection due to STAT and IRF3 binding sites in its promoter (26). Interestingly, type I IFN upregulates ZAP-S and ZAP-M protein expression but has a minimal effect on ZAP-L and ZAP-XL expression levels, indicating that, in addition to transcription, alternative splicing or polyadenylation is also regulated by IFN (6–8, 27). Structural and interaction studies suggest that ZAP isoforms bind RNA as either homo- or heterodimers, and as each ZAP molecule binds only one CpG, multiple ZAP molecules may form an oligomer on a target RNA (4, 10, 14, 15, 24, 28).

ZAP INHIBITS A DIVERSE RANGE OF VIRUSES

ZAP's antiviral activity was first identified in a genetic screen in which ZAP-S was shown to inhibit replication of the gammaretrovirus murine leukemia virus (MLV) (4). Since its initial discovery as an antiviral factor, ZAP has been shown to restrict a wide range of viruses through at least two potentially linked mechanisms: targeted RNA degradation and inhibition of messenger RNA (mRNA) translation (4, 29–32) (**Figure 2**). While there is a more mechanistic understanding for how ZAP interacts with cellular cofactors to mediate viral RNA degradation, ZAP-mediated translation repression may precede and be required for RNA degradation (31). Because research on ZAP now spans almost two decades, there are a few technical considerations for the experimental approaches used to analyze its antiviral activity. Most of the initial reports used ZAP overexpression to determine whether and how it inhibited viral replication, and this approach is still used. However, depending on the experimental conditions, ZAP overexpression may lead to nonphysiological levels of ZAP or an altered isoform ratio. In addition to transgenic knockout mice, several protein depletion technologies, including RNA interference and CRISPR-mediated genome editing, can substantially decrease or eliminate endogenous ZAP expression in cell lines. These approaches allow for the antiviral activity of endogenous ZAP to be determined. They also allow the expression of individual ZAP isoforms or ZAP with specific mutations to be expressed in the absence of the wild-type protein. Analyzing ZAP functional determinants in knockout/knockdown cells avoids potential oligomerization between endogenous ZAP and ectopically expressed ZAPs, which could confound experimental interpretation. Furthermore, in some cases, reporter constructs containing only a portion of a viral genome have been used to characterize ZAP antiviral activity. Below, we differentiate between experimental systems in which ZAP has been overexpressed or depleted (**Table 1**) and focus on findings using full length, infectious virus when possible.

Overexpression and depletion studies have shown that both ZAP-L and ZAP-S inhibit MLV replication by targeting the viral RNA for degradation in the cytoplasm (4, 9, 23, 29, 33, 34). Furthermore, ZAP antiviral activity against this virus can be stimulated by type I IFN (34). During

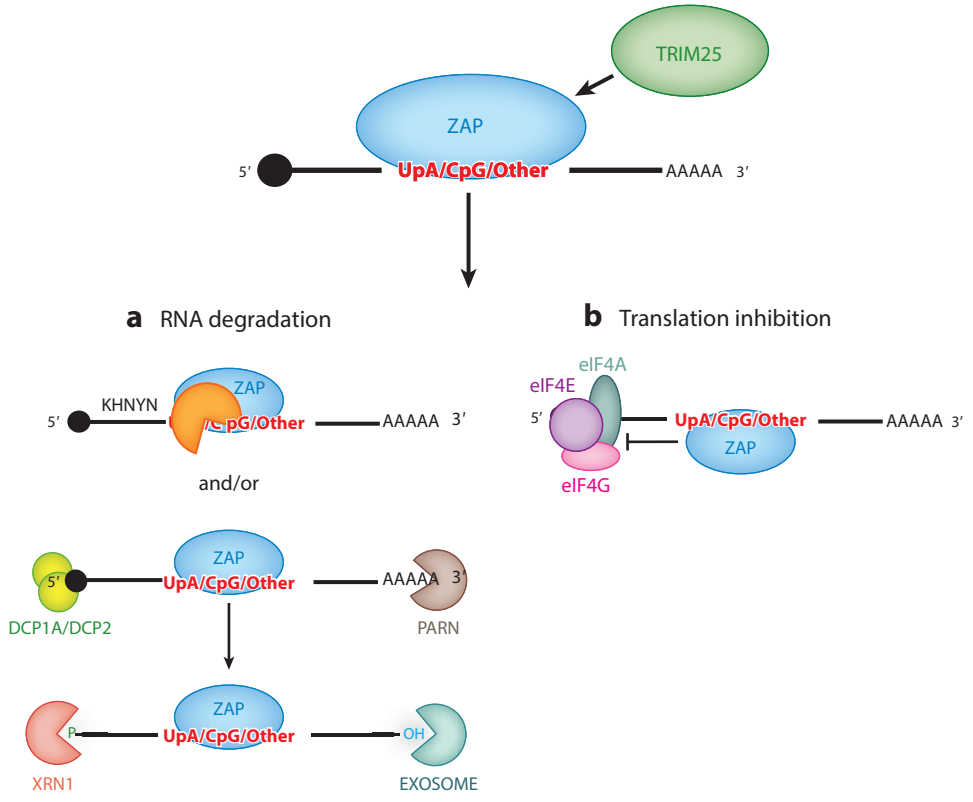

Figure 2

Mechanisms of ZAP-mediated repression of viral gene expression. ZAP binds to ZRE-containing viral RNA and, along with its cofactor TRIM25, can either (*a*) mediate RNA degradation by interacting with the putative endonuclease KHNYN and/or through recruiting components of the 5′–3′ and 3′–5′ RNA degradation machinery or (*b*) repress messenger RNA translation by inhibiting the interaction between the translation initiation factors eIF4A and eIF4G. Abbreviations: ZAP, zinc finger antiviral protein; ZRE, ZAP-response element.

