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ZBP1 as a sensor of viral and cellular Z-RNAs: walking the Razor's Edge

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Abstract

Z-form nucleic acid binding protein 1 (ZBP1) detects viral Z-form RNAs (Z-RNAs), activates Receptor Interacting Protein Kinase 3 (RIPK3), and triggers cell death during both RNA and DNA virus infections. Such cell death promotes virus clearance by eliminating infected cells and galvanizing antiviral immunity, and is thus often targeted for evasion by virus-encoded suppressors. Recent evidence demonstrates that ZBP1 can also be activated by cellular Z-RNAs transcribed from endogenous retroelements within mammalian genomes. These cellular Z-RNAs, if not edited and neutralized by Adenosine Deaminase RNA Specific1 (ADAR1), trigger ZBP1-dependent cell death and inflammation, which may drive disease in Aicardi-Goutière's Syndrome and related interferonopathies. Thus, while well-controlled activation of ZBP1 by viral Z-RNAs during infections is beneficial, the same pathway can have harmful consequences when inappropriately triggered by cellular Z-RNAs in other disease settings.

Keywords

ZBP1; Z-RNA; Z-DNA; DAI; RIPK3; MLKL; cell death; necroptosis; influenza A virus; vaccinia virus; poxvirus; herpesvirus

Introduction.

Multicellularity affords metazoans the luxury of sacrificing virus-infected cells by deploying dedicated programmed death pathways, an altruistic decision that martyrs the few for the

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Declaration of interest statement.

S.B. is listed as a co-inventor on US Patent Application Serial No. 63/339,860, entitled Combination Of Curaxins And Immune Checkpoint Inhibitors For Treating Cancer. The other authors declare no financial interests.

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many. During infection, well-controlled cell death is an effective means of halting virus spread and instigating an adaptive immune response that will eventually result in virus clearance. But when cell death is not well controlled, or is aberrantly activated outside settings of an acute infection, it often drives pathology. ZBP1-initiated death signaling in vertebrates provides an exemplar of this paradigm, and several recent studies illustrate just how beneficial, and dangerous, activating ZBP1 can be.

ZBP1 (also called DAI or DLM-1) was initially described as a sensor of cytosolic double-stranded (ds) DNA and thought to play a role in activating the Type I interferon (IFN) response [1]. Later studies showed that this function was primarily carried out by cyclic GMP/AMP Synthase (cGAS) [2]. Instead, ZBP1 was found to specifically detect the left-handed conformation of nucleic acids – referred to as Z-form nucleic acids (Z-NAs) – produced during viral infections. These Z-NAs are structurally different from the classic and more common right-handed A-form nucleic acid duplexes (Fig. 1). Highly specific Zα domains carried by Z-NA binding proteins allow cells to reliably detect these Z-NAs over their right-handed counterparts. Following binding of these Z-form ligands, ZBP1 recruits RIPK3 via their shared RIP homotypic interaction motifs (RHIMs), activating the protein kinase RIPK3 and cumulating in apoptosis and necroptosis [3,4].

ZBP1 is a remarkably potent initiator of antiviral host defense, as illustrated by mouse models of influenza A virus (IAV), vaccinia virus (VACV) and certain herpesvirus infections [3,5]. More recent reports, however, have demonstrated that ZBP1 can also sense Z-RNA species arising from mammalian genomes even in the absence of viral infection, especially when these are not first quelled by the Zα containing p150 isoform of ADAR1 [6–9]. In this case, ZBP1 activation is deleterious, triggering lethal autoinflammatory pathology in an otherwise ‘sterile’ context.

In this review, we summarize exciting new advances in our understanding of how ZBP1 senses Z-RNAs of viral and cellular origin, and how its activation leads to either beneficial or immunopathological outcomes.

ZBP1 as sensor of viral Z-RNA.

ZBP1 possesses two tandem N-terminal Zα domains, called Zα1 and Zα2 (Fig. 2). The Zα domain was first discovered in the ADAR1 p150 isoform (the p110 isoform does not contain a Zα domain; see Fig. 2), and shown to bind both Z-DNA and Z-RNA in a structure-specific manner, without any particular sequence dependence or base-specific contacts [10,11]. ZBP1 and ADAR1 are the only known mammalian proteins with a Zα domain. The observations that (1) ADAR1 p150 and ZBP1 are encoded by IFN-inducible genes, and (2) the E3 virulence proteins of poxviruses possess a related Zα domain, indicated a role for Z-form nucleic acid sensing in IFN-dependent antiviral immunity [12,13].

