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Abstract | CRISPR–Cas is a widespread adaptive immune system in bacteria and archaea that protects against viral infection by targeting specific invading nucleic acid sequences. Whereas some CRISPR–Cas systems sense and cleave viral DNA, type III and type VI CRISPR–Cas systems sense RNA that results from viral transcription and perhaps invasion by RNA viruses. The sequence-specific detection of viral RNA evokes a cell-wide response that typically involves global damage to halt the infection. How can one make sense of an immune strategy that encompasses broad, collateral effects rather than specific, targeted destruction? In this Review, we summarize the current understanding of RNA-targeting CRISPR–Cas systems. We detail the composition and properties of type III and type VI systems, outline the cellular defence processes that are instigated upon viral RNA sensing and describe the biological rationale behind the broad RNA-activated immune responses as an effective strategy to combat viral infection.

Mobile genetic elements
(MGEs). Simple genetic organisms (such as phages, plasmids, conjugative elements, transposons, introns and phage-inducible chromosomal islands) capable of horizontal transfer within or between genomes.

Since the appearance of primordial microorganisms, life has been in an intricate dance with death. Parasites are major contributors to this: 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, defence systems evolved that enabled the host to survive by preventing parasitic propagation. But complete parasite eradication did not happen either: contemporary bioinformatics demonstrates that virtually all life forms harbour diverse evolved parasites, such as plasmids, viruses and transposons, often termed 'mobile genetic elements' (MGEs)[2]. The wide range of MGEs is thought to have resulted from early parasites that evolved counterdefence to protect against host defence, upon which the host evolved counter-counterdefence, 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 bacterium encodes five anti-MGE systems[5,6]. Although these systems 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 bacteria and archaea discovered is CRISPR–Cas. 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]. A spacer corresponds to a segment of a previously encountered virus, known as a protospacer[10], and the CRISPR array thus provides a genetic memory bank of past invasions. The Cas proteins are often encoded in the neighbourhood 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 (crRNA) molecule, which is further processed to free individual short crRNAs[15,16]. The crRNAs subsequently assemble with Cas proteins to form ribonucleoproteins, called 'effector complexes', which monitor the cell in search of 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: direct dismantling of the invading DNA via crRNA-guided cleavage; and interference with the invader's replication cycle, often involving the activation of downstream immune proteins. The first strategy is used by effector complexes that belong to the DNA-targeting CRISPR–Cas systems (types I, II, IV and V). In this case, 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

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systems (types III and VI). RNA-activated type III and type VI effectors set in motion a broad, collateral response that often goes far beyond merely cleavage of the bound RNA target; typical immune outcomes include cellular dormancy.

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

Recognition of RNA rather than DNA

Upon DNA genome ejection into the cytoplasm, the infecting virus will encounter not only the transcription and translation machinery required for its propagation but also immune proteins that aim to stop it. DNA-targeting immune proteins (for example, 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 are often dubbed the 'first intracellular line of immunity'[6,18,19] (FIG. 1a). But viruses have evolved various ways of escaping this first line of defence, including deploying anti-immune proteins (for example, regulation disruptors, anti-CRISPR proteins, RecBCD inhibitors and antirestriction proteins[20–23]), changing recognition sequences (for example, mutating protospacer sequence and the protospacer adjacent motif (PAM)[24]), chemically modifying the DNA (for example, glycosylation and non-canonical nucleotide incorporation[25–27]) and physically protecting the DNA (for example, genome compartmentalization[28,29]) (FIG. 1b). Moreover, even in the case of successful targeting, viral genome replication may already have occurred to the point of outrunning the activity of DNA-targeting 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 these systems could thus be regarded as a secondary line of immunity[30,31] (and, potentially, a first line of defence against RNA viruses[32,33]) (FIG. 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[28,34–38]. Self-RNA cleavage as a result of this permissive nature is less detrimental compared with 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[39] — cannot occur with RNA-targeting effectors. Only the RNA

resulting from antisense transcription of the CRISPR array can be recognized as a target. This, however, will not lead to severe self-immunity, as progression of the immune response is warranted only when the crRNA of the effector is sufficiently mismatching a region on the target called the 'protospacer flanking site' (PFS)[40–42] (FIG. 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.

The co-occurrence of type I and type III CRISPR–Cas loci[19] and the suggested sharing of spacers between type I and type 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]. Crosstalk between RNA-targeting and DNA-targeting CRISPR–Cas systems was also observed on the level of CRISPR adaptation, whereby 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-targeting and DNA-targeting systems can also complement each other. An intriguing example of defence complementation was discovered in *Pseudomonas* and *Serratia* bacteria, in which infecting jumbo viruses build a nucleus-like proteinaceous compartment to shield their genome from various DNA-targeting defences[28,29,47] (FIG. 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[27,29,47]. In other words, RNA-targeting CRISPR–Cas is not only able to temporally back up DNA-based immunity evasion but also does so spatially. Further studies will undoubtedly uncover more fascinating insights into the biological and ecological interactions between co-occurring RNA-targeting 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 suppressing only toxic lytic gene transcripts[49,50] (FIG. 1c). This enables the host to use viral gene content, while preventing the virus from becoming a threat.