MLV infection, ZAP localizes to RNA granules with characteristics of both stress granules and processing bodies (23). ZAP also inhibits the deltaretrovirus human T-lymphotropic virus type-1 (HTLV-1) and the alpharetrovirus avian leukosis virus (35–38). For both viruses, ZAP appears to target viral RNA transcripts for degradation. While ZAP overexpression inhibits primate lentiviruses, including human immunodeficiency virus type I (HIV-1), endogenous ZAP exhibits only modest antiviral activity against HIV-1 (14, 30, 34, 39–43). However, this may affect virus fitness and is discussed below. ZAP-S and ZAP-L inhibit the reverse-transcribing hepatitis B virus by promoting viral RNA decay (44, 45). In addition, ZAP inhibits long interspaced element-1 (LINE-1) and Alu element retrotransposition by specifically depleting retroelement RNA in the cytoplasm (22, 24). This suggests that, beyond its antiviral role, ZAP may be a crucial regulator of human retrotransposons that could protect the human genome from mutations due to their replication.

ZAP antiviral activity is best characterized for members of the positive-strand RNA virus *Togaviridae* family, which include Sindbis virus (SINV), Ross River virus, and Semliki Forest virus (SFV) (6, 9, 32, 46, 47). Interestingly, the predominant mechanism by which ZAP inhibits their

Table 1 Viruses targeted by zinc finger antiviral protein (ZAP)

Virus(es)	Viral family	ZAP antagonist(s)	Experimental evidence	Reference(s)
Porcine reproductive and respiratory syndrome virus	*Arteriviridae*	Nsp4 protease	Overexpression and depletion	53, 54
Severe acute respiratory syndrome coronavirus 2	*Coronaviridae*	–[a]	Overexpression and depletion	55
Ebola virus and Marburg virus	*Filoviridae*	–	Overexpression	6, 56
Japanese encephalitis virus	*Flaviviridae*	–	Overexpression and depletion	50
Hepatitis B virus	*Hepadnaviridae*	–	Overexpression and depletion	6, 44, 45
Human cytomegalovirus, herpes simplex virus, and murine gammaherpesvirus 68	*Herpesviridae*	Herpes simplex virus UL41, murine gammaherpesvirus 68 replication and transcription activator (RTA)	Overexpression and depletion	32, 60–63, 68
Influenza A virus	*Orthomyxoviridae*	PB1 and NS1	Overexpression and depletion	57–59
Sendai virus and Newcastle disease virus	*Paramyxoviridae*	–	Overexpression and depletion	27, 38
Coxsackievirus B3 and enterovirus A71	*Picornaviridae*	Enterovirus A71 3C protease	Overexpression and depletion	51, 52
Vaccinia virus	*Poxviridae*	C16	Depletion	64
Murine leukemia virus, human immunodeficiency virus, simian immunodeficiency virus, human T cell leukemia virus, avian leukosis virus	*Retroviridae*	–	Overexpression and depletion	4, 23, 30, 33, 35, 36, 40–43
Sindbis virus, Semliki Forest virus, and Ross River virus	*Togaviridae*	–	Overexpression and depletion	32, 46, 47

[a]– indicates that there is no known ZAP antagonist for this virus.

replication is preventing translation of the incoming viral genome (primary translation), although RNA degradation may also play a role (32, 47, 48). ZAP potently inhibits SINV replication, and ectopic expression of ZAP-L or ZAP-S suppresses SINV replication in ZAP knockout mouse embryonic fibroblasts (32, 47). ZAP-L is more active than ZAP-S against SINV and SFV because the PARP-like domain and the S-farnesylation motif at its C terminus are necessary for potent antiviral activity (5–7, 17). This may be because the S-farnesyl modification mediates ZAP-L localization to endocytic membranes, allowing it to target the incoming virus (7, 17). NAD^+ is the substrate for PARP proteins, and the NAD^+ binding site in the ZAP PARP-like domain has evolved changes that prevent its binding (5, 49). Mutation of conserved amino acid residues in this region to those predicted to restore catalytic activity reduced SINV restriction by ZAP-L, demonstrating the importance of the PARP-like domain for full ZAP antiviral activity (49). In addition, ZAP localization to stress granules correlates with optimal antiviral function against SINV (21), although whether ZAP-L localizes to stress granules and membranes simultaneously is unclear.

