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Highlights

*Seminars in Cell & Developmental Biology xx (2011) xxx–xxx*

## Zalpha-domains: At the intersection between RNA editing and innate immunity

Alekos Athanasiadis*

►The A to I RNA editing modification has a regulatory role in innate immunity. ►The RNA editing enzyme ADAR1 increases viral proliferation. ►Distinguishing feature of ADAR1 is a Z-DNA/Z-RNA binding domain, Zalpha. ►Zalpha domains characterize proteins of the interferon response involved in the recognition of foreign nucleic acids.

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Review

# Zalpha-domains: At the intersection between RNA editing and innate immunity

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ABSTRACT

The involvement of A to I RNA editing in antiviral responses was first indicated by the observation of genomic hyper-mutation for several RNA viruses in the course of persistent infections. However, in only a few cases an antiviral role was ever demonstrated and surprisingly, it turns out that ADARs – the RNA editing enzymes – may have a prominent pro-viral role through the modulation/down-regulation of the interferon response. A key role in this regulatory function of RNA editing is played by ADAR1, an interferon inducible RNA editing enzyme. A distinguishing feature of ADAR1, when compared with other ADARs, is the presence of a Z-DNA binding domain, Zalpha. Since the initial discovery of the specific and high affinity binding of Zalpha to CpG repeats in a left-handed helical conformation, other proteins, all related to the interferon response pathway, were shown to have similar domains throughout the vertebrate lineage. What is the biological function of this domain family remains unclear but a significant body of work provides pieces of a puzzle that points to an important role of Zalpha domains in the recognition of foreign nucleic acids in the cytoplasm by the innate immune system. Here we will provide an overview of our knowledge on ADAR1 function in interferon response with emphasis on Zalpha domains.

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**Contents**



## 1. Introduction

In biology what you see is not always what you get. The discovery of RNA editing in Xenopus eggs in 1988 [1] and extensive research since have challenged a deeply rooted belief that the sequence of transcribed nuclear RNA is a faithful copy of the genomic sequence. This RNA modification was found to be present in all metazoans and was termed A to I RNA editing because it consists in the hydrolytic deamination of adenosines (A) to inosine (I) within RNAs. Inosine base-pairs with cytosine instead of thymine and so for any biological process that involves recognition of the RNA sequence through base pairing, inosine is an equivalent of guanosine. As a consequence, A to I editing is able to recode aminoacid sequences [2], to alter splice sites [3], create miRNA binding sites and change the specificity of miRNAs themselves [4].

Responsible enzymes for this RNA modification are adenosine deaminases called ADARs that show limited target sequence

*Abbreviations:* ADAR, adenosine deaminase acting on RNA; A to I, adenosine to inosine; PKR, protein kinase RNA depended; DAI, DNA depended Activator of IRFs; IFN, interferon; CNS, central nervous system; eIF2a, eukaryotic initiation factor 2a; HIV, human immunodeficiency virus.

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specificity [5]. Instead, their specificity is determined primarily by the secondary structure of the RNA substrate [6]. While ADARs modify a perfect RNA duplex in a promiscuous manner leading up to 50% of modified adenosines, the modification can be highly specific towards complex RNA structures containing bulges and internal loops [6].

The discovery that this RNA modification leads to functionally critical aminoacid changes in vertebrate neurotransmitter receptors like the GluR-2, GluR-5 and -6 subunits of AMPA receptors, the Serotonin-2C (reviewed in [2,7]) and the γ-amino butyric acid (GABA) receptors [8] as well as in invertebrate chloride channel subunits [9], nicotinic acetylcholine receptor (nAChR) subunits [10] and several other proteins involved in fast synaptic transmission [11], led to the notion that the primary role of A to I RNA editing is to provide the much needed diversity in the proteome of the animal central nervous system (CNS). This notion however left unexplained the observed abundance of RNA editing activity outside of the CNS.

Indeed, in recent years transcriptome analysis has shown that ADARs, the A to I RNA editing enzymes, are responsible for extensive modification of 3′UTR and intronic sequences of thousands of human genes [12–15] targeting secondary structures often created by pairs of inverted repetitive elements. These findings indicate that any extensive and stable pre-mRNA stem-loop structure is accessible to ADARs and a potential target for RNA editing and have led to a wide rethinking regarding the primary role of this post-transcriptional modification.

