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RNA-targeting CRISPR-Cas systems

Sam P. B. van Beljouw[1,2], Jasper Sanders[1,2], Alicia Rodríguez Molina[1,2], Stan J. J. Brouns[1,2]

[1]Department of Bionanoscience, Delft University of Technology, Delft, the Netherlands

[2]Kavli Institute of Nanoscience, Delft, the Netherlands

Abstract

CRISPR-Cas is a widespread adaptive immune system in prokaryotes that protects against viral infection by targeting specific invading nucleic acid sequences. While some CRISPR-Cas systems sense and cleave viral DNA, CRISPR-Cas type III and VI systems sense RNA resulting from viral transcription. The sequence specific detection of viral RNA evokes a cell-wide response that typically involves global damage to halt the infection. How to make sense of an immune strategy that encompasses broad, collateral effects rather than specific, targeted destruction? This review summarizes the current understanding on RNA-targeting CRISPR-Cas systems. It details the composition and properties of type III and VI systems, outlines the cellular defense processes that are instigated upon viral RNA sensing and describes the biological rationale behind the broad RNA activated immune responses as an effective strategy to combat viral infection.

Introduction

Since the appearance of primordial microorganisms, life has been in an intricate dance with death. Major contributors to this are parasites: their emergence is thought to be inevitable in even the simplest of replicator systems[1]. Parasites prey on host machinery for their propagation, often at the expense of host viability. However, the simple observation of a rich biosphere around us teeming with life indicates that the first hosts did not simply collapse under parasitic pressure. Instead, defense systems evolved that allowed the host to survive by preventing parasitic propagation. But complete parasite eradication did not happen either: contemporary bioinformatics demonstrate that virtually all life forms harbor diverse evolved parasites, such as plasmids, viruses and transposons, often termed mobile genetic elements (MGEs)[2]. The wide range of MGEs are thought to have resulted from early parasites that evolved counter-defense to protect against host defense, upon which the host evolved counter-counter-defense, and during this perpetual host-parasite arms race[3], functionalities are often swapped and recruited horizontally[4]. The host-parasite co-evolution is a main driver of increasing biological complexity, with the enormous variation and ingenuity in microbial immune systems as a prime example.

An average prokaryote encodes five anti-MGE systems[5,6]. Although they can be exploited by various MGEs to resolve conflict beyond traditionally emphasized host-virus interactions[7], they are often studied as cellular immune systems against viral invaders. In this context, interference of the viral infection can occur at various stages, from blocking the initial penetration at the cell membrane to preventing access to resources by initiating cell death[8]. The only form of adaptive immunity in prokaryotes discovered is the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) with CRISPR-associated (Cas) proteins. A hallmark feature of CRISPR-Cas systems is the presence of a CRISPR array, which consists of repeat sequences that are separated by variable sequences, termed spacers[9]. Spacers correspond to a segment of a virus, known as protospacer[10], which has previously been encountered and thus provide a genetic memory bank of past invasions. The Cas proteins are often encoded in the neighborhood of CRISPR arrays and are involved in various stages of CRISPR-Cas immunity[11–14].

Transcription of the CRISPR array generates a multi-spacer precursor CRISPR RNA molecule, which is further processed to free individual short CRISPR RNAs (crRNAs)[15,16]. The crRNAs subsequently assemble with Cas proteins to form ribonucleoproteins, called effector complexes, that surveil the cell in search for nucleic acids that base pair with the loaded crRNA[17]. Successful complementary binding indicates the presence of an intruder, whereupon the effector complex initiates a protective response. Depending on the type of effector complex, two general strategies intended to abrogate infection are discernible: (i) direct dismantling of the invading DNA via crRNA-guided cleavage and (ii) interference with the invader's replication cycle, often involving the activation of downstream immune proteins. The first strategy is employed by effector complexes belonging to the DNA-targeting CRISPR-Cas systems (type I, II, IV and V). Here, target recognition is coupled to DNase activity initiated by the effector complex architecture. This leads to rapid degradation of the bound DNA to abolish infection without necessitating further action. The second strategy is used by effector complexes from the RNA-targeting CRISPR-Cas systems (type III and VI). Activated type III and type VI effectors set a broad, collateral response in motion that often goes far beyond merely cleavage of the bound target; typical immune outcomes include cellular dormancy, an anti-viral tactic akin to a lockdown.

In this review, we discuss the biological principles underpinning RNA-targeting CRISPR-Cas systems. We first look at the properties of RNA recognition and some intrinsic differences between RNA- and DNA-targeting systems. Then we compare and contrast the molecular architecture of type III and VI CRISPR-Cas and describe how the sensing of RNA can lead to cellular states that facilitate viral immunity. Lastly, we focus on cellular dormancy induced by RNA-targeting CRISPR-Cas systems and its implications for both host and virus.

Recognition of RNA rather than DNA

Upon DNA genome injection into the cytoplasm, the infecting virus will not only encounter the transcription and translation machinery required for its propagation, but also immune proteins that aim to stop it. DNA-targeting immune systems (e.g. restriction enzymes, RecBCD, and type I, II, IV and V CRISPR-Cas effectors) can become active immediately upon entry of viral DNA into the cell and given their high abundance in prokaryotic genomes, they are often dubbed the first intracellular line of defense[5,6,18] (**Figure 1A**). But viruses have evolved various ways of escaping first line immunity, including chemically modifying the DNA (e.g. glycosylation and non-canonical nucleotide incorporation[19–21]), changing recognition sequences (e.g. mutating protospacer sequence and the protospacer adjacent motif (PAM)[22]), deploying anti-immune proteins (e.g. regulation disruptors, anti-CRISPR proteins, RecBCD inhibitors and anti-restriction proteins[23–26]), and physically protecting the DNA (e.g. genome compartmentalization[27,28]) (**Figure 1B**). Moreover, even in case of successful targeting, viral genome replication may already have occurred to the point of outrunning the activity of DNA-acting systems. A failed or slow DNA-targeting response results in progression of the infection, beginning with the accumulation of viral transcripts. This is when the RNA-targeting CRISPR-Cas systems can come to the rescue.

A prerequisite for activation of the RNA-targeting CRISPR-Cas systems is the presence of viral transcripts and could thus be regarded as secondary line of defense[29,30] (and, potentially, a first line of defense against RNA viruses[31,32]) (**Figure 1C**). At this stage, the cumulative effect of error-prone viral replication and transcription may have led to some viral RNA diversity within the cell. To make the occurrence of escape mutations less likely, relaxed targeting specificity has evolved in RNA-targeting effectors[27,33–37]. Self-RNA cleavage as a result of this permissive nature is less detrimental compared to self-DNA cleavage, as a transcript is easily replenished. Moreover, direct self-targeting on the CRISPR array –a major concern for DNA-targeting systems and a driving pressure for the evolution of the PAM requirement[38]– cannot occur with RNA-targeting effectors. Only the RNA resulting from antisense transcription of the CRISPR array can be recognized as target. This, however, will not lead to severe self-immunity, as progression of the immune response is only warranted when the crRNA of the effector is sufficiently mismatching a region on the target called the protospacer flanking site (PFS)[39–41] (**Figure 1C**). The PFS of the antisense CRISPR array transcript is complementary to the crRNA, thus signifying self, protecting the host from toxic incorrect immune activation (see **Dissection of CRISPR-Cas type III and VI**).

The co-occurrence of type I and type III CRISPR-Cas loci[42] and the suggested sharing of spacers between type I and III effectors[43,44] points at the possibility of cooperation between CRISPR-Cas systems targeting DNA and RNA. Indeed, it was found that viral escape from the DNA-targeting type I-F system was overcome through the targeting by a co-occurring type III-B system that used the type I-F crRNAs[45]. Cross-talk between RNA- and DNA-targeting CRISPR-Cas systems was also observed on the level of adaptation, where a native type VI-B locus was enriched with functional spacers that were acquired by the machinery of a co-occurring type II-C system[46]. Besides cooperating synergistically, RNA- and DNA-targeting systems can also complement each other. An intriguing example of defense complementation was discovered in *Pseudomonas* and *Serratia* bacteria, where infecting jumbo viruses build a nucleus-like proteinaceous compartment to shield their genome from various DNA-acting defenses[27,28,47] (**Figure 1B**). RNA-targeting CRISPR-Cas was shown to still provide protection, as viral transcripts are translated outside the protective barrier, where they are exposed to type III and type VI effectors[27,28,47]. In other words, RNA-targeting CRISPR-Cas is not only able to temporally, but also spatially back up DNA-based immunity evasion. Further studies will undoubtedly uncover more fascinating insights of the biological and ecological interactions between co-occurring RNA- and DNA-targeting systems.