The first evidence that ZBP1 sensed viral RNA came from work with orthomyxoviruses. Influenza A and B viruses (IAV and IBV) were shown to activate RIPK3-dependent cell death, but how RIPK3 was activated by these viruses was not clear [14]. A breakthrough came with the discovery that ZBP1 was the host sensor which detected replicating

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orthomyxoviruses and activated RIPK3 [15,16] (Fig. 2). This finding was in itself a surprise because ZBP1 was until then considered a DNA sensor, while orthomyxoviruses have RNA genomes. We showed that the second Zα domain (Zα2) in ZBP1 was essential for activating cell death signaling during IAV infection, and mutating key Z-NA contact amino acids (N122D and Y126A) in Zα2 completely abolished death signaling [17]. These results strongly suggested that ZBP1 directly sensed IAV RNAs, and our subsequent analyses showed that this was indeed the case. We discovered that ZBP1 bound several viral genomic RNA species, including Defective Viral Genomes (DVGs), which are truncated RNAs often generated in large amounts during the replication of RNA viruses. DVGs form when the viral polymerase falls off its template RNAs but re-engages further downstream along the same RNA strands, producing sub-genomic RNAs with intact 5' and 3' ends but harboring large internal deletions. The 5' and 3' ends of IAV gene segments are semi-complementary to each other, allowing DVGs to form double-stranded structures such as panhandles and corkscrews. Indeed, when DVG dsRNAs adopt the A-conformation (i.e., form right-handed duplexes) they are activating ligands for RIG-I-like receptors (RLRs) [18]. In 2020, we showed that IAV DVG RNAs may also form Z-RNA, which can act as ligands for ZBP1 [4]. Orthomyxoviral Z-RNAs were primarily localized to the nucleus, and probably adopt the Z-conformation as a consequence of negative supercoiling induced by processive enzymes such as the viral RNA-dependent RNA polymerase or cellular helicases [19].

Z-RNAs have now been implicated as necroptosis-activating ZBP1 ligands in cells infected with several viruses, including those with DNA genomes (Fig. 3). For example, in MCMV and Herpes Simplex Virus-1-infected cells, blocking RNA synthesis prevents activation of ZBP1, suggesting that Z-RNA, not Z-DNA, are the dominant ZBP1 ligands produced during these herpesvirus infections [20–22]. VACV infections also generate Z-RNA species, which accumulate in the cytoplasm and activate ZBP1, but only when the virus-encoded E3 protein is mutated. This is because E3 possesses an N-terminal Zα domain, which outcompetes ZBP1 for Z-RNA [23]. *In vivo,* ZBP1-RIPK3 signaling was found to be crucial for host defense against IAV, MCMV, and VACV, underscoring the importance of this pathway to antiviral innate immune responses [15,24,25]. More recently, SARS-CoV-2 has been shown to produce Z-RNA; these may arise from viral ORF1a and ORF1b genomic regions [26].

Overall, while the identity of the dsRNA species which form Z-RNA in most of these scenarios is still unclear, and while our mechanistic understanding of how ZBP1 senses Z-RNA and activates RIPK3 remains incomplete, the discovery of Z-RNAs as *bona fide* ZBP1 ligands during virus infections provides a much-needed explanation for the biological significance of Zα proteins in the antiviral host defense.

ZBP1 as sensor of cellular Z-RNA.

Virus infections are not the only source of Z-NA. Evidence of naturally occurring Z-NAs in mammalian cells began to accrue in the early 1980s, when antibodies to the left-handed conformation of nucleic acid duplexes were found in sera of lupus-afflicted mice and humans, and Z-prone DNA sequences were reported to be dispersed throughout the human genome [27–29]. How these Z-NAs form, where they arise from, and how mammalian cells

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regulate them was unclear. It was not until 1997, with the discovery of the Zα domain in the IFN-induced p150 isoform of ADAR1, that answers to these questions began to emerge [11].