Double stranded RNA in the cytoplasm is known to represent a danger signal indicating viral infection and several antiviral pathways are triggered by dsRNA including the RNA interference pathway in invertebrates [16] and the interferon response in vertebrate cells [17]. Through the introduction of mismatches in RNA duplexes, A to I RNA editing can reduce their double stranded nature and mark such duplexes with Inosine. Could nuclear A to I RNA editing prevent the exposure of cellular mRNAs containing dsRNA structures to such antiviral pathways? Is RNA editing in the cytoplasm responsible for limiting the duration and/or the intensity of active antiviral responses? While evidence linking A to I RNA editing to antiviral responses dates back to its discovery, in the recent years several lines of research converge on an unexpected role for the editing machinery in the modulation of cellular responses to foreign nucleic acids.

## 2. Links between A to I RNA editing and immunity

The first evidence for a role of A to I RNA editing in immunity came with the discovery of modifications in several viral RNA genomes and viral transcripts. These modifications were shown to take the form either of genomic hyper-editing in the course of persistent viral infections or more specific RNA editing events in structured viral mRNAs. A second and independent line of evidence for a role of RNA editing in innate immunity comes from work implicating the interferon inducible ADAR1 editing enzyme in the regulation of the antiviral interferon response.

### 2.1. Modification of viral nucleic acids

Viral nucleic acids have been among the first known substrates of ADARs: the discovery of hyper-mutated RNA genomes of measles virus [18] and other members of the *paramyxoviridae* family was followed by similar observations for Vesicular Stomatitis Virus and mRNAs of the mouse polyoma DNA-virus reviewed in [19–21]. More recently similar hypermutation was observed in the invertebrate sigma RNA-virus [22] involving the ADAR enzyme of drosophilids. These findings were taken to suggest an antiviral role of ADAR mediated RNA editing, mechanistically based on scrambling the information content of viral nucleic acids, not unlike the role of the APOBEC3 family of cytidine deaminases in retroviral restriction. However, in only few cases an antiviral role of ADARs has been demonstrated and even in these cases it still remains unclear if this is a direct result of hyper-editing of viral nucleic acids or the result of the influence of ADARs on elements of the innate immune system. Indeed, knockdown of ADAR1 in an ADAR2$^{-/-}$ background in cell culture is shown to alter the cytopathic effects of polyoma virus infection [23] in a manner independent of the modification of viral RNA.

Specific editing within a well-defined sequence and structure context of viral nucleic acids was first demonstrated for Hepatitis D virus (HDV) [24]. Surprisingly, HDV was shown to make use of the cellular RNA editing machinery for its own needs: a stop codon in Hepatitis delta antigen is being changed to a tryptophan codon by RNA editing allowing the switch from the replication stage to the packaging stage, thus enabling, viral proliferation. Interestingly, HIV replication is also shown to be enhanced in cells over-expressing ADAR1, and HIV transcripts of Rev and Tat coding sequences as well as the TAR RNA are shown to be edited by ADAR1. However, it remains unclear whether the increased replication is editing depended [25–27]. Specific RNA editing has also been found in transcripts of the Kaposi sarcoma associated virus (KSHV) [28,29], where again RNA editing appears to be adapted by the virus as the levels of editing correlate with the replicative state of the virus [29].

In summary, for both the hyper-editing and the more specific editing events observed in viral nucleic acids there is no clear evidence that these base modifications overall represent an antiviral action of ADARs. On the contrary, for viruses like Hepatitis D, RNA editing represents a valuable cellular contribution.

### 2.2. ADAR1 as a regulator of antiviral responses

In vertebrate species we find two genes encoding for active dsRNA dependent adenosine deaminases: ADAR1 and ADAR2. ADAR1 was the first to be discovered and expression of an N-terminally extended form of the protein was shown to be up-regulated during viral infection. Indeed, ADAR1 comes in two isoforms: a short, constitutively expressed and nuclear form (P110) and a longer, mainly cytoplasmic form (P150) which is transcribed from a different promoter responsive to type I and type II interferons [30] (Fig. 1A). The homozygous knockout of the ADAR1 gene in mice is embryonic lethal and its phenotypic characterization shows that its lethality is associated with extensive apoptosis in the hematopoietic tissue [31,32]. Further characterization of hematopoietic stem cells derived from an inducible ADAR1 gene knockout in mice shows a global upregulation of interferon responsive genes during embryonic development [33] pointing to a role of ADAR1 as a suppressor of interferon signaling. Selective knockout of the interferon controlled isoform P150 [34] suggests that this isoform is responsible for the observed embryonic lethality, although there is an ongoing debate on this matter.