Instead of rapid eradication of the virus, tolerating an invader can be an advantageous cellular strategy. Temperate viruses are capable of integrating into the host chromosome, often bringing new genes that potentially benefit host fitness[48]. Whereas systems targeting DNA destroy the virus independent of its benefits, RNA-targeting systems can conditionally tolerate viral presence by only suppressing the toxic lytic gene transcript[49,50] (**Figure 1C**). This allows the host to use viral gene content, while preventing the virus to become a threat.

Thus, while the DNA-targeting CRISPR-Cas systems display characteristics of a first line of defense strategy (e.g. immediate targeting, genome clearance and high specificity), RNA-recognizing CRISPR-Cas systems can be viewed as a second line strategy with various advantages. (i) Recognition on the RNA level circumvents hindrance by DNA modifications or other genome protecting mechanisms, giving the host an additional route to protect itself against foreign nucleic acids. (ii) RNA-recognizing effectors are generally lenient toward mutations in the target sequence,

making the chance of escape mutants less likely. (iii) Due to the transient nature of RNAs, an instance of autoimmunity through self-RNA cleavage is less detrimental to the host compared to self-targeting of DNA, and a full immune response is often not mounted when a self-RNA is recognized. (iv) Being reliant on transcription enables conditional tolerance of viral presence, allowing the host to potentially benefit from the additional genetic baggage. (v) Different cellular locations of DNA and RNA allows RNA-recognizing CRISPR-Cas systems to back up evaded DNA-targeting systems, as observed in defense against jumbo viruses.

Dissection of CRISPR-Cas type III and VI

Although a shared feature of CRISPR-Cas type III and type VI –and some type II and type V systems (**Box 1**)– is the ability to sense target RNA, they are evolutionarily distant and structurally unrelated, thus differing greatly in terms of immune activity (**Table 1**). Here, we go into depth on the molecular anatomy and mechanistic functioning of both systems.

CRISPR-Cas type III

CRISPR-Cas type III is believed to be the oldest member of the CRISPR-Cas family[51,52]. Up to now, six different type III subtypes have been identified: III-A to III-F[53]. The effector complexes are typically composed of multiple subunits (Cmr1 and Cmr3-6 in type III-B and III-C, Csm2-5 in type III-A and III-D) with signature subunit Cas10 being the largest component[54–57]. Cas10 is a multi-domain protein harboring a nuclease and a cyclase/polymerase domain, providing the effector complex with DNase activity as well as the capacity to generate cyclic second messengers from adenosine triphosphate (ATP). While effectors of type III-C have an apparent inactivated cyclase/polymerase domain, effectors of type III-D and various type III-B lack the nuclease domain[58,59]. The other type III subunits are responsible for either complex formation with the Cas6-processed crRNA[15,60], recruitment of host factors that promote crRNA maturation[61] and nucleic acid clearance[62], assisting in binding a complementary RNA or cleavage of the bound target[55]. The type III-E and III-F effectors have notably different architectures: the former has the subunits fused together while lacking the Cas10 subunit[63,64], whereas the latter contains only one Cas7-like protein with a Cas10 subunit that lacks the active cyclase/polymerase configuration[53]. Although type III systems can protect against an RNA virus under experimental conditions[64,65] and spacer matches to RNA viruses have been discovered[31,32], early analyses of the CRISPR arrays indicated spacer matches with DNA viruses[29,66] and thus viral transcript targeting by type III systems has been primarily studied.

The type III effector complexes surveil the cell to detect RNA molecules complementary to the crRNA (**Figure 2A**). Initial target RNA pairing occurs at the 3´ end of the crRNA, setting a conformational change in motion within the effector complex that allows for base-pairing the crRNA and target RNA[67]. In order to verify the origin of the bound transcript, complementarity is checked between the repeat derived crRNA portion (also known as 5´ tag) and the PFS (also known as 3´ anti-tag) of the suspected RNA invader[37,40,41,66]. Base pairing in this region indicates the binding of a self-RNA. When such false alarm occurs, Cas10 is kept locked in an inactive state to inhibit the immune response and reduce the toxic effects[41,66–69]. Target cleavage, on the other hand, does still occur: the RNase domains embedded in the backbone Cmr4/Csm3 subunits of a target RNA bound effector complex are exposed to the substrate and cleave it with a 6 nucleotide periodicity[63,65,70–74]. Cleaved RNA fragments dissociate from the crRNA[75], which is believed to recycle the effector complex for binding of a new target (**Figure 2A**).

When there is both sufficient base pairing in the spacer portion of the crRNA (particularly in the seven nucleotides at the 5´ side, also known as CAR, or Cas10 Activating Region[67]) and sufficient mismatches in the PFS region, the bound RNA is most likely of invader origin (**Figure 2A**). Conformational changes are relayed in the complex[69], unleashing nuclease and cyclase/polymerase catalytic activities within Cas10. The Cas10 nuclease activity is exerted by an histidine-aspartate (HD) domain and is able to degrade single-stranded DNA (ssDNA)[41,66,68,76]. Whereas the exact role of Cas10 ssDNA activity has to be established, several hypotheses are plausible: ssDNA cleavage might promote immunity via degradation of (i) ssDNA at or near the viral transcription bubble[76,77] (**Figure 2A**), (ii) the R-loops that arise during viral transcription elongation[78] or (iii) single-stranded replication intermediates of viruses and plasmids[78]. It also has been proposed that (iv) Cas10 nuclease activity promotes host mutagenesis through the induction of host chromosomal lesions[79]. Whereas the DNA cleavage can be sufficient for viral protection in case of abundant viral transcription[80], signal amplification is needed when transcription is limited. This is where the Cas10 cyclase/polymerase activity comes into play: two Palm domains facilitate ATP binding[81,82], whereupon a GGDD motif in one of the Palm domains catalyzes the conversion of bound ATP molecules into cyclic oligoadenylate (cOA) second messengers. This is achieved by 3´-5´ joining of adenosine monophosphates (AMPs) to form rings ranging between two (cyclic di-adenyate, or cA_2) to six (cyclic hexa-adenylate, or cA_6) AMP units[81,83–85] (**Figure 2A**). The dispersed cOA molecules in turn bind CRISPR-associated Rossmann Fold (CARF) domains of proteins often found in or near the CRISPR-Cas type III loci[81,84–87]. CARF is a nucleotide-binding domain that allosterically activates an attached effector domain upon binding the cOA nucleotide ligand, releasing its immune enzymatic activity[88,53].

Genomic neighborhood analysis of CRISPR-Cas type III loci have revealed numerous CARF family proteins, amongst others nucleases, transcription factors, proteases, deaminases, nitrilases and membrane associated proteins[86,87], suggesting a plethora of potential intriguing cOA-based defense pathways. Four nuclease-type CARF proteins have been experimentally characterized: Csm6/Csx1[81,84,88–92], Can1[93] and Card1[94]/Can2[95] (**Figure 2B**). Upon cA_4 or cA_6 binding to a Csm6/Csx1 homodimer, conformational changes bring together the Higher Eukaryotes and Prokaryotes Nucleotide-binding (HEPN) domains to constitute a promiscuous RNase pocket that degrades both viral and host transcripts. Similarly, TTHB144 was shown to harbor HEPN mediated non-specific, HEPN mediated ribonuclease activity after activation by cA_4[96]. Viral transcript levels during some infections drastically outnumber those of the host[97–99], so in these cases the indiscriminate RNA degradation will primarily affect the virus. However, it is generally believed that the desired outcome of viral suppression is through induction of cellular dormancy due to depleted host transcripts levels (see **Dormancy as an immune strategy**). Can1, containing two CARF-domains, a nuclease domain and a nuclease-like domain, was shown to nick supercoiled DNA upon cA_4 activation. This is thought to destabilize replication forks as they are stalled at the nicked sites, which could in turn interfere with viral replication. Card1/Can2 was found to possess both ssRNase and ssDNase activities upon activation by cA_4, presumably achieving its protective function through two separate, but perhaps synergistic, processes: cellular dormancy due to transcript depletion and direct invading genome destruction through cleavage of ssDNA intermediates in DNA replication. It was later found that the Can1 and Can2 nucleases not only respond to cA_4, but also cA_3, resulting in different substrate specificities (i.e. ssRNA or dsDNA) depending on the bound cOA[100]. As a single type III effector complex is capable of synthesizing multiple cOA species[101,102], encoding a multi-pronged ancillary nuclease –or, alternatively, multiple ancillary proteins that bind different cOAs[93]– might allow further fine-tuning an appropriate immune response. Another nuclease that is activated by cOA, albeit in a CARF-independent manner, is NucC[59,103,104] (**Figure 2B**). NucC was initially studied as part of the cyclic oligonucleotide-based anti-phage signaling system (CBASS), but some homologs of NucC are associated with type III loci and were demonstrated to be activated by Cas10 generated cA_3. The activation of NucC involves the assembly of two NucC trimers into a homohexamer upon cA_3 binding, leading to complete destruction of the bacterial chromosome to cause cell death.