DsRNAs are generally very immunogenic, and mammalian cells attempt to limit their abundance so they do not trigger autoimmunity. ADAR1 is a workhorse of this regulatory process. In addition to a Zα domain, the ADAR1 p150 isoform also contains three tandem dsRNA binding domains (dsRBDs) which sense A-RNA, the right-handed RNA double helix seen commonly in nature. ADAR1 p150 can thus limit the accumulation of A-RNA and Z-RNA by binding and introducing destabilizing adenosine-to-inosine (A-to-I) edits in both forms of dsRNA [11,30] (Figs. 2 and 3). If not modified by ADAR1, endogenous dsRNAs are highly immune stimulatory. For example, dampened ADAR1 activity in humans causes Aicardi-Goutières syndrome (AGS), a severe inflammatory disease that is invariably fatal [31]. Thus, editing of dsRNAs by ADAR1 prevents their accumulation, limiting inappropriate inflammation.

But what, if any, is the contribution of Z-RNAs to the inflammation seen in AGS patients? And what mechanisms initiate such inflammation? Previous studies have shown that AGS patients manifest a chronic type I IFN signature which (in the mouse model) is driven by the A-RNA sensor melanoma differentiation-associated protein 5 (MDA-5) (encoded by *Ifih1*) and its downstream adaptor mitochondrial antiviral-signaling protein (MAVS) [32]. However, whereas MDA-5 or MAVS deficiency rescues the embryonic lethal phenotype of *Adar*-deficient animals, mice with combined *Adar/Ifih1* or *Adar/Mavs* deficiency succumb within a few weeks of birth [32]. These results suggested that ADAR1 p150 limits the pathogenic activation of additional RNA sensors. Notably, two of the human AGS Mendelian variants (N173S and P193A) map to the Zα domain in ADAR1 p150, suggesting that a Z-RNA sensing protein might be an effector of AGS pathology. As ZBP1 is the only other mammalian protein known to contain a Zα domain, it was an obvious candidate for investigation [33] (Fig. 2). The question thus became whether there are cellular sources of Z-RNA which, if not quenched by ADAR1 p150, can activate ZBP1 and trigger inflammatory pathology.

The first evidence that ZBP1 bound cellular RNAs came in 2017, when overexpressed ZBP1 was reported to associate with endogenous RNAs in HEK293T cells [22]. But from where exactly were these endogenous Z-RNAs coming? Early insight into this question came when loss of RIPK1, which is known to inhibit ZBP1 dependent cell death via RHIM-RHIM interactions, was shown to trigger the spontaneous generation of dsRNA species, likely arising from endogenous retroelements (EREs). Sensing of these dsRNAs by ZBP1 resulted in necroptosis-mediated skin inflammation and perinatal lethality [34–36]. As with loss of RIPK1, loss of Fas-associated death domain (FADD) or caspase 8 also resulted in the production of cellular Z-RNAs and activation of ZBP1 [37]. How loss of RIPK1, FADD, or caspase 8 results in the spontaneous generation of cellular Z-RNAs is not clear, but derepressing EREs by ablating the epigenetic regulator SET Domain Bifurcated Histone Lysine Methyltransferase 1 (SETDB1) triggered ZBP1-dependent necroptosis *in vivo* [38]. Similar epigenetic mechanisms may account for reawakening of EREs when RIPK1, FADD, or caspase 8 are absent. Whatever the underlying mechanism(s) might be, these studies indicated that EREs may be sources of Z-RNA forming cellular ligands.

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Strong supporting evidence for this idea came in 2021, when four groups independently generated knock-in mice harboring either AGS-derived or biochemically-informed mutations in the Zα domain of ADAR1 p150 [39–42]. By examining mRNAs whose A→I editing is decreased in the mutant mice compared to control animals, these groups showed that the bulk of cellular Z-RNAs quenched by ADAR1 could be mapped to the 3'UTRs of host mRNAs, and were significantly enriched for a class of EREs called Short Interspersed Nuclear Elements (SINEs) [39–41]. In related studies, ADAR1 p150 was found to bind and edit dsRNAs formed from inverted Alu SINEs harboring Z-forming motifs, consistent with the *in vivo* findings [19,43].