The mechanism through which ADAR1 exerts control over innate immune responses has not been yet clarified. Clues however, have been obtained in studies showing that ADAR1 can antagonize protein kinase R (PKR) [35,36], a key protein of interferon response that mediates shutdown of cellular translation through the phosphorylation of eukaryotic initiation factor eIF2a during infection. PKR is activated by dsRNA and ADAR1 can inhibit its activation either through the sequestration of dsRNA or by rendering such dsRNA unrecognizable by PKR through modification. Indeed, ectopic ADAR1 expression results in a general increase in protein translation through the inhibition of PKR [37,38] and possibly of other dsRNA activated effector proteins of the interferon response

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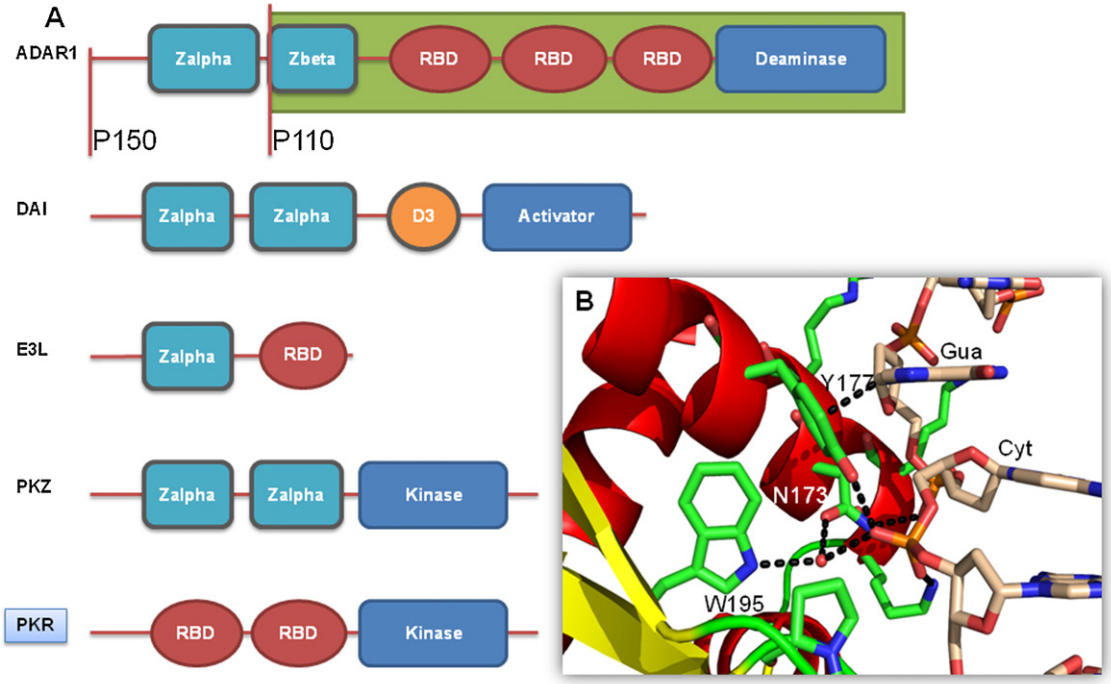


**Fig. 1.** Domain organization of proteins with Zalpha motifs (A). ADAR1 isoforms P150 and P110 are indicated. RBD stands for dsRNA binding domain. The domain organization of PKR is shown for comparison with PKZ. Conserved interactions between Zalpha domains and Z-DNA (B). Ty177, Asn173 and Trp195 positioned to recognize the characteristic Z-DNA phosphate backbone and a guanosine in Syn conformation.

such as the 2′-5′-oligoadenylate synthetase (OAS). A key question that arises is whether and to what extend cellular transcripts containing dsRNA can erroneously activate dsRNA dependent pathways and whether the observed extensive editing in such cellular RNA duplexes can have a preventive role. Experiments aiming to answer this question show that a single transcript containing a long dsRNA hairpin activates interferon response only at abnormally high expression levels [39]. However, given that there are possibly thousands of genes producing transcripts with embedded dsRNA structures, the activation of interferon response by cellular RNAs seems possible.