ZAP also restricts other positive-strand RNA viruses, including the flavivirus Japanese encephalitis virus and the picornavirus coxsackievirus B3, in overexpression and depletion experiments by inhibiting viral RNA translation and stability (50, 51). ZAP-L overexpression only moderately inhibited viral titer and RNA levels for the picornavirus enterovirus A71, likely due to its 3C protease cleaving ZAP within the central domain (52). Overexpression and depletion experiments showed that ZAP inhibits the arterivirus porcine reproductive and respiratory

syndrome virus (PRRSV) at an early stage of viral replication (53). ZAP interacts with PRRSV viral RNA polymerase Nsp9 through its N-terminal domain to inhibit genome synthesis, although the viral 3C-like serine proteinase Nsp4 partially antagonizes ZAP by cleaving it (53, 54). The pandemic severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is responsible for coronavirus disease 2019 (COVID-19), is restricted by endogenous ZAP upon IFN treatment (55). While both ZAP-L and ZAP-S overexpression reduced the levels of spike and nucleoprotein mRNAs, ZAP-L also inhibited the accumulation of full-length SARS-CoV-2 RNA and therefore inhibited viral replication more potently than ZAP-S.

ZAP has been shown to restrict several negative-strand RNA viruses. ZAP overexpression inhibits Ebola virus by targeting the mRNA encoding the viral polymerase (L protein) for degradation (6, 56). However, the role of endogenous ZAP has not been determined for this virus. ZAP has at least two direct mechanisms for inhibiting influenza A virus (IAV). The ZAP-L PARP-like domain interacts with the PA and PB2 polymerase proteins, leading to their poly(ADP-ribosyl)ation, ubiquitylation, and proteasomal degradation (57). IAV PB1 counteracts this by binding to the WWE subdomains in ZAP and promoting its dissociation from PA and PB2 proteins, allowing them to escape degradation (57). As ZAP lacks PARP activity, it must recruit unknown cofactors to post-translationally modify PA and PB2. ZAP-S also inhibits viral protein expression by binding and reducing the abundance of specific IAV transcripts (58). IAV nonstructural protein 1 (NS1) counteracts ZAP-S by preventing it from binding the viral RNA (58). The two separate mechanisms counteracting ZAP restriction result in only moderate inhibition of IAV infection by endogenous ZAP levels (57–59).

In addition to targeting reverse-transcribing, positive- and negative-strand ssRNA viruses, ZAP inhibits DNA virus replication. ZAP-L and ZAP-S inhibit human cytomegalovirus by targeting specific viral mRNAs for degradation (60, 61). Two other herpesviruses have been shown to evade efficient ZAP restriction by expressing factors that counteract ZAP antiviral activity (62, 63). Murine gammaherpesvirus 68 partially evades ZAP-mediated antiviral activity through the viral replication and transcription activator (RTA) protein (10, 63). Herpes simplex virus 1 evades ZAP-mediated restriction by degrading ZAP mRNA in infected cells through the viral nuclease UL41 (32, 62). Modified vaccinia virus Ankara (MVA) is an orthopoxvirus and an attenuated smallpox vaccine created through the serial passage of the parental vaccina virus strain Ankara in chick embryo fibroblasts. The restricted replication of MVA in human cells is partly due to ZAP antiviral activity, and depletion of endogenous ZAP increases MVA replication (64). Vaccinia virus C16 protein (strain Copenhagen), which is disrupted in MVA, antagonizes ZAP by sequestering it in cytoplasmic punctate (64). In the absence of C16, ZAP inhibits MVA replication by interfering with the assembly of infectious virions without obvious effects on viral RNA or protein expression.

Overall, ZAP-L appears to be the predominant isoform inhibiting viral protein synthesis, although ZAP-S also has antiviral activity (4, 5, 7, 17). The relative activity of ZAP-L and ZAP-S may be influenced by the experimental system used to analyze them because they can oligomerize with endogenous ZAP isoforms if overexpressed in wild-type cells. The higher antiviral activity for ZAP-L is consistent with its constitutive expression in most cell types, and the residues under position selection are found in this isoform (5, 6). While ZAP restricts a diverse range of RNA and DNA viruses, it shows selectivity in its antiviral activity and, unlike some dsRNA PRRs such as RIG-I or MDA5, does not induce a general antiviral state. In addition to the viruses discussed above, viruses from multiple families, including *Flaviviridae* (dengue virus, yellow fever virus, Zika virus), *Rhabdoviridae* (vesicular stomatitis virus), *Togaviridae* (chikungunya virus), *Picornaviridae* (poliovirus, echovirus 7), and *Parvoviridae* (minute virus of mice), evade restriction by ZAP (6, 32, 50, 65, 66). Viruses that escape ZAP-mediated antiviral activity could lack ZAP-response elements (ZREs) (discussed next) in viral RNA or encode countermeasures against it.

Viral factors that inhibit ZAP antiviral activity could be targets for novel antiviral therapeutics, and continued research on the viral mechanisms used to evade ZAP restriction will shed light on complex host-pathogen interactions.