Thus, although the DNA-targeting CRISPR–Cas systems display characteristics of a first line of defence strategy (for example, immediate targeting, genome clearance and high specificity), RNA-targeting CRISPR–Cas systems can be viewed as a second-line strategy with various advantages. First, 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. RNA-targeting effectors are also generally lenient towards mutations in the target sequence, making the

DNA targeting

a First line of immunity
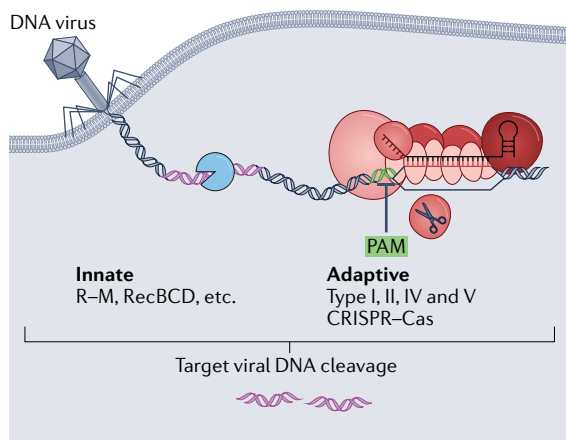
b Strategies to evade first line of immunity
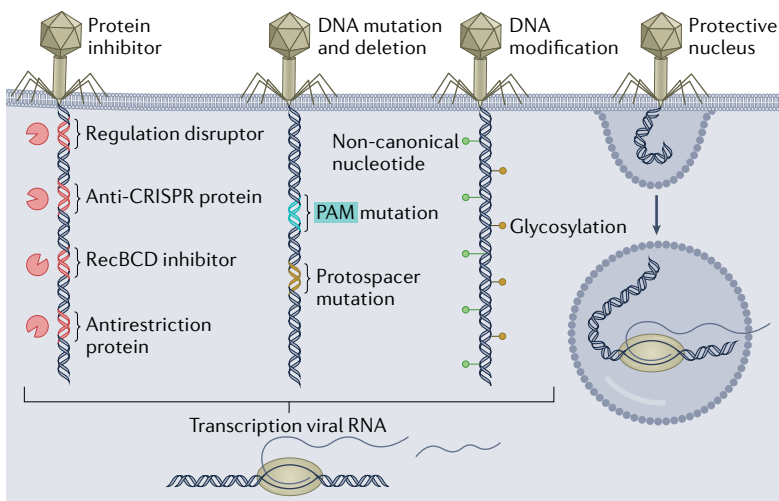
RNA targeting

c Second line of immunity
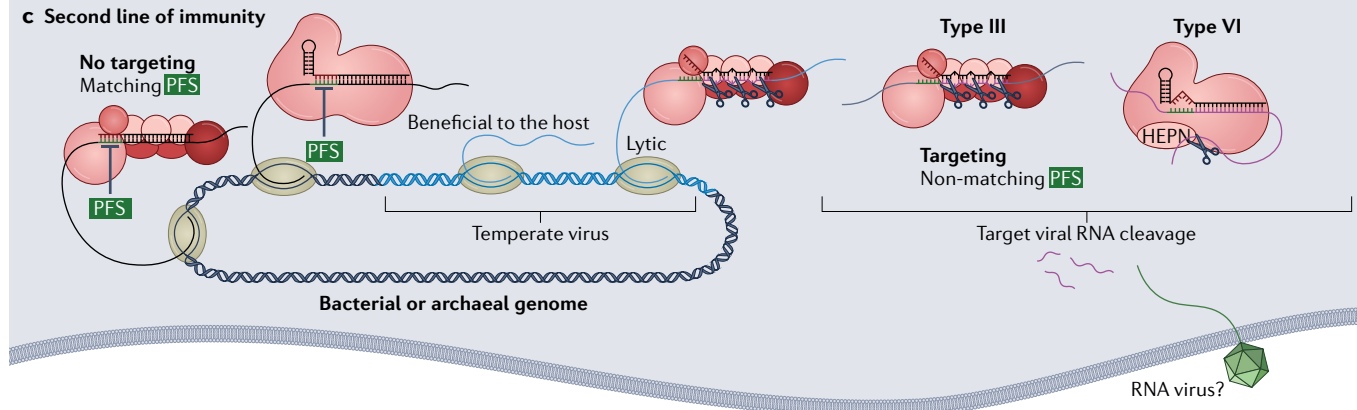
Fig. 1 | **DNA-targeting and RNA-targeting host defence systems and viral evasion strategies. a** | Upon entering of the viral genome, DNA-targeting systems (for example, restriction–modification (R–M) systems, RecBCD and type I, II, IV and V CRISPR–Cas) are immediately able to attack the target DNA (violet) once it has been identified as non-self (for example, 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, antirestriction proteins and use of protective nuclei. Additionally, PAM and protospacer mutations as well as DNA modifications (for example, non-canonical nucleotide incorporation and glycosylation) can evade efficient DNA targeting, resulting in progress of the viral life cycle 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 types III and VI. These effectors have lenient target base-pairing requirements to enable 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 enables toleration of integrated temperate viruses by restricting only lytic transcripts.