Another dsRNA dependent pathway that acts as an antiviral response in invertebrates but also present in vertebrates, is RNA interference (RNAi). An interaction between ADARs and the RNAi pathway was first suggested in experiments in *Caenorhabditis elegans* showing that the ADAR knockout phenotype can be rescued by the ablation of components of the RNAi machinery [40]. More recent work also in *C. elegans* provides support for a specific role of ADARs to protect an extensive population of dsRNA in cellular transcripts from RNAi [41]. RNAi of dsRNA in mammalian cellular transcripts has been recently demonstrated in oocytes [39] as well as the ability of ADARs, and in particular of cytoplasmic ADAR1 P150 to bind siRNA and limit the efficacy of the RNAi pathway [42].

Thus, in vertebrates ADAR1 is shown to antagonize both RNA interference and the interferon response. In doing so, ADAR1 may limit dsRNA dependent responses not only against cellular RNA but also against viral RNA. Indeed, in a survey for the effects of 380 IFN-regulated genes on viral proliferation, is shown to significantly enhance the replication rates of all tested viruses [43] supporting a pan-viral mechanism of enhancement.

An attractive model based on the available data ascribes a dual function of ADAR1: in the nucleus, it keeps under control the amount of cellular dsRNA that can reach dsRNA sensors in the cytoplasm while upon prolonged activation of the interferon pathway the P150 isoform migrates to the cytoplasm where it down-regulates antiviral responses by limiting the available dsRNA. This model establishes ADAR1 as a central node within a feedback mechanism that limits runaway antiviral responses and their cytopathic effects.

What are the molecular details of ADAR1 action in the cytoplasm and what are its interactions with the cytoplasmic nucleic acids sensors? The answer to these questions must largely be found at the 296 aminoacids (in humans) long extension at the N-terminus of the interferon inducible P150 isoform. The unique recognizable and conserved feature of this segment of ADAR1 is a DNA/RNA binding domain of so far unknown biological function, the Zalpha.

## 3. The ADAR1 Zalpha domain

The N-terminal domain of ADAR1 was the surprising yield of an in vitro screen for Z-DNA binding domains in blood nuclear extracts [44] and from this discovery derives its name: Zalpha. It was shown that this 78aa domain of ADAR1 binds with high affinity [45] to CpG repeats and that the bound dsDNA forms a left-handed Z-DNA helix, an alternative DNA conformation originally described more than 30 years ago [46]. While in vitro formation of Z-DNA requires high salt concentrations, the Zalpha domain is shown to stabilize this conformation on CpG repeats under physiological salt concentrations. This makes Zalpha the first known specific Z-DNA binding protein domain.

### *3.1. The ADAR1 Zalpha domain structure and its nucleic acids complexes*

The crystal structure of the ADAR1 Zalpha domain with a $T(CG)_3$dsDNA complex showed that it belongs to the larger family of winged-Helix-Turn-Helix domains (wHTH), a common DNA binding motif used in this case in a unique configuration [47]. A key role in the recognition of the Z-DNA backbone is played by a triplet of residues: Tyr177, Asn173 and Trp195 (all aminoacid references are based on human ADAR1-Zalpha numbering), which are all highly conserved among Zalpha domains (Fig. 1B). Tyr177 forms

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a π-bond with the guanosine in the Syn conformation characteristic for Z-DNA, the only direct base contact made by the protein and which contributes to the discrimination between the B-DNA and Z-DNA conformations. Mutation of any residue in this triplet eliminates DNA binding [48]. The remaining protein/DNA contacts are with the characteristically shaped Z-DNA phosphate backbone, allowing further discrimination of the two DNA-conformers. Interestingly, two Zalpha domains interact with the DNA duplex without forming a physical dimer suggesting a cooperative mode of DNA-binding, a feature that is so far seen in all structures of Zalpha domains.