Although most of the type III associated immune proteins are uncharacterized, domain function inference and experimental data suggest that ensuring an anti-viral response through non-specific, debilitating action on both host and virus is a common theme. These are obviously damaging measures for the host when insufficiently controlled. Therefore, tight and specific regulation on ancillary protein activation has evolved: CRISPR-Cas type III appears to be an intricately regulated signaling system, displaying properties such as signal amplification, self-regulation and tuning of signaling molecule concentrations. Recognition of just a single RNA molecule can potentially generate about a thousand cOA molecules, which in turn allows enzymatic activation of many immune proteins[75,105]. The cOA concentration increases proportionally to the viral load, for example in case of multiple co-occurring infections or internally replicating viruses, as more viruses generally means more transcripts. This ensures scaling of the immune response to the severity of infection[75,105]. To limit the detrimental effects to the host after invader clearance, the cOA concentration can be tuned down by shutting off new production as well as destroying excess cOA. New cOA production is stopped through cleavage of the target RNA, as this returns Cas10 to an inactive state[75]. It has been hypothesized that Cas10 inactivation, rather than the protective effect of direct degradation of viral transcripts, is the primary role of target RNA cleavage by the effector complex[68]. Removal of excess cOA is achieved by dedicated CARF containing ring nucleases, which cleave cOA rings into inactive linear di-adenylate species[105–107] (**Figure 2C**). Furthermore, some of the CARF effectors themselves have been shown to degrade cOA via a CARF domain[89,96], HEPN domain[101,108,109] or fusion to a ring nuclease[110], thereby acting as an intrinsic timer to regulate their own activity[111]. Also a family of membrane-associated nucleases was shown to possess cOA degradation capacity[112], as well as the virus encoded ring nuclease AcrIII-1[105,113]. The potent AcrIII-1 binds cA_4 at a higher affinity than Csx1 and degrades it at a faster rate than host ring nucleases. This effectively reduces the number of activated Csx1 molecules, leading to suppression of the immune response to safeguard cellular integrity until viral release.

In some prokaryotes, proteins in association with type III systems possess a domain called SAVED (SMODS-associated and fused to various effectors domains) instead of CARF[114]. Structural insights suggest that the SAVED domain evolved through fusion of two CARF protein subunits, broadening the range of cyclic nucleotide molecules that can be detected to activate a fused effector domain[115]. An intriguing example of this is CRISPR-Lon[116], a protease encoded in close proximity to a CRISPR-Cas type III-B system. CRISPR-Lon has an integrated SAVED domain and forms a strong complex with a MazF-like protein, called CRISPR-T (**Figure 2B**). The protease is activated upon binding cA_4 and cleaves off CRISPR-T, presumably to degrade RNA in a MazF-like manner. The fact that also putative families of genes without nucleotide-sensing domains were identified associated with type III modules, including nucleases, proteases,

peptidases and ATPases, suggest that type III signaling goes beyond the usage of cOA second messengers[86,87]. As an alternative, physical association of the accessory protein with the type III effector complex might function as a means of regulation. Accordingly, it was found that TPR-CHAT, a caspase-like peptidase commonly encoded in type III-E loci, associates with the type III-E effector complex to form the multi-subunit Craspase (CRISPR-guided Caspase) complex[63] (**Figure 2B**). Craspase is able to recognize and cleave target RNA, potentially to serve as a physical on and off switch for TPR-CHAT activity. Elucidating the mechanism of action of CRISPR-Cas type III clusters lacking nucleotide-sensing domains will likely expand our knowledge of type III intermolecular communication routes beyond cOA dependency.

The wide pool of functions inherent to the type III systems –RNA-targeting, second messenger signaling and the availability of large repertoire of ancillary proteins– raises the intriguing possibility that their functionality extends beyond immune defense[86]. An example of type III cOA signaling transcending direct anti-viral defense is found in the CARF-containing transcription factor Csa3 from a co-occurring type I-A system, which appears to be involved in transcriptional regulation[117–119]. Certain CRISPR-Cas type III proteins were also shown to be secreted by the bacterial pathogen *Mycobacterium tuberculosis* to function as virulence factors[120]. Deciphering the full range of CRISPR-Cas type III biology, with its many uncharacterized proteins and functionalities, will provide molecular biologists with experimental challenges for years to come as well as potential for new biotechnological applications (**Box 2**).

CRISPR-Cas type VI

In CRISPR-Cas type VI, the functionalities for crRNA processing, invader recognition and immune response are contained in a single effector protein: Cas13[16,121–123]. Up to now, six different type VI subtypes have been identified: types VI-A to VI-D, Cas13X and Cas13Y[53,124]. The Cas13 proteins across subtypes are distantly related, only sharing the presence of two HEPN domains[53]. Cas13 adopts a bilobed structure, with one lobe being responsible for RNA target recognition and the other for the RNA nuclease activity. Initially, Cas13 was demonstrated to be capable of targeting RNA viruses in an experimental setting[123]; newer insights showed that type VI spacer sequences match the genome of DNA viruses, indicating binding of viral transcripts[125,126]. Cas13 has the remarkable capacity of reaching femtomolar sensitivity in finding a target RNA in a population of non-target RNAs[127]. Upon target RNA loading, a conformational shift in the nuclease lobe accommodates the two HEPN domains to form a stable composite RNase pocket that mediates target RNA cleavage as well as hydrolysis of by-stander RNA[121,128,129], leading to inhibition of the invading DNA virus[27,129–133]. Unloaded Cas13 is inactive, indicating the presence of an auto-inhibited conformation that is released upon target recognition.

Initial binding of a target RNA to Cas13 occurs at the 'central seed region', a solvent-exposed part in the center segment of the crRNA[122,123,128,134] (**Figure 3A**). This region is most sensitive to mismatches, as it initiates RNA duplex formation. The nucleotides in the crRNA at the 5´ side of the seed region are known as the 'HEPN-nuclease switch region' (**Figure 3A**), because imperfect base-pairing in this portion prevents HEPN-nuclease activation[128]. Extensive base-pairing between the repeat derived crRNA portion and PFS also blocks the formation of the HEPN catalytic pocket, likely to prevent autoimmunity[39,135]. Bound target RNA is cleaved by Cas13 only when there is sufficient base-pairing in the switch region and non-complementarity between 5´ tag and PFS (**Figure 3A**). Although genomes of Cas13 escaper viruses were found to contain deletions of tens to hundreds of bases[27,133], one would expect that strict matching requirements in the seed and switch regions also allows viruses to escape by point mutations.

In contrast to other CRISPR-Cas effector proteins, in which the catalytic sites are buried deep inside the protein, the HEPN catalytic site is located at the solvent exposed external surface of Cas13[136]. This leads to RNA cleavage outside of the target RNA binding region (**Figure 3A**), with different homologs of Cas13 displaying varying ribonucleotide cleavage preferences[137,138]. The fact that the crRNA-bound portion of the target RNA is not cleaved seems to suggest that the RNA:RNA duplex stays intact, perhaps preventing target release. This could in turn mean that Cas13 is not able to sequentially bind new targets and that Cas13 collateral cleavage remains activated upon target RNA cleavage. Pioneering work on Cas13 demonstrated that when Cas13 is guided towards early-expressed transcripts, viral DNA does not accumulate, probably because host and viral transcript depletion early in the lytic cycle prevents genome replication[129]. Extensive host transcript depletion interferes with vital cellular pathways, whereupon cells enter dormancy. This state of hibernation is maintained as long as active virus continue to produce target RNA. Inhibition of target transcription –signifying a defeated virus, for example through viral DNA elimination by co-existing restriction-modification systems[139]– was found to reverse dormancy even after 9 hours, implying that cells stay alive during the process[129]. Direct cleavage of the target RNA seems to be less important for the antiviral response, as pre-

activation of Cas13 with non-viral RNA is sufficient to clear a virus infection. This indicates that Cas13, once activated, is able to provide immunity against co-infecting escaper viruses whose transcripts cannot be directly recognized. Indeed, cross-protection was shown to be a feature of Cas13 targeting, establishing broad and nonspecific immunity[129].