ZAP DIRECTLY INTERACTS WITH VIRAL RNA TO RESTRICT REPLICATION

As discussed above, ZAP appears to inhibit most viruses by directly binding viral RNA (4, 9, 67). ZREs were initially characterized in MLV and SINV (9). However, no consensus or homologous sequence was found between these viruses or ZREs later identified in other viruses, although they appeared to be length dependent (9, 33, 45, 51, 56, 63, 68). A breakthrough in understanding ZAP target specificity was the discovery that it binds to CpG dinucleotides in HIV-1 (40). Endogenous ZAP has only a small effect on wild-type HIV-1 (30, 34, 40–43). However, increasing the CpG content in the 5′ region of *env* using synonymous mutations resulted in significant inhibition of viral replication by ZAP through cytoplasmic viral RNA degradation (34, 40–42). Cross-linking-immunoprecipitation sequencing (CLIP-seq) and structural analyses demonstrated that ZAP directly bound CpG dinucleotides in viral RNA (14, 15, 40). Importantly, residues in the ZAP RBD that interact with a CpG are required for its full antiviral activity against CpG-enriched HIV-1 and SINV (14, 15). Therefore, at least some virus ZREs appear to be formed by abundant CpG dinucleotides over long stretches of RNA, although other motifs may also be important.

Supplemental Material >

Many vertebrate RNA viruses have genome-wide suppression of CpG dinucleotides (41, 69–72) (**Figure 3**; **Supplemental Table 1**). There are three rough clusters of viruses based on their

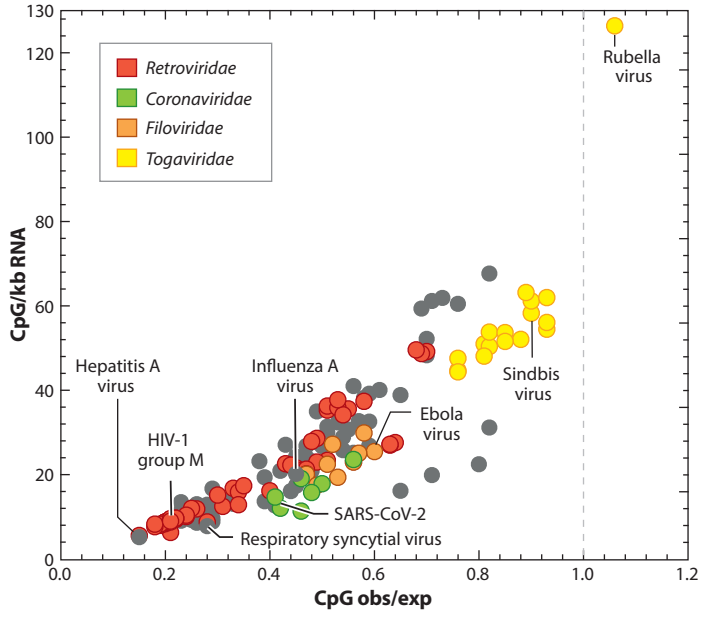

Figure 3

CpG abundance is highly suppressed in many RNA viruses that infect vertebrates. The number of CpG/kb and the CpG observed/expected ratio were calculated for vertebrate RNA viruses (41). Members of the *Retroviridae* (*red*), *Coronaviridae* (*green*), *Filoviridae* (*orange*), and *Togaviridae* (*yellow*) families are highlighted. Abbreviations: HIV-1, human immunodeficiency virus type I; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2. Figure adapted from Reference 41.

genomic CpG content. The cluster with the lowest CpG abundance includes hepatitis A virus, primate lentiviruses such as HIV-1, and respiratory syncytial virus. A cluster with moderate CpG suppression includes human coronaviruses such as SARS-CoV-2, filoviruses such as Ebola virus, and IAV. The third cluster showing small or no CpG suppression includes several togaviruses such as SINV. CpGs have been demonstrated to be directly deleterious for several different viruses, and a higher CpG abundance in HIV-1 *env* has been linked to a slower rate of disease progression (42, 73–75). Supporting the hypothesis that CpG dinucleotides sensitize viral RNA to ZAP, introducing CpGs into viruses normally not restricted can make them ZAP sensitive. In addition to HIV-1, CpG introduction into the picornavirus echovirus 7 or the parvovirus minute virus mediates ZAP-dependent restriction (34, 40, 41, 65, 66, 76). Furthermore, ZAP targets the high CpG content in HTLV-1 mRNAs and binds a region with high CpG abundance in the Japanese encephalitis virus 3′ untranslated region (UTR) (35, 50). Vertebrate genomes are depleted in CpGs, at least partly due to CpG DNA methylation to 5-methyl-cytosine, followed by spontaneous deamination to form a thymidine (77). This may have created an opportunity for ZAP to evolve the ability to distinguish viral RNA from cellular RNA based on CpG content (3, 40). Therefore, CpG suppression in RNA viruses could have evolved, in part, to evade ZAP restriction, with this adding selective pressure on CpG abundance in transcribed regions of mammalian genomes (3). However, it should be noted that introducing CpGs into HIV-1 and other viruses can inhibit viral replication through ZAP-independent mechanisms, such as altering precursor mRNA (pre-mRNA) splicing or codon pair deoptimization (40, 41, 78–80).