Formation of Z-DNA requires a dinucleotide repeated unit of alternating purine/pyrimidine bases suggesting that Zalpha could interact with CA/TG repeats in addition to CG. Such repeats however show lower propensity of Z-DNA formation and the co-crystal structure of Zalpha with these type of sequences had to wait until recently [49]. How can Zalpha bind CpG repeats in the context of a larger DNA segment that cannot be entirely in the left handed conformation? The answer to this question came with the crystal structure of a Zalpha domain bound to a B–Z junction formed at the boundaries of the left handed helix [50] demonstrating that such junctions are formed by the extrusion of a single base pair at the junction site. Similarly, Z–Z junctions are formed when Zalpha binds to imperfect CpG repeats with disruptions of the dinucleotide repeat [51]. These crystal structures document the molecular details on how Zalpha domains are able to bind Z-DNA forming segments within natural DNA molecules of variable sequence.

CpG repeats in dsRNA also adopt a left-handed helical conformation [52] known as Z-RNA and consequently, this raises the possibility that the *in vivo* target of Zalpha domains could be dsRNA. Indeed, CD-spectroscopy confirmed the interaction of Zalpha with RNA [53] and the crystal structure of ADAR1 Zalpha with an RNA $(CG)_3$ oligonucleotide revealed a Z-RNA structure [54] with significant similarities to Z-DNA, making Zalpha one of the rare if not unique domains capable to bind dsDNA and dsRNA of the same sequence.

This extensive structural analysis of the recombinant ADAR1 Zalpha domain demonstrated a consistent behavior and conserved interactions of the domain with CpG sequences in a variable nucleic acids context.

### 3.2. Interferon response related proteins with Zalpha domains

Sequence based identification of Zalpha domains in other proteins led to the delineation of the Zalpha domain family (SMART ID: SM00550), a subfamily of the winged-Helix-Turn-Helix domains. Interestingly, all proteins sharing this motif were found to be involved in the interferon response pathway, and particularly components of this antiviral pathway that relate to the intracellular recognition of foreign nucleic acids. Two Zalpha domains are found in DNA-dependent activator of IFN-regulatory factors (DAI), a cytoplasmic receptor for foreign nucleic acids and activator of the production of type I interferons, in Z-DNA dependent protein kinase (PKZ), a fish ortholog of the mammalian PKR, and the pox-viral inhibitor of interferon response E3L (Fig. 1A).

Intriguingly, isolated Zalpha domains from each of these proteins were not only shown to share high affinity for CpG repeats but also a highly conserved mode of interaction with Z-DNA as demonstrated by the crystal and NMR structures of both DAI Zalpha domains [55–57], of Yatapoxvirus E3L [58] and biochemical data from the Zalpha domain of PKZ [59]. Such results point to a common function of Zalpha domains and a functional interconnection of the proteins that contain them.

DAI (also known as DLM-1 and ZBP-1) was shown to act as a sensor for dsDNA in the cytoplasm activated by TpA and to a lesser extent by CpG repeats [60]. A third DNA binding domain (D3) was shown to be primarily responsible for the recognition of TpA repeats and its two Zalpha domains expectedly play no role in its ability to bind B-form DNA, nevertheless they appear to be required for the activation of the signaling pathway through the interferon regulatory factor 3 (IRF3) [61]. The function of DAI seems to be specialized, playing roles in specific cell types and for viral infections by cytomegalovirus [62] and Herpes simplex virus [63] but not for example by intracellular bacteria as *Legionella pneumophila* [64].

PKZ is another nucleic acids sensor with two N-terminal Zalpha domains. This protein was found so far only in fish species like zebrafish, Atlantic salmon and goldfish and is an ortholog of the dsRNA dependent protein kinase PKR. PKZ shows a conserved and functional kinase domain but the dsRNA binding domains found in PKR are replaced by Zalpha domains [59]. This kinase appears to maintain the ability to phosphorylate eIF2a like PKR [65] and to block cellular translation. Unlike PKR however, its activity depends on the presence of CpG dsDNA and not poly I:C dsRNA [65]. While both DAI and PKZ appear responsive to CpG DNA the ability of CpG dsRNA to activate these proteins has not been tested, leaving open the possibility that these sensor proteins may respond to both DNA and RNA.