Given these observations, several groups sought to test if loss of ADAR1 p150 expression, or mutation of its Zα domain, resulted in the accrual of endogenous Z-RNAs capable of activating ZBP1. In 2022, we showed that ablating *Adar* in MEFs did indeed result in the spontaneous generation and accumulation of Z-RNA [9]. Exposing *Adar*-ablated cells to Type I IFN strongly boosted Z-RNA accrual, and sequencing these Z-RNAs following their immunoprecipitation with a Z-NA-specific antibody demonstrated that the vast majority (~90%) of Z-forming RNA sequences mapped to mRNAs, including many mRNAs that were interferon-stimulated gene (ISG) products. In agreement with previous results, almost all Z-prone sequences mapped to the 3' UTRs of these mRNAs and fell into two major categories: inverted SINEs, and simple (e.g., GU-type) repeats. While SINE-derived dsRNAs have previously been shown to adopt the A-conformation and activate RLR-initiated Type I IFN responses, inverted SINEs can also form Z-RNA [9,39,40,44]. Together, these endogenous Z-RNAs arising from inverted SINEs, GU-repeats, and other less-abundant sources are potent activators of ZBP1, triggering ZBP1/RIPK3-dependent cell death when ADAR1 p150 is absent or its Zα domain is mutated.

In the same year, three other groups found that either knocking out *Adar,* or mutating Z-NA contact residues in the Zα domain, lead to activation of ZBP1 in mice[6–8]. The Pasparakis group showed that mice harboring mutations (N175D/Y179A) in the Zα domain of ADAR1 p150 spontaneously manifested high levels of ERE-derived RNAs with strong dsRNA-forming potential, including those arising from Long Terminal Repeats (LTRs), Long Interspersed Nuclear Elements (LINEs), and SINEs [8]. High-coverage sequencing done by the Maelfait group uncovered similar results in IFN-stimulated primary lung fibroblasts from *Adar* Zα mutant mice, as well as in human HEK293 cells [7]. Murine (e.g., B2 and B4 family) and human (Alu) SINEs, respectively, made up the bulk of their Z-RNA hits [7]. Importantly, Alu-derived Z-RNA forming sequences (from the 3' UTRs of *NICN1* and *BPNT1* mRNAs) were able to robustly activate ZBP1-dependent cell death when transfected into human HT-29 cells, and such cell death was prevented by mutating the ZBP1 Zα domain [7]. Altogether, these results indicate that cellular Z-RNAs predominantly arise from EREs, and these Z-RNAs activate ZBP1 when not edited and/or sequestered by ADAR1 p150. Of note, telomeric RNAs and mitochondrial DNA have also been shown to activate ZBP1; whether these contribute to ZBP1-initiated pathology in autoimmune settings remains to be seen [45,46].

There are clear ZBP1-driven pathological consequences to accrual of endogenous Z-RNAs. Mice engineered to express ADAR1 p150 mutants deficient in enzyme function or Z-NA

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binding capacity develop AGS-like pathology, with a spontaneous IFN signature, increased ZBP1 expression, and post-natal lethality. Ablating *Zbp1* in these mice significantly reduced pathology, diminished the IFN signature, and extended animal survival [6–8]. The exact mechanism by which ZBP1 initiates pathogenesis when ADAR1 cannot quench endogenous Z-RNAs remains unclear. In cell culture experiments, ablating *Adar* results in robust ZBP1 dependent apoptosis (driven by RIPK3-caspase 8 signaling) and necroptosis (mediated by a RIPK3-MLKL axis) (Fig. 3). *In vivo*, however, eliminating either necroptosis or apoptosis signaling (or both) did not rescue *Adar* Zα mutant mice [6,8]. ZBP1 may therefore drive inflammation independently of its capacity to trigger cell death.

Concluding Perspectives.

ZBP1/RIPK3-mediated cell death pathways are essential for limiting virus spread and promoting virus clearance, as evidenced by results from mouse models of IAV, VACV, MCMV, and other virus infections. Notably, some poxviruses encode Zα domain containing decoys, and herpesviruses (such as MCMV, HSV-1, and HSV-2) produce RHIM-containing proteins, either of which can interfere with ZBP1-initiated cell death signaling [3,47]. Indeed, evidence suggests that increased ZBP1 activity may confer an evolutionary advantage during virus infections. For example, ADAR1 p150 Mendelian variants with reduced Z-RNA binding capacity (e.g., ADAR1 $p150^{N173S}$ and $p150^{P193A}$) are hemizygously present in 0.2% of all humans, increasing to 0.3% in northern European populations [33]. In these populations, reduced ADAR1 activity may result in heightened ZBP1-driven antiviral signaling, as more viral Z-RNA is now available to activate ZBP1. This increased activity of ZBP1 may improve the immune response against viral infections, allowing positive selection of ADAR1 variants over time as humans began living in larger communities, and as viral epidemics became increasingly commonplace.