Studies in HIV-1 and other primate lentiviruses have suggested that the position, RNA structure, and overall context of CpG dinucleotides may be determinants for optimal ZAP antiviral activity (41, 42). CpG dinucleotides in the 5′ region of the HIV-1 *env* are particularly sensitive to ZAP-mediated restriction, while large numbers of CpGs in other regions of the HIV-1 genome confer only moderate ZAP sensitivity. Furthermore, inserting reporter genes with high CpG content, such as *Renilla* luciferase or green fluorescent protein, into the 3′ end of the HIV-1 genome does not sensitize it to ZAP antiviral activity (40, 41). Likewise, the CpG dinucleotide frequency in alphavirus genomes and human cytomegalovirus mRNAs does not predict their susceptibility to ZAP-mediated restriction (6, 61). In vitro binding studies for the ZAP RBD and different CpG-containing sequences identified C(n7)G(n)CG as a high-affinity sequence (15). The CpG motif is essential for in vitro binding, while the additional C and G nucleotides further enhance affinity. RNA immunoprecipitation experiments showed that the 5′ and 3′ nucleotides immediately flanking the CpG affect the interaction between viral RNA and ZAP (76). Specifically, a UCGU sequence promotes ZAP binding and antiviral activity in CpG-recoded echovirus 7, while the ACGA sequence had the opposite effect. These results suggest that the sequence context surrounding a CpG dinucleotide in viral RNA may modulate its sensitivity to ZAP. ZAP may also bind non-CpG sequences in viral RNA. Mutation of ZAP residues that directly interact with the CpG did not abrogate RNA binding in a living cell (14). Furthermore, ZAP has been shown to interact with echovirus 7 RNA with either increased CpG or UpA abundance, possibly through separate binding sites (65, 76). It remains unclear whether or how ZAP directly binds UpA at a structural level.

Togaviruses have high CpG abundance compared to most vertebrate RNA viruses (**Figure 3**), and an open question is why they are unaffected by ZAP evolutionary pressures. One possibility is that they highjack the ZAP antiviral pathway to promote their replication in an animal host, with the neurovirulent SINV reported to use the ZAP antiviral system to evade the innate immune response (46). While SINV has increased viremia and higher mortality in suckling ZAP knockout mice, in weaning mice, ZAP repression allows it to evade initiating a potent antiviral response (46, 47). This allows SINV to disseminate to the brain of the weaning mice, leading to higher mortality in wild-type mice than ZAP-knockout mice.

CELLULAR COFACTORS ARE REQUIRED FOR ZAP ANTIVIRAL ACTIVITY

Supplemental Material >

The interaction of ZAP with other cellular proteins can positively or negatively regulate its activity. ZAP is a component of a large complex in uninfected cells (81), although the full complement of cellular proteins essential for its antiviral activity remains unclear. Large-scale interactome studies have identified more than 250 cellular proteins that interact with ZAP (**Supplemental Table 2**) (24, 82, 83). Many of the identified ZAP-interacting proteins are RNA binding proteins, and RNA may be bridging the interaction. Notably, the interactome studies have not been performed in the context of viral infection or type I IFN treatment, so whether the identified interactomes represent an accurate picture of ZAP interacting proteins in the context of viral infection is unknown. To date, only a few of these proteins have been functionally validated and shown to be bona fide ZAP cofactors.

TRIM25 is an ISG and E3 ubiquitin ligase that regulates the innate immune response against viral infection (84). It interacts with ZAP and is required for ZAP antiviral activity against SINV, human cytomegalovirus, and HIV-1 containing introduced CpG dinucleotides (3, 34, 40, 60, 85, 86) (**Figure 2**). TRIM25 consists of a RING domain that mediates ubiquitylation, a coiled-coil domain that enables dimerization, and a SPRY domain that interacts with ZAP (3, 84) (**Figure 1**). Similar to ZAP, TRIM25 localizes to stress granules (25). TRIM25 also binds RNA, potentially through two motifs, and this enhances its E3 ubiquitin ligase activity (87–89). It interacts with the N-terminal domain of ZAP, and there is species specificity for ZAP-TRIM25 interaction in highly divergent orthologs (3). While it remains unclear how TRIM25 controls ZAP activity, TRIM25 has been reported to regulate ZAP pre-mRNA splicing and enhance ZAP binding to SINV RNA (60, 86). The enzymatic activity of TRIM25 is necessary for it to regulate ZAP activity (85, 86). ZAP-L and ZAP-S are ubiquitylated at multiple sites (24, 85, 86, 88). TRIM25 depletion reduced ZAP ubiquitylation, and TRIM25 overexpression significantly increased ZAP ubiquitylation levels (85, 86). Surprisingly, while mutation of all shared ubiquitylation sites in ZAP-S and ZAP-L eliminated ZAP ubiquitylation, this did not affect ZAP's ability to restrict SINV replication or retroviral reporter constructs (85, 86). Therefore, the functional role for TRIM25 ubiquitin ligase activity could be autoubiquitylation, which is a key feature of TRIM family members (90). Alternatively, TRIM25 could ubiquitylate other proteins in the ZAP interactome to control ZAP activity. Identifying a complete repertoire of TRIM25 ubiquitylation targets in the context of ZAP-sensitive virus infection and/or IFN treatment will be essential to determine how it regulates ZAP activity.