Given the role of Zalpha domains in antiviral responses, it should not come as a surprise that a Zalpha domain is found in E3L, a viral inhibitor of interferon response. E3L is a small two-domain protein found in all poxviruses whose C-terminal dsRNA binding domain blocks PKR activation and is required for viral proliferation. Its N-terminal Zalpha domain appears dispensable for viral replication in cell culture but crucial for pathogenicity in mice [66]. Interestingly, it is shown that replacement of the E3L Zalpha domain with that of ADAR1 or DAI fully supports viral pathogenicity, suggesting that Zalpha domains are functionally interchangeable [66]. TheE3L Zalpha domain could function through competition for nucleic acids with DAI similar to the mechanism proposed for the inhibition of PKR by its C-terminal dsRNA binding domain. Indeed micro-array analysis shows that the Zalpha domain of E3L blocks a distinct part of the interferon signaling cascade [67] which overlaps with the IRF3-TANK-binding kinase 1 (TBK1) signaling pathway shown to be activated by DAI [60].

### 3.3. The non-conforming Zbeta

The uniform behavior of Zalpha domains has a single exception: A domain homologous to Zalpha is also present in the constitutively expressed isoform of ADAR1. It was named Zbeta reflecting its position within ADAR1. This domain in isolation did not demonstrate any affinity for CpG repeats and in mammalian ADAR1 Zbeta domains, the critical aminoacids corresponding to Zalpha Tyr177 and Asn173 are mutated. The crystal structure of Zbeta [68] confirmed that this domain shares the same architecture with the Zalpha domain but it also shows that Zbeta cannot form interactions with DNA equivalent to Zalpha. It is unfortunate that in proteins that contain more than one Zalpha domain (DAI, PKZ) the term Zbeta has been historically used to describe the second domain although the mammalian ADAR1 Zbeta appears to have unique properties not shared by any other member of the family. Indeed, unlike the ADAR1 Zbeta domain the second Zalpha domain of DAI does interact with CpG repeats as it is described in the structures of its complexes with DNA [56,57].

## 4. In search for the Zalpha domain target and its biological function

Despite the very extensive and successful work characterizing the nucleic acids binding properties of Zalpha domains, the *in vivo*

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target(s) of these domains remain elusive. This is partly due to the dynamic and transient nature of Z-DNA formation and partly because the involvement of these domains in innate immunity suggests that their nucleic acids target may only be present during specific host–pathogen interactions. The information available so far comes from *in vivo* cross linking experiments and the effects of mutations of the Zalpha domains on the localization of the protein.

### 4.1. Nucleic acids bound by Zalpha in vivo

Pull-down experiments of genomic DNA segments probed for Zalpha binding reveal an enrichment in centromeric repeats [69] and probably reflect regions of Z-DNA formation rather than a functional association between the mostly cytoplasmic proteins and centromers.

Similar experiments where Zalpha is cross-linked *in vivo* to cellular RNA identified specific regions of ribosomal RNA of both bacterial and eukaryotic ribosomes [70] associated with the domain. Zalpha mediated binding of ADAR1 to the ribosome is shown to inhibit translation and the authors propose that the translation of ADAR1-bound mRNAs may be specifically inhibited this way. Such an interference with the translation machinery would be in agreement with the finding that the Zalpha domain of DAI is responsible for an association of DAI with stress granules that contain stalled translation preinitiation complexes [71]. How such action could be reconciled with a proviral role of ADAR1 and the general upregulation of translation through the modulation of PKR activity remains to be seen.

The accumulation of negative supercoiling introduced by RNA polymerases during transcription plays a key role in the stabilization of Z-DNA *in vivo* [72]. In recent years it has been shown that detection of certain types of dsDNA in the cytoplasm proceeds through its transcription by RNA polymerase III and subsequent recognition of the resulting 5′-triphosphate-RNA by the RIG-I helicase [73]. It is conceivable that Pol III transcription can be the source of negative supercoiling that gives rise to Z-DNA formation and the site of attachment for Zalpha-containing proteins. Although E3L is shown to inhibit RIG-I mediated activation of interferons against poly-AT DNA, this activity appears to be independent from its N-terminal Zalpha domain [74]. The study of other types of DNA activator sequences maybe necessary to reveal an involvement of Zalpha domains on the Pol III pathway.

## 5. Concluding remarks

The universal chemical and structural nature of nucleic acids presents a great challenge for cells that have to distinguish between self and non-self. CpG sequences and their unique structural features appear to have a central role in this distinction both for Toll mediated recognition and for cytoplasmic receptors where Zalpha domains may have a central role. Deregulation of this recognition and of the RNA editing process not only can affect the progress of pathogen-induced disease but may also have a crucial role in autoimmune diseases and cancer.

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