Although the biotechnological potential of Cas13 in RNA-based applications has gained substantial attention (**Box 2**), the study of its biology has received relatively little attention. Recent studies, however, increased the resolution of our Cas13 understanding. The paradigm of strict non-specific RNA-targeting by Cas13 was challenged when Cas13a was found to have a bias towards cleavage of specific RNA molecules, most substantially tRNAs[140] (**Figure 3B**). Massive tRNA cleavage results in compromised translation and dormant behavior of the cell, limiting the success of the virus in the cell population. Interestingly, it was found that next to Cas13 mediated tRNA cleavage, certain mRNAs and the 16S rRNA were also cleaved upon Cas13 activation, although not by Cas13 itself. Instead, presumably RNases present in the cell get activated by ribosome stalling as a consequence of tRNA inactivation, leading to the observed additional RNA cleavage (**Figure 3B**). It is interesting to speculate on the possibility that Cas13 generated tRNA fragments can serve as signaling molecules –akin to cOA second messengers in type III– for activation of downstream pathways[140], as broadly observed in eukaryotic systems[141]. Cas13-induced downstream protein activation has recently been shown for the type VI accessory protein Csx28[126], which forms a membrane pore to enhance anti-viral defense through membrane depolarization[132] (**Figure 3B**). This indicates that Cas13 has the capacity to also act as a signal relay, besides directly interfering with the viral lifecycle through RNA cleavage. Although the details have to be established, this feature would bring type VI closer to type III in terms of sensor capabilities.

Given the profound cellular consequences of Cas13 activity, the nuclease has to be tightly controlled. Besides low tolerance of mismatches in the central seed region, another layer of Cas13 control is provided by accessory proteins with regulation capacity in some type VI CRISPR loci. The accessory Csx27 is found in type VI-B and functions as an inhibitor, possibly by steric interference of its transmembrane domains with Cas13b, decreasing interference up to 5 orders of magnitude[126,136]. Another possibility is that Csx27 localizes in the membrane where it keeps Cas13 bound in an inhibited state, to perhaps release active Cas13 for local suppression of transcription during DNA uptake or viral infection[142,143] (**Figure 3C**). An additional regulatory protein was identified in type VI-D loci, where WYL1 was shown to interact with Cas13d to stimulate its collateral cleavage capabilities, perhaps through allosteric modulation[125,144,145]. Alternatively, because WYL1 possesses affinity for single-stranded RNA, it is hypothesized that WYL1 acts as an RNA sponge that upregulates Cas13d cleavage by confining RNA close to its active pocket (**Figure 3C**). For both Csx27 and WYL1, the exact mechanistic functioning as well as the biological implications are still unclear. As it is fair to assume that fine-tuned regulation of the toxic Cas13 is important for the cell's viability, future studies may uncover more sophisticated regulatory processes to ensure both cell safety and immune specificity.

	Type III	Type VI
Class	1	2
Abundance	25% and 34% of total CRISPR-Cas loci in bacteria and archaea, respectively[58]	Rare in bacteria, absent in archaea[146]
Recognition nucleic acids	RNA	RNA
Effector composition	Multi-subunit (type III-A to III-D and III-F) and single-subunit (type III-E)	Single subunit (Cas13)
Pre-crRNA processing	External (Cas6), internal (gRAMP/Cas7-11)	Internal (Cas13)
Target RNA cleavage	Csm3/Cmr4	Internal HEPN
Location of seed region in crRNA	At the 3´ end of the spacer	In the center of the spacer
Location of target RNA cleavage	In the crRNA binding region	Outside the crRNA complementary region
Cleavage specificity	6 nt periodicity	Preference for certain (di)nucleotide motifs (Lsh-Cas13a and Lbu-Cas13a show preferred cleavage at U[137]; Lwa-Cas13a, Cca-Cas13b, Lba-Cas13a and Psm-Cas13b cleave efficiently at AU, UC, AC and GA, respectively[138])
Self/non-self discrimination	crRNA tag/anti-tag pairing	crRNA tag/anti-tag pairing
crRNA tag/anti-tag paired	RNA target cleavage, no Cas10 activation	No RNA target cleavage, no Cas13 collateral activity
crRNA tag/anti-tag unpaired	RNA target cleavage, Cas10 activation	RNA target cleavage, Cas13 collateral activity
Second messenger	cOA (Palm domain in Cas10)	tRNA fragments*
DNase activity	Yes (HD domain in Cas10)	No
Secondary effector proteins**	NucC, Card1/Can2, Can1, Csm6/Csx1, TTHB144, TPR-CHAT, CRISPR-Lon, Csa3	Csx28
Secondary effector target	Indiscriminate RNA or DNA degradation, protease activity*	Indiscriminate RNA degradation and membrane depolarization
Secondary effector regulation	cOA regulation (ring nuclease, target cleavage)	Cas13 regulation (Csx27, WYL1)
Induction of dormancy	Yes	Yes

Table 1. Comparison of various key aspects of CRISPR-Cas type III and VI. *: posed as a hypothesis in literature. **: studied experimentally.

Dormancy as an immune strategy

Prokaryotes have the capability to go into a physiological state of low metabolism known as dormancy, facilitating survival in unfavorable conditions. Dormancy allows utilization of energy and resources for processes that attempt to sustain, repair and prevent, rather than those that aim at growth and propagation[147]. When conditions become more favorable, the cell can re-emerge and continue regular metabolism. In scenarios of sustained stress, the cell accumulates so much damage that it cannot go back. Cell death may follow and can thus be defined as the final stage of cell dormancy. Circumstances that may induce dormancy phenotypes include scarcity of nutrients, extremes of temperature, damage to vital components, oxidative stress, the presence of toxic compounds or parasitic invaders[148].

Many immune systems, including CRISPR-Cas type III and VI, use dormancy as a strategy to halt viral invasion[30]. The broad activities (e.g. DNase, RNase, membrane depolarization, proteolysis) responsible for dormancy induction in CRISPR-Cas type III and VI are known or predicted, but the exact cellular pathways involved in generating the dormancy phenotype are often obscure (**Table 2**). Especially the downstream effects of global RNA degradation are to be investigated in detail. Cas13 collateral RNase activity has recently been specified against primarily tRNAs, presumably resulting in dormancy through ribosome stalling[140]; for collateral RNase activity in CRISPR-Cas type III, it is not well known whether there is a bias towards degradation of certain transcripts (e.g. mRNA, rRNA, tRNA, RNA toxin components of toxin-antitoxin systems). Although the terms 'dormancy' and 'cell death' are often used interchangeably, reports on cell death caused by the action of RNA-targeting CRISPR-Cas systems are limited. Cas13 was shown to cause cell death when targeted towards an ampicillin resistance gene in cells under ampicillin conditions[149], but there is no data on Cas13 causing cell death during an actual infection. For type III secondary effectors, only NucC has shown a clear cell death phenotype[103,104].

An intuitive biological rationale for why halted cellular activity facilitates immunity is the generation of an inhospitable environment for the infecting virus, with limited access to essential host processes such as replication, transcription and translation. This results in at least two discernible and likely synergistic scenarios to prevent viral success (**Figure 4**): (i) interference with the viral lifecycle buys time for the already present DNA-targeting enzymes to destroy the foreign genomes[150,151], and (ii) viruses are trapped in the cytosol of the dormant cell, preventing it from completing its lifecycle and spreading to neighboring cells, implying a kin-selection strategy[150,151]. The exact processes required for exiting dormancy after viral clearance are not elucidated and remain an interesting topic of research, but restoration of the damage and replenishment of depleted cellular components are expected to occur. Furthermore, it has been hypothesized that for Cas13, certain RNAs required for restarting cellular processes are protected from degradation through dedicated proteins or tertiary conformations in the RNA[152].