ZAP has been proposed to target viral RNA for degradation through several potentially complementary mechanisms (**Figure 2**). First, ZAP has been reported to interact with components of the 5′–3′ and 3′–5′ RNA degradation pathways, including DCP1A-DCP2, XRN1, PARN, and the exoribonuclease exosome complex (24, 29, 30). The 3′–5′ decay pathway is initiated by the recruitment of cellular deadenylases to shorten the poly(A) tail followed by exosome-mediated degradation. ZAP interacts with the deadenylase PARN, and depletion of PARN reduced RNA degradation by overexpressed ZAP (30). ZAP also interacts with the exosome subunits EXOSC5 (Rrp46p) and EXOSC7 (Rrp42p), and depletion of EXOSC4 (Rrp41p), EXOSC5, or EXOSC7 in cells overexpressing ZAP reduced its ability to target RNA for degradation (24, 29, 30, 50). While depletion of exosome subunits increased Japanese encephalitis virus infectious virus production, depletion of several subunits of this complex did not substantially increase wild-type or CpG-enriched HIV-1 infectious virus production, indicating that other factors may also be involved for some viruses (40, 50). ZAP indirectly interacts with components of the 5′–3′ degradation pathway, including the decapping complex DCP1A-DCP2 and the 5′–3′ exoribonuclease XRN1 (30). Depletion of DCP1A, DCP2, or XRN1 reduced the activity of overexpressed ZAP to degrade

retroviral RNA (30). However, XRN1 depletion did not affect ZAP restriction of Japanese encephalitis virus, suggesting that there may be virus-specific ZAP requirements for components of the RNA degradation machinery (50).

ZAP also interacts with the RNA helicases DDX17 (also known as p72) and DHX30 (24, 30, 91, 92). Overexpression of DDX17, but not a catalytically inactive mutant, increased ZAP activity, and depletion of endogenous DDX17 or DHX30 decreased ZAP-mediated viral inhibition (91, 92). DCP1A-DCP2 and XRN1 coimmunoprecipitated with DDX17 in an RNase-dependent manner, suggesting that ZAP indirectly recruits 5′–3′ degradation machinery through DDX17 (30). Thus, RNA helicases may facilitate the recruitment of mRNA degradation machinery by ZAP and disrupt RNA secondary structure to allow for ZAP-mediated RNA decay.

A second mechanism for how ZAP interacts with cellular proteins to mediate viral RNA degradation is by recruiting the paralogs KHNYN or N4BP1 (34, 43). Of the two, KHNYN is better characterized as a ZAP cofactor. As its name implies, KHNYN contains an N-terminal extended di-KH-like domain and an NYN domain, which likely has endoribonuclease activity (34, 93, 94) (**Figure 1**). It also has a cullin binding domain associating with NEDD8 (CUBAN) domain that selectively binds NEDD8 over ubiquitin (95). KHNYN interacts with ZAP in yeast-two-hybrid, coimmunoprecipitation, BioID, and affinity purification-mass spectrometry experiments (34, 82, 83) (**Supplemental Table 2**). Depleting endogenous KHNYN increases CpG-enriched HIV-1 infectious virion production as well as MLV Gag expression and virion production, phenocopying the effect of depleting endogenous levels of ZAP (34). Although nuclease activity has not been experimentally demonstrated for the NYN domain, all of the known catalytic residues in paralogous active domains are conserved in KHNYN, and mutation of conserved residues putatively required for endonuclease activity abrogated its antiviral function (34). KHNYN requires ZAP for antiviral activity against CpG-enriched HIV-1, and it is hypothesized that ZAP is required for KHNYN to target retroviral RNA and mediate endonucleic cleavage (34, 41). Of note, this would produce viral RNA targets for 5′–3′ and 3′–5′ exonucleic decay machinery. KHNYN or N4BP1 depletion did not substantially affect replication of the togaviruses SINV or SFV, respectively (34, 43). One explanation for this is that ZAP predominately inhibits retrovirus gene expression by degrading viral RNA while togaviruses are restricted primarily through translation inhibition (4, 9, 29, 32, 48). Another possibility is that, for some viruses, the two paralogs are functionally redundant. While N4BP1 is an ISG, KHNYN is not, and it remains unknown whether they have different relative activities as ZAP cofactors in different cell types or after IFN treatment (34, 43, 55, 96).

Supplemental Material >

The OAS3/RNAse L pathway has been reported to contribute to ZAP-mediated restriction of echovirus 7 with increased UpA or CpG content (65, 76). RNase L, an endoribonuclease, is activated by 2′–5′-linked oligoadenylate produced by OAS1–3 upon binding dsRNA and preferentially cleaves ssRNA at sites with UpU and UpA dinucleotides (97). Inhibition of E7 replication due to high UpA or CpG content was abrogated in ZAP, OAS3, and RNAse L knockout cell lines but was unaffected by OAS1 depletion (65, 76). ZAP and OAS3 colocalize and coimmunoprecipitate (76). Interestingly, viral RNA decay rates for ZAP-sensitive echovirus 7 with introduced CpGs or UpAs were not affected by ZAP, OAS3, or RNaseL knockout, suggesting that the ribonuclease activity of RNAse L or the ZAP antiviral pathway is not involved in the specific degradation of these viral RNAs (65). The specific role of OAS3 and RNAse L in ZAP antiviral activity remains to be elucidated.