But the same hemizygous allelic variations in *ADAR* that are potentially beneficial during viral epidemics can become lethal when the intact wild type allele is lost, enabling cellular Z-RNAs to activate ZBP1 and drive sterile autoinflammation, as seen in AGS. The elegant mouse and human genetics studies described in this review now provide mechanistic insight into such aberrant ZBP1 signaling, underscoring the importance of ensuring that ZBP1 activation is limited to beneficial host innate immune responses.

While activation of ZBP1 can provoke dangerous autoinflammation when triggered systemically, such inflammation may be useful as an adjuvant for cancer therapy if deployed in a localized manner within the tumor mass. Indeed, ADAR1 is a major determinant of unresponsiveness to immune checkpoint blockade (ICB)-based immunotherapy, in part because it prevents ZBP1 activation in tumors [9,48,49]. ADAR1 inhibitors, epigenetic modulators, or agents (such as CBL0137) which generate Z-NA in cells may lead to the selectively activation of ZBP1-initiated necroptosis within tumors [9]. Once activated, such inflammatory cell death may then reawaken ICB responsiveness in therapeutically cold tumors. These strategies are already showing promise in preclinical models and represent potentially game-changing options for improving immunotherapeutic outcomes in human patients [50,51].

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There is thus a Jekyll-and-Hyde quality to ZBP1 activation: it is a highly effective antiviral mechanism, and potentially of great benefit in oncological settings, but one that can quickly become pathogenic when activated by endogenous Z-RNAs in sterile contexts. Notably, the same conflicts exist for Z-prone genomic DNA sequences, which risk activating ZBP1 if allowed to 'freeze' in the left-handed conformation for long enough, or if liberated from heterochromatin, where they are typically silenced [52,53]. This dichotomy may explain why ZBP1 – and indeed, the entire necroptosis machinery - is poorly conserved through evolution. For example, carnivores do not encode MLKL, and birds do not express ZBP1 or RIPK3 [54]. In these cases, the hyper-inflammatory consequences of Z-NA sensing by ZBP1 may have outweighed its benefits.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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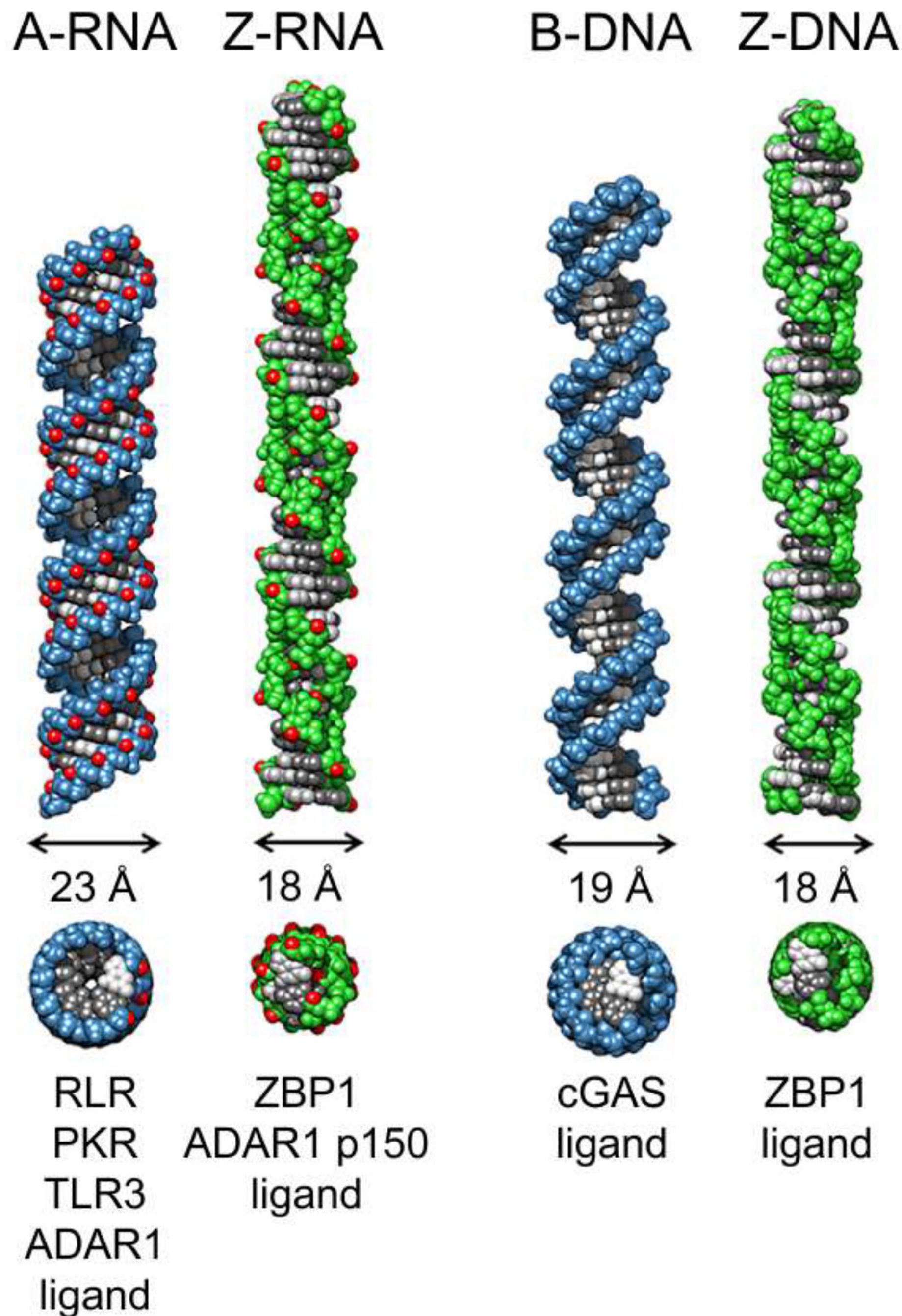