Buying time

The events that happen after viral infection, including viral genome replication and protein synthesis, occur rapidly[153,154]. Moreover, cells can be infected with multiple viruses at the same time[155] and the DNA target sequence can be in a genomic region that is late-expressed in the infection cycle[29]. This could quickly lead to accumulation of viral DNA and a cellular state in which the viral genome copy number outruns the DNA-acting systems[156]. Induction of dormancy slows down the viral replication processes, effectively buying time for DNA-targeting systems that were initially too slow to halt the viral genomes (**Figure 4**). Additionally, dormant cells can potentially use the extra time to acquire new spacers from the invading genomes, which can then be immediately used against it during a CRISPR-Cas defense. Although this effect was not studied directly in infected cells, growth inhibited bacteria were shown to have increased spacer acquisition[157]. Kinetic studies on the relations between viral replication and the activity of co-occurring DNA- and RNA-acting systems could shed experimental light on the buying time concept, which remains to this day mostly hypothetical.

Kin protection

At first glance, unicellular programmed cell death seems paradoxical. Whereas prokaryotic immune systems often confer benefits to the cell carrying them, systems evoking death are clearly not advantageous to the enacting individual. Instead, impeding viral development through abortion of cellular processes results in fewer progeny released and thus effectively lowers the viral epidemic (**Figure 4**). This decreases the chance for the infection to spread to neighboring cells, which are often closely related kin, and thus likely also susceptible to the infecting virus[158]. Additionally, the sacrificed cell may leak valuable cellular resources into the population[159], perhaps even cOA

signaling molecules to prime defense in neighboring cells. So whereas the individual cell does not benefit from suicide, protection of kin makes the maintenance of suicide genes and pathways evolutionary advantageous[160]. Moreover, co-infecting viruses that are resistant to other forms of defense are also taken down in the process, further highlighting why acting on the host and virus simultaneously instead of the invader only can be beneficial[129,152].

Blindfolded self/non-self discrimination

The use of nucleic acids for biological information storage is a universal feature of life. Therefore, to prevent accidental targeting of the prokaryotic genome by DNA-targeting systems, distinguishing features have evolved to discern self and non-self DNA. Two principles for self/non-self discrimination are well-described: (i) mask self and damage non-self, and (ii) recognize non-self and damage non-self. The first principle is employed in type II restriction-modification systems, where the own genome is masked by methylation sites[161]. This prevents accessibility of the restriction enzymes, such that only the unmethylated viral genomes are detected and restricted. The second principle is found in DNA-targeting CRISPR-Cas systems, which only act on genetic elements which carry a PAM next to the target[162]. The combination of a PAM and target sequence in the own genome is scarce, preventing the recognition of self.

The success of RNA-targeting CRISPR-Cas immunity depends on inflicting damage to both host and virus, without strictly discriminating self from non-self. Due to intrinsic differences between the lifestyles of host and virus, such as the capacity to sustain when important resources are not readily available, the host is often able to survive the global damage whereas the virus is not. So, during RNA-targeting CRISPR-Cas immunity, another form of self/non-self discrimination seems to apply: damage both self and non-self, outlive non-self. As opposed to pointing directly at non-self prior to the immune response, this principle acts without actively seeking out who is who and effectively differentiates only in hindsight which was self (i.e. the biological unit that survived) and which was non-self (i.e. the biological unit that perished). We therefore term it 'blindfolded self/non-self discrimination' (**Figure 5A**). For example, the activity of various RNA-targeting CRISPR-Cas proteins (e.g. Cas13, Csm6/Csx1, Card1/Can2) is guided towards RNA non-specifically. The virus is programmed for fast replication, so depleted RNA levels (e.g. tRNA, rRNA, mRNA) and resulting protein scarcity (e.g. transcription and translation machinery, structural proteins) can lead to logistical problems in the viral lifecycle. In contrast, the host enters a dormant state, during which slowed metabolic processes do not require fine-tuned RNA and protein concentrations. When the viral processes have been disorganized to the point of virus neutralisation, the host can replenish RNA and protein levels to resume normal life. Another example is presented by the effector protein Can1, which nicks supercoiled DNA that could result in the collapse of replication forks in both the virus and the host[93]. This is detrimental to the virus, where many replication forks are present due to its fast replication cycle. The host, on the other hand, prevents catastrophic damage by entering a dormant state, ensuring few replication forks. Upon clearance of the virus, the host can repair broken DNA and continue normal metabolism. So one can say that autoimmunity –a phenomenon that is generally thought to be avoided– is used in the cell's benefit during blindfolded self/non-self discrimination.

It is interesting to speculate that the integration of the viral genome into the host genome, called lysogeny, presents a counter to blindfolded self/non-self discrimination. Instead of rapid replication, the virus goes "dormant" upon host integration (**Figure 5B**). By doing so, it effectively mimics the cell in terms of its slow lifestyle, circumventing "detection" by blindfolded self/non-self discrimination. If viral lysogeny indeed functions as such counter strategy, one might expect lysogenic viruses to be overrepresented in prokaryotes that employ dormancy as an immune response.

Effector protein	CRISPR-Cas type	Target	Cellular pathway	Phenotype
NucC	III	dsDNA	Host chromosome degradation	Cell death
Card1/Can2	III	ssRNA, ssDNA, dsDNA	Unknown	Dormancy
Can1	III	dsDNA, ssRNA	Replication fork destabilization through DNA nicking*	Dormancy
Csm6/Csx1	III	RNA	Unknown	Dormancy
TPR-CHAT	III	Protein*	Unknown	Unknown
CRISPR-Lon	III	RNA*	Unknown	Unknown
Cas13	VI	RNA	Ribosome stalling*	Dormancy
Csx28	VI	Cell membrane	Perturbed membrane integrity	Dormancy or cell death*

Table 2. Experimentally described CRISPR-Cas type III and type VI proteins able or expected to cause dormancy phenotypes. *: posed as a hypothesis in literature.

Conclusion

CRISPR-Cas immune strategies have far exceeded the “simple” paradigm of cleaving invader nucleic acids. CRISPR-Cas type III and type VI systems provide immunity through sensing invader transcripts, whereupon a plethora of broad and often rigorous responses are initiated. Mechanisms include collateral RNA degradation, own chromosome destruction, replication fork collapse, tRNA inactivation and membrane depolarization. But although the general workings of CRISPR-Cas type III and VI are understood, many of the details remain obscure and various open questions are to be answered (see Open questions). Since prokaryotes have a long history with virus outbreaks, numerous surprises in RNA-targeting CRISPR-Cas immunity undoubtedly await discovery.

Open questions

- Under what circumstances does CRISPR-Cas type III and VI immunity lead to cell death?
- Which cellular pathways generate the dormancy phenotype during CRISPR-Cas type III and VI immunity?
- To which extent is cellular dormancy induced by viral infection reversible, and which processes are responsible?
- Do cOA signaling molecules leak into the environment upon suicide induced by the CRISPR-Cas type III immune response, and are they capable of priming defense in neighboring cells?
- Are there other systems in the cell that are activated by the cOA produced during the CRISPR-Cas type III immune response?
- How is the synergy between the DNA- and RNA-targeting immune systems kinetically orchestrated?
- Can viral lysogeny be considered a counter to ‘blindfolded self/non-self discrimination’?
- How prevalent is Cas13-based downstream activation of ancillary proteins?
- How, and to which extent, is Cas13 activity regulated by ancillary proteins?
- How does signal relay occur in CRISPR-Cas type VI?
- What is the effect of viral RNA modifications (e.g. modified nucleosides, secondary structures) on RNA-targeting CRISPR-Cas effectors?
- Have RNA-targeting CRISPR-Cas systems evolved to protect from RNA viruses?
- Besides adaptive RNA-targeting immune systems, do prokaryotes also employ innate immune systems that act on RNA?