In addition to mediating viral RNA degradation, ZAP has been shown to inhibit translation initiation by interfering with the interaction between eIF4A and eIF4G (31) (**Figure 2**). Translation initiation requires recognition of the 5′ cap on mRNAs by the cap-binding protein eIF4E, the scaffold protein eIF4G, and the RNA helicase eIF4A (98). ZAP has been proposed to inhibit the interaction between eIF4A and eIF4G to prevent translation initiation (31). However,

specific mutations in ZAP that eliminate this activity have not been identified, making it difficult to determine this mechanism's contribution to the restriction of different viruses.

There is often an interplay between positive and negative regulatory factors to modulate the activity of antiviral proteins. MATR3 was identified as a negative regulator of ZAP-mediated retroviral restriction (39). MATR3 has an RNA-dependent interaction with ZAP. In cells overexpressing ZAP, MATR3 overexpression decreased ZAP restriction against retroviral reporter constructs, while knockdown of MATR3 increased overexpressed ZAP-mediated restriction of MLV and HIV-1 transcripts. It has been hypothesized that MATR3 partially shields viral nucleic acids from recognition by ZAP. However, whether MATR3 is a general negative regulator of ZAP activity is unclear.

ZAP antiviral activity is regulated by phosphorylation (65, 99). The serine/threonine kinase GSK3B was reported to phosphorylate rat ZAP (99). Treatment of ZAP-expressing cells with a specific GSK3B inhibitor or depletion of GSK3B decreased the antiviral activity of ZAP against retroviral reporter constructs, while overexpression of GSK3B increased ZAP-mediated restriction (99). In cells treated with a GSK3B inhibitor, ZAP's ability to degrade reporter mRNA was unaffected (99). However, reporter protein expression was inhibited, suggesting that it modulates ZAP's ability to restrict viral translation but not ZAP-mediated RNA degradation. Treatment of cells with a broad kinase inhibitor or GSK3B-specific inhibitor led to increased replication of echovirus 7 with introduced CpGs or UpAs, demonstrating that phosphorylation of human ZAP is also required for optimal antiviral activity (65). The structural subunit of serine/threonine-protein phosphatase 2A, PR65A, and its catalytic subunit PPP2CA coimmunoprecipitated with ZAP in an RNA-independent manner, with depletion of PR65A decreasing ZAP-mediated antiviral activity against a retroviral reporter construct (24, 100). While PR65A may regulate ZAP activity by controlling its phosphorylation status, it is unknown whether it dephosphorylates the same residues modified by GSK3B.

Overall, identifying and characterizing the full complement of ZAP cofactors and how they regulate its antiviral activity is critical to understanding the ZAP antiviral system. One of the challenges is that specific point mutations in ZAP that abrogate its interaction with individual cofactors have not been defined and characterized using multiple viruses. Therefore, the specific contribution of each potential cofactor for ZAP antiviral activity is often unclear. Furthermore, it is unclear which proteins directly interact with ZAP and which are bridged by other macromolecules (proteins, RNA, membranes). Critically, little is known about how the ZAP-L and ZAP-S interactomes differ, especially in the context of their different subcellular localization.

ZAP REGULATES CELLULAR GENE EXPRESSION

It is essential for ZAP to be able to target specific transcripts because nonspecific binding could broadly repress cellular gene expression. The CpG depletion in vertebrate genomes has been proposed to allow for the evolution of ZAP to discriminate between self and nonself RNA (3, 40). Nonetheless, in addition to directly binding viral RNA, ZAP also regulates the expression of cellular genes, possibly to modulate the innate immune response. ZAP depletion in HeLa cells was shown to alter the abundance of a subset of transcripts enriched in genes encoding proteins with a signal peptide and members of the IFN immune response pathway (18).

Both ZAP-L and ZAP-S directly bind cellular mRNAs and can regulate cellular gene expression by targeting them for degradation (18). ZAP-L represses TRAILR4 expression, which encodes a decoy receptor for the proapoptotic cytokine TRAIL, by binding its 3′ UTR and targeting it for exosome-mediated degradation (18, 101). Thus, ZAP depletion increases sensitivity to TRAIL-mediated apoptosis. Whether other ZAP cofactors, such as TRIM25 or KHNYN, are

required for TRAILR4 degradation is unknown. ZAP-S has been reported to regulate type I and III IFN production, although this is contested. One study found that ZAP directly bound AU-rich elements in the 3′ UTR of IFN mRNAs, and its depletion led to increased IFN expression (7). In contrast, other studies showed that ZAP is required for IFN production by promoting RIG-I oligomerization and downstream signaling events (27, 59). Furthermore, other reports showed no effect on IFN production in ZAP knockout mouse cells (23, 47). Therefore, a comprehensive analysis of how ZAP binding sites in cellular RNAs correlate with changes in gene expression, especially in the context of viral infection, will be essential to understand how ZAP binding to cellular transcripts regulates their expression.