Figure 1. Structures of A-RNA, Z-RNA, B-DNA and Z-DNA.

Right-handed (A-RNA, B-DNA) double helices are shown in blue, and left-handed Z-conformations of dsRNA and dsDNA are depicted in green. Bases are shown in gray, and red spheres denote 2’ hydroxyls only found in RNA. Cross-sections and diameters are shown below each double-helical conformer. A-RNAs are ligands for RIG-like receptors (RLRs), Toll-like receptor 3 (TLR3), protein kinase dsRNA-dependent (PKR), Adenosine deaminase RNA specific1 (ADAR1), and other innate immune sensors. Z-RNAs are selectively detected by Z-form nucleic acid binding protein 1 (ZBP1) and ADAR1 p150

in mammals. B-DNA, the Watson-Crick double helix, is sensed by cGAS when present in the cytosol. ZBP1 can also detect Z-DNA in cells.

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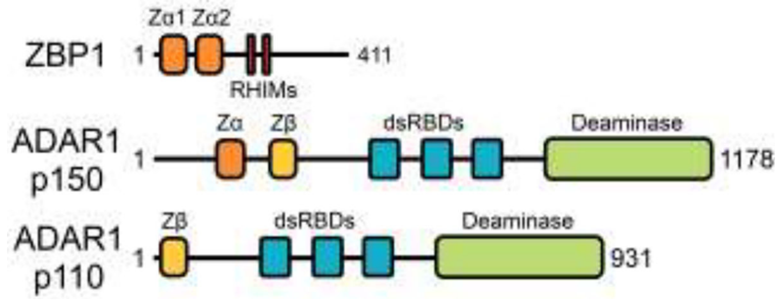

Figure 2. Domain architecture of ZBP1 and ADAR1 isoforms.

ZBP1 and the ADAR1 p150 isoform share a similar Zα domain, represented by orange boxes. The ADAR1 p110 isoform contains only the Zβ domain, shown in yellow. Both ADAR1 isoforms contain A-RNA binding domains (dsRBDs), represented by blue boxes, and a deaminase domain which catalyzes A-to-I editing (green box). ZBP1 harbors RIP homotypic interaction motifs (RHIMs, shown in red boxes), which mediate interactions with other RHIM containing proteins.