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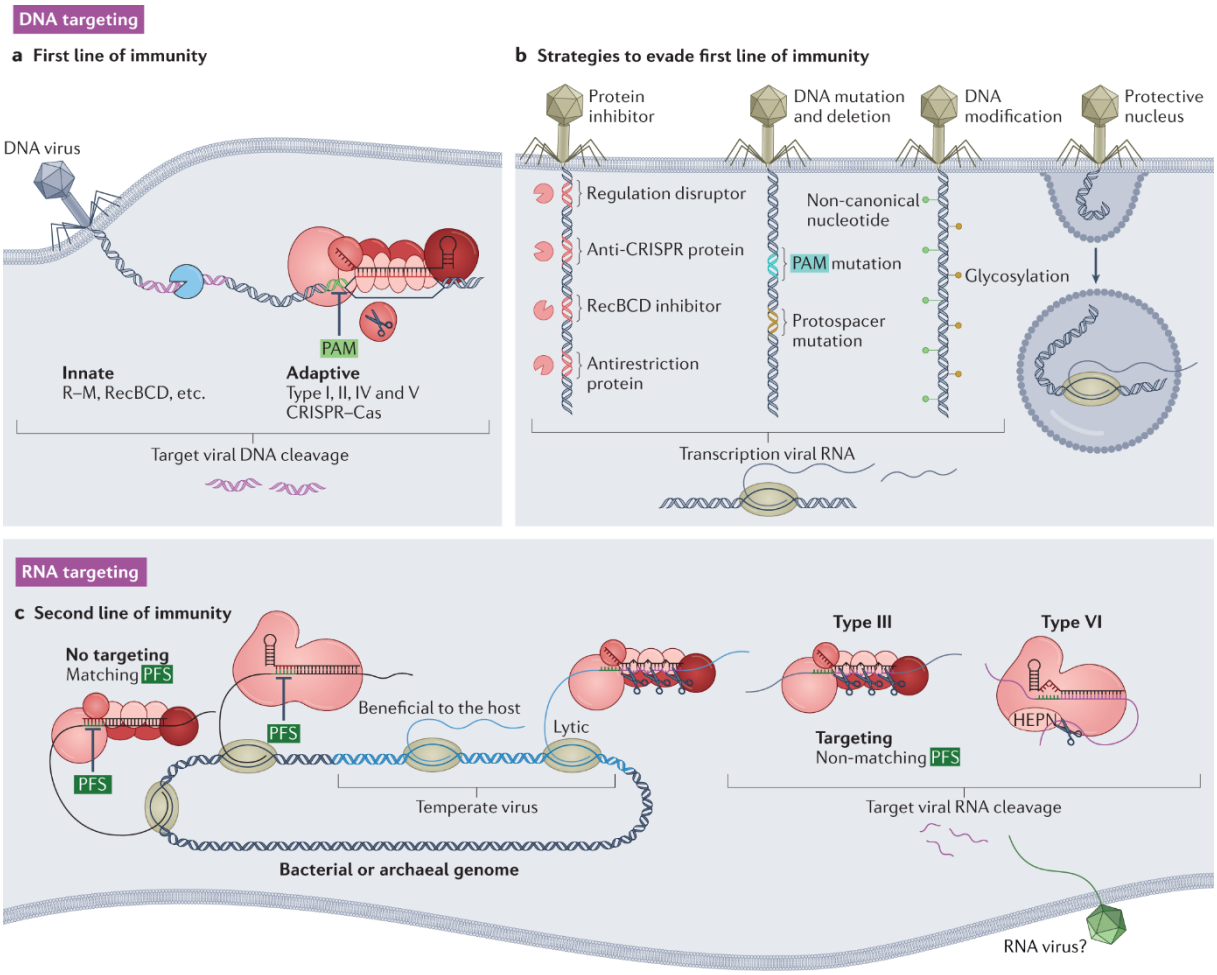

Figure 1. Prokaryotic lines of defense based on targeting invading DNA or RNA. (A) Upon entering of the viral genome, DNA-targeting systems (e.g. R-M, RecBCD, CRISPR-Cas type I, II, IV and V) are immediately able to attack the target DNA (violet) once identified as non-self (e.g. through protospacer adjacent motif (PAM) recognition) and hence form a first line of immunity. (B) Viruses have evolved various ways to evade the first line of immunity, including regulation disruptors, anti-CRISPR proteins, RecBCD inhibitors, anti-restriction proteins and usage of protective nuclei. Additionally, PAM and protospacer mutations as well as DNA modifications (e.g. non-canonical nucleotide incorporation and glycosylation) can evade efficient DNA-targeting, resulting in progress of the viral lifecycle to transcription. (C) The presence of viral transcripts (or, perhaps, viruses with an RNA genome) is sensed by systems of the second line of immunity, including CRISPR-Cas effectors of type III and VI. These effectors have lenient target base pairing requirements to allow for targeting and cleavage of transcripts from mutated viruses. To prevent self-targeting, the RNA-targeting CRISPR-Cas effectors test pairing in the protospacer flanking site (PFS) to distinguish self from non-self. Targeting RNA instead of DNA allows for toleration of integrated temperate viruses by only restricting lytic transcripts.

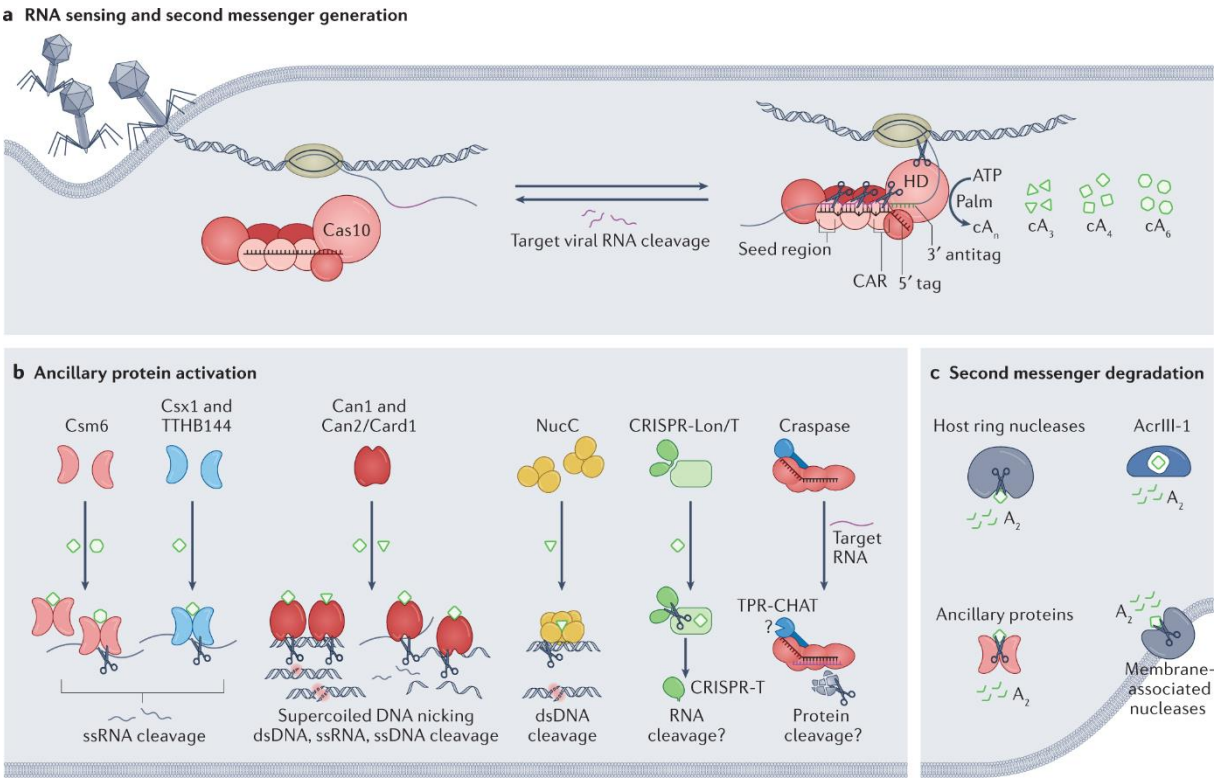

Figure 2. Schematic of CRISPR-Cas type III immunity. (A) Transcription of a viral target RNA (violet) is sensed by the multi-subunit CRISPR-Cas type III effector complex. Target recognition is initiated at the seed region in the 3′ end of the crRNA, and sufficient binding in the Cas10 activating region (CAR) with mismatching 5′ tag (red) at the 3′ anti-tag (green) results in Cas10 activation. CRISPR-Cas type III displays features of a sensor system, where recognition and cleavage of a target transcript leads to activation and deactivation of the Cas10 domains, respectively. The HD domain is capable of cleaving single-stranded DNA, perhaps close to the transcription bubble, whereas the Palm domain employs cyclase/polymerase functionality to generate cyclic oligoadenylates molecules (cOA) from adenosine triphosphate (ATP). (B) Various ancillary proteins in CRISPR-Cas type III clusters are activated by different cOA species (cA_n, n = 3-6), or perhaps through direct interaction with a target bound CRISPR-Cas type III effector. The enzymatic activities of ancillary proteins are guided towards different forms of nucleic acid moieties and perhaps protein. (C) Degradation of cOA into linear di-adenylate species (A_2) through ring nuclease activity of host or viral proteins can defuse cOA molecules and as such dampen or shut-off the ancillary protein immune response in case of infection alleviation.