ZAP also regulates cellular gene expression by inhibiting microRNA-mediated silencing. MicroRNAs target the RNA-induced silencing complex (RISC) to mRNAs to repress their translation and target them for degradation (102). ZAP interacts with several components of RISC (**Supplemental Table 2**), including argonaute (AGO) proteins (20, 103). Upon cellular stress or viral infection, both ZAP and AGO proteins are poly(ADP-ribosoyl)ated. ZAP is required for AGO poly(ADP-ribosyl)ation, although the active PARP proteins that mediate this are unknown (20, 103). This inhibits microRNA-mediated repression for a subset of ISG mRNAs, including those associated with proliferation and cell death, which promotes their expression (103). Therefore, ZAP directly and indirectly regulates antiviral gene expression, and comprehensive analysis of cellular genes that are post-transcriptionally controlled by ZAP will help elucidate how it controls the innate immune response in addition to its direct antiviral activity.

Supplemental Material >

THERAPEUTIC POTENTIAL

Understanding the molecular biology of the ZAP antiviral system may allow several translational applications. Cost-effective chemical synthesis of long DNA segments has allowed the rapid development of synthetic virology in which large numbers of changes can be made throughout a viral genome. An example of this is synonymous genome recoding, in which synonymous mutations are added to viral genes to change the nucleotide sequence without affecting the amino acid sequence (104, 105). There are several approaches for synonymous genome recoding, including altering codon, codon pair, or dinucleotide frequencies to create novel live attenuated virus vaccine candidates. Inserting substantial numbers of CpGs into viral genomes through synonymous mutations may sensitize them to ZAP, attenuating their replication while maintaining their ability to provide active acquired immunity (106–109). One advantage of this approach could be high genetic stability for live attenuated virus vaccines because each CpG may be only slightly inhibitory, and many independent mutations would have to occur for reversion to virulence. Another advantage is that recoded genomes can be quickly designed and produced for emerging viruses. IAV and Zika virus have been attenuated through the synonymous introduction of CpGs (107, 108). Both viruses were attenuated in vitro and in vivo, and yet they elicited robust immune responses and protected mice when challenged with wild-type virus. However, the role of ZAP in restricting these CpG-recoded viruses is unclear, and producing synonymous recoded viruses that are restricted by ZAP remains a challenge due to limited knowledge of optimal ZAP binding sites. Characterizing sequences for high-affinity ZAP binding and defining their optimal spacing to allow multiple ZAP molecules to be targeted to an RNA is essential because CpG dinucleotides can inhibit viral replication through ZAP-independent mechanisms, and introducing CpGs, at least in some contexts, is not sufficient for IAV attenuation (41, 78, 80).

Anticancer therapies could be another application for the ZAP antiviral pathway. Some types of cancer, including liver, colon, and bladder, have lower ZAP expression than adjacent noncancer tissue, and this is associated with poor disease progression and survival (101, 110–112). While it

remains unclear how ZAP downregulation in specific types of cancer cells affects their transcriptome and proteome, dysregulated TRAILR4 expression may be one of the mechanisms by which ZAP regulates cancer growth (18, 101). Alphavirus M1 is an oncolytic virus that replicates preferentially in cancer cells with low ZAP abundance (112). This virus showed selective cancer cell killing through ER stress-induced apoptosis in vitro and high tumor specificity correlating with ZAP abundance. Alphavirus M1 had potent antitumor activity in ex vivo cancer tissues and several preclinical in vivo tumor models. In addition, a CpG-recoded Zika virus selectively replicates in glioblastoma stem cells and inhibits tumor growth (113). Therefore, ZAP deficiency may serve as a biomarker for personalized oncolytic therapy.

A better understanding of how ZAP binds to a transcript to inhibit its translation and/or target it for degradation will facilitate the development of optimal ZREs that can be incorporated into therapeutics or define RNA sequences that should be avoided, such as applications in which high transgene expression is required. As discussed in the sections above, there are several critical open questions regarding how to build a synthetic ZRE. First, while each ZAP molecule binds one CpG dinucleotide through ZnF2, how the local sequence context and RNA secondary structure surrounding the CpG affect ZAP binding must be better defined (14, 15, 41, 42, 76). Second, ZAP dimerizes and multiple ZAP molecules can bind an RNA with several CpG dinucleotides (10, 15, 28). However, the frequency and spacing of CpGs in a target RNA that efficiently mediate ZAP antiviral activity is unclear. Furthermore, whether there is a threshold of ZAP abundance on an RNA for antiviral activity or if higher-order ZAP-RNA structures are formed remains to be determined. It is also unclear if the specific position of the ZRE in a transcript, such as its location relative to the 5′ cap, poly(A) tail, exon-junction boundary, or other feature, affects ZAP binding and downstream activities. Third, while both CLIP-seq and structural studies support CpG dinucleotides as critical components of the ZRE, the role of other sequences such as UpAs must be resolved to fully understand how ZAP targets viral RNA (14, 15, 40, 65, 76). Finally, how ZAP interacts with other cellular proteins to mediate antiviral activity and how the binding sites for some of these cofactors that also bind RNA, such as TRIM25, overlap with ZAP on a target transcript remain to be elucidated. Overall, a complete understanding of the molecular and cell biology underlying the ZAP antiviral system will allow it to be utilized to promote human health.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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