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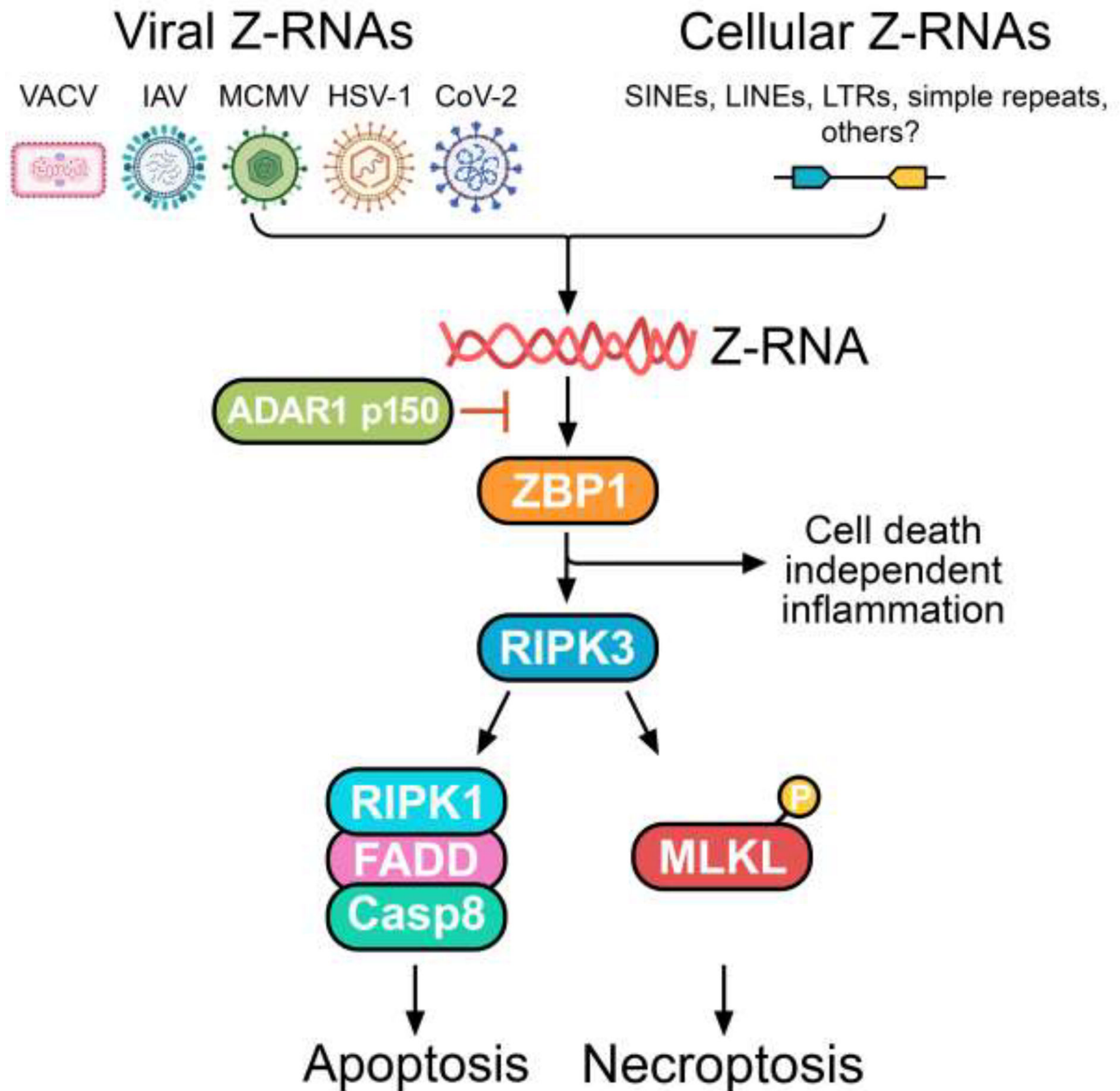

Figure 3. Viral and cellular Z-RNAs activate ZBP1.

Z-RNAs produced during infections by numerous viruses (left) are ZBP1 ligands, trigging beneficial antiviral responses which clear infected cells and promote adaptive immune responses. Cellular Z-RNAs (right) also activate ZBP1, for example when ADAR1 p150 is mutated or lost. ZBP1 activated in such 'sterile' contexts can promote autoinflammatory pathology. Whether activated by viral or cellular Z-RNAs, ZBP1 drives twin pathways of apoptosis and necroptosis, as well as cell death-independent inflammation.