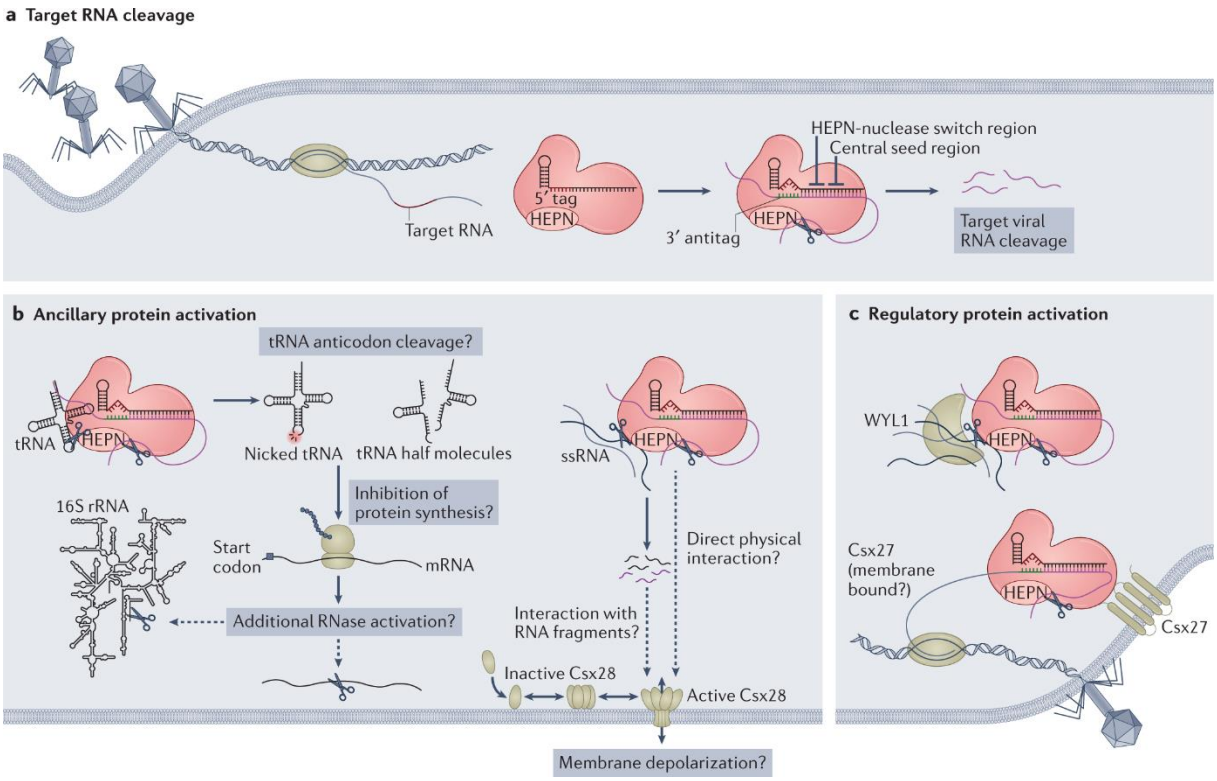

Figure 3. Schematic of CRISPR-Cas type VI immunity. (A) Target RNA recognition by Cas13, the effector protein in CRISPR-Cas type VI systems, occurs at the central seed region and leads to the activation of a RNase pocket formed by higher eukaryotes and prokaryotes nucleotide-binding (HEPN) domains when the HEPN-nuclease switch region matches the corresponding target sequence and the 5′ tag (red) mismatches the 3′ anti-tag (green). The activated RNase catalytic site is capable of degrading bound target RNA as well as by-stander RNA. (B) Some activated Cas13 variants are able to cleave transfer RNA (tRNA) molecules in the anti-codon loop, which can result in ribosome stalling and subsequent activation of additional RNases to establish a dormant phenotype, hampering viral propagation. Target bound Cas13 is also able to activate the ancillary protein Csx28, perhaps by target RNA degradation products or through physical interaction, which form pores in the membrane to instigate membrane depolarization. (C) Csx27 and WYL1 are believed to regulate Cas13 activity, although exact functionalities are not elucidated. Csx27 is thought to anchor Cas13 to the membrane for localized target RNA degradation. Alternatively, physical interaction of Csx27 with Cas13 might downregulate its cleavage activity. WYL1 is believed to upregulate Cas13 activity by confining RNA close to the RNase pocket or by allosterically boosting its cleavage efficiency.

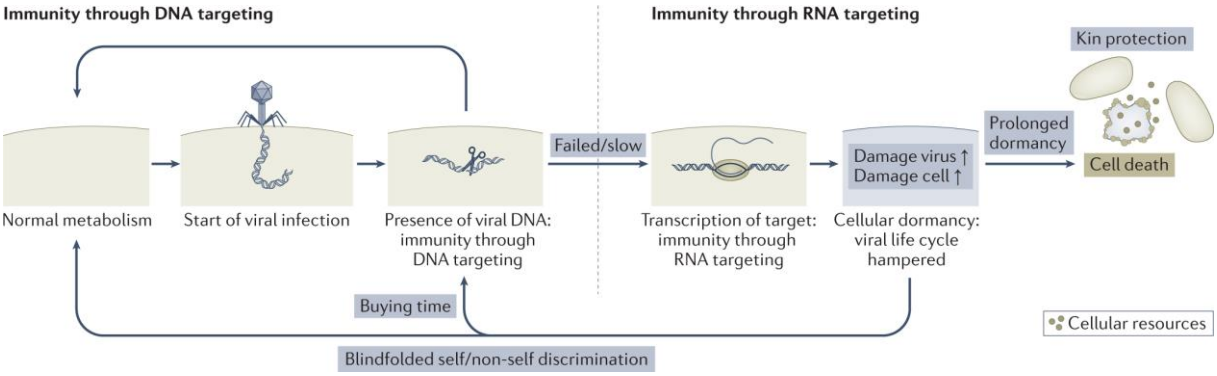

Figure 4. Model of the dormancy strategy employed by RNA-targeting CRISPR-Cas type III and VI systems. Infection by a virus is signified by the presence of viral DNA inside the cell. DNA-targeting systems are able to provide first line immunity by immediately attacking the invader genomes, usually through genomic DNA cleavage. When the anti-DNA response is unsuccessful (e.g. viruses circumventing DNA-targeting mechanisms or viral genome replication outrunning genome cleavage), the viral lifecycle progresses to transcription. Target transcripts are recognized by RNA-targeting CRISPR-Cas type III and VI effector proteins, typically leading to an immune response that involves global damage to both host and virus. The resulting cellular dormancy and hampered progression of the viral lifecycle effectively buys time for initially too slow DNA-targeting immune systems to inactivate remaining viral genomes. The cell can exit from dormancy through restoration of the inflected self-damage. Alternatively, or synergistically, as the fast viral processes are more severely affected compared to the slow processes of a dormant cell, self is discriminated from non-self through the capacity to recover from the induced damage (blindfolded self/non-self discrimination; see **Figure 5**). In case of sustained dormancy due to prolonged infection, the cell accumulates damage to reach a point of no return: cell death. The invader, requiring a living host for its propagation, is taken down in the process, making cellular suicide an altruistic act to protect kin. Additionally, cellular components leak in the environment to provide nutritional aid to neighboring cells.

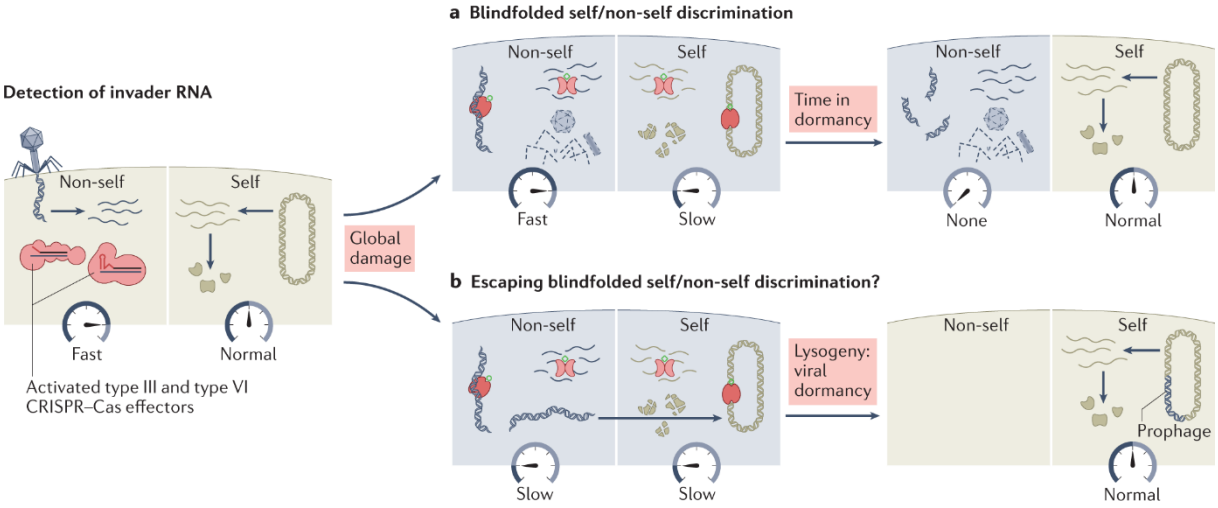

Figure 5. Blindfolded self/non-self discrimination. Upon viral infection, unsuccessful immediate clearance of the virus leads to progression of the viral lifecycle to transcription (rate of metabolism is indicated by the speed-o-meters in the bottom of the cell). Target transcripts are recognized by the CRISPR-Cas type III and VI effector proteins that typically initiate a global immune response. (A) Target recognition typically includes damage to both host and virus, as exemplified by the activity of RNase Csm6 (pink) and ssDNA nickase Can1 (red), affecting the processes of replication, transcription and translation. In order to sustain this stress, the host enters a state of dormancy; the virus, on the other hand, will pursue their rapid lifecycle and eventually collapse due to the lack of cellular means. In this way, self is discriminated from non-self as if wearing a blindfold, i.e. the induced indiscriminate damage can be overcome by self, but not by non-self. (B) A way of viral escape from blindfolded self/non-self discrimination could be through mimicking the host in terms of metabolism. Instead of following a fast lifecycle, temperate viruses have the capability of integrating into the host genome as a prophage during the process of lysogeny. This presents a form of viral dormancy, slowing the viral processes down such that they cannot be “detected” by blindfolded self/non-self discrimination.

Supplement

Box 1. RNA-targeting Cas9 and Cas12g

Cas9, the CRISPR-Cas type II effector protein, is well-known for its RNA-guided DNase activity in genome engineering[1], but some Cas9 nucleases are also capable of targeting RNA. Cas9 from *Streptococcus pyogenes* can be programmed to bind and cleave RNA targets in vitro when annealed to a DNA moiety that contains the PAM sequence[2]. Other studies identified RNA-targeting Cas9 variants in *Neisseria meningitidis*[3]*, Staphylococcus aureus*[4]*, and Campylobacter jejuni*[4] that possess RNase activity even without the addition of PAM oligo's. Although the physiological relevance of Cas9 RNase activity in a natural context is not completely understood, it is speculated to help in clearing invading transcripts during infection[3] and demonstrated to be capable of conferring protection against an RNA virus[4] as well as repressing gene expression in a heterologous host[4]. Accordingly, Cas9 from *Francisella novicida*[5] and *C. jejuni*[6] act as such natural gene regulators, as they were found to target endogenous transcripts to suppress protein expression. The RNA-targeting capacity of Cas9 was exploited to visualize[7] and remove[8] RNAs in human cells, as well as for inhibition of the RNA virus hepatitis C in eukaryotic cells[9].

Cas12g, a CRISPR-Cas type V RNA-guided endonuclease, recognizes RNA substrates[10,11]. Target RNA binding by Cas12g leads to target RNA cleavage as well as collateral RNase and single-stranded DNase activities. Various characteristics of Cas12g –no PAM restrictions, small size and high thermal stability– make it an interesting candidate for RNA-specific biotechnological applications[12].

Box 2. Biotechnological applications of CRISPR-Cas type III and VI

RNA-targeting CRISPR-Cas systems are now being exploited for various technologies[13], including RNA knock-down, RNA editing, RNA imaging, RNA splice modifications, RNA-protein interaction mapping, counter selection in genome engineering and molecular diagnostics in nucleic acid detection. The natural RNA interference functionality of Cas13 can be guided to a desired target by programming it with the complementary crRNA to knockdown transcript levels[14–17], which allowed for implementation of Cas13 as an antimicrobial agent[18] and a selection tool for virus engineering[19,20]. Cas13 is used for tagging proteins in the vicinity of specific cellular RNAs to study native protein-RNA interactions in methods called RPL[21] (RNA proximity labelling), CRUIS[22] (CRISPR RNA-unified interaction system) and CAPRID[23] (CRISPR-CasRx-based RNA-targeting and proximity labeling). Genetic fusions of a dead variant of Cas13 (dCas13) yielded various tools: dCas13-GFP is used for fluorescence-based localization of target RNA in a cell[24], dCas13 fused to mRNA splicing repressors can skew protein isoform ratios[16], dCas13 fused to a deaminase domain is used to edit a specific adenosine to an inosine (RNA Editing for Programmable A to I Replacement; REPAIR)[25,26] or a cytosine to uracil (RNA Editing for Specific C-to-U Exchange; RESCUE)[26,27], and dCas13 fused to a demethylase was shown to successfully demethylate targeted mRNA in cells[28]. The collateral cleavage capability of Cas13 is exploited for nucleic acid detection in SHERLOCK[29,30] (Specific High-sensitivity Enzymatic Reporter unLOCKing), SHINE[31] (Streamlined Highlighting of Infections to Navigate Epidemics), SATORI[32] (CRISPR-based amplification-free digital RNA detection), CREST[33] (Cas13-based, rugged, equitable, scalable testing), CARMEN[34] (Combinatorial Arrayed Reactions for Multiplexed Evaluation of Nucleic acids) and droplet microfluidics[35], which are all based on quenched reporter RNAs that become fluorescent upon degradation by target bound Cas13. The same principle been exploited in APC-Cas (Allosteric Probe-initiated Catalysis and CRISPR-Cas13a) for the detection of bacterial pathogens[36]. Also a Cas13 platform using solution turbidity caused by liquid-liquid phase separation as a readout has been employed for RNA detection[37]. Various variations on the theme have been developed for the detection of microRNA[38–41]. The Cas13-based diagnostics tools vary in terms of sensitivity and specificity and the utility during the SARS-CoV-2 outbreak[42–44].

Also type III systems are engineered for nucleic acid detection: cOA-activated RNases for target-induced reporter cleavage are deployed in SCOPE[45] (Screening using CRISPR Oligoadenylate Perceptive Effectors) and MORIARTY[46] (Multipronged, One-pot, target RNA-Induced, Augmentable, Rapid, Test sYstem), and

dedicated SARS-CoV-2 detection methods that additionally make use of pyrophosphates and protons generated by Cas10 upon target detection[47]. Moreover, the Can2[48] and NucC[49] nucleases have been demonstrated to be capable of cOA-activated reporter cleavage for RNA diagnostics. To enhance sensitivity of RNA detection, a pull-down method for capturing and concentrating target RNA from heterogeneous samples was developed using a type III effector complex[48]. The FIND-IT (Fast Integrated Nuclease Detection In Tandem) approach combines Cas13 target recognition with Csm6 RNase activity to enable RNA detection[50]. Sequence-specific RNA-targeting by the type III-E gRAMP/Cas7-11 single-unit effector can be employed for RNA editing[51], as well as RNA knockdown in vitro[51,52], in bacteria and in mammalian cells[51].

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