chance of escape mutants less likely. Moreover, due to the transient nature of RNAs, an instance of autoimmunity through self-RNA cleavage is less detrimental to the host compared with self-targeting of DNA, and a full immune response is often not mounted when a self-RNA is recognized. Furthermore, being reliant on transcription enables conditional tolerance of viral presence, which enables the host to potentially benefit from the additional gene content. Finally, different cellular locations of DNA and RNA enable RNA-targeting

CRISPR–Cas systems to back up evaded DNA-targeting systems, as observed in defence against jumbo viruses.

Dissection of type III and type VI CRISPR–Cas
Although a shared feature of type III and type VI CRISPR–Cas systems, and some type II and type V systems (Supplementary 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). In this section, we review the molecular

anatomy and mechanistic functioning of type III and type VI CRISPR–Cas systems.

Type III CRISPR–Cas. Type III CRISPR–Cas is believed to be the oldest member of the CRISPR–Cas family[51,52]. Until 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, Cmr3, Cmr4, Cmr5 and Cmr6 in types III-B and III-C, and Csm2, Csm3, Csm4 and Csm5 in types III-A and III-D), with signature subunit Cas10 (Cmr2 in types III-B and III-C, and Csm1 in types III-A and III-D) being the largest component[54–58]. Cas10 is a multidomain protein harbouring a nuclease and a cyclase (also known as a polymerase; from here on 'cyclase/polymerase') domain, providing the effector complex with DNase activity as well as the capacity to generate cyclic second messengers from adenosine triphosphate (ATP). Whereas effectors of type III-C have an apparent inactivated cyclase/polymerase domain, effectors of type III-D and various type III-B effectors lack the nuclease domain[59,60]. The other type III subunits are responsible for complex formation with the Cas6-processed crRNA[15,61], recruitment of host factors that promote crRNA maturation[62] and nucleic acid clearance[63], assisting in binding a complementary RNA, or cleavage of the bound target[55]. The type III-E and type III-F effectors have notably different architectures: the former comprise subunits that are fused together, while lacking the Cas10 subunit[64,65],

whereas the latter contain only one Cas7-like protein and a Cas10 subunit that lacks the active cyclase/polymerase configuration[53]. Although type III systems can protect against an RNA virus under experimental conditions[65,66] and spacer matches to RNA viruses have been discovered[32,33], early analyses of the CRISPR arrays indicated spacer matches with DNA viruses[30,67], and thus viral transcript targeting by type III systems has been primarily studied.

The type III effector complexes monitor the cell to detect RNA molecules complementary to the crRNA (FIG. 2a). Initial target RNA pairing occurs at the 3′ end of the crRNA, which induces a conformational change within the effector complex that enables base-pairing between the crRNA and target RNA[68]. To verify the origin of the bound transcript, complementarity is checked between the repeat-derived crRNA portion (also known as a 5′ tag) and the PFS (also known as a 3′ anti-tag) of the suspected RNA invader[38,41,42,67]. Base-pairing in this region indicates the binding of a self-RNA. When such a false alarm occurs, Cas10 is kept locked in an inactive state to inhibit the immune response and reduce the toxic effects[42,67–70]. However, target cleavage does still occur: the RNase domains embedded in the Csm3 (in types III-A and III-D) and Cmr4 (in types III-B and type III-C) subunits of a target RNA-bound effector complex are exposed to the substrate and cleave it with a six-nucleotide periodicity[64,66,71–75]. Cleaved RNA fragments dissociate from the crRNA[76], which is believed

**Table 1 | Key aspects of type III and type VI CRISPR–Cas**

Feature	Type III	Type VI
Class	1	2
Abundance	25% of total CRISPR–Cas loci in bacteria and 34% in archaea[59]	Rare in bacteria, absent in archaea[162]
Recognized nucleic acid	RNA	RNA
Effector composition	Multiple subunits (types III-A, III-B, III-C, III-D and III-F) and single subunit (type III-E)	Single subunit (Cas13)
Pre-crRNA processing	External (Cas6), internal (gRAMP)	Internal (Cas13)
Target RNA cleavage	Csm3 and Cmr4	Internal HEPN domain
Location of seed region in crRNA	At the 3′ end of the spacer	In the centre of the spacer
Location of target RNA cleavage	In the crRNA-binding region	Outside the crRNA complementary region
Cleavage specificity	6-nucleotide periodicity	Preference for certain nucleotide or dinucleotide motifs (Lsh-Cas13a and Lbu-Cas13a show preferred cleavage at U[138]; Lwa-Cas13a, Cca-Cas13b, Lba-Cas13a and Psm-Cas13b cleave efficiently at AU, UC, AC and GA, respectively[139])
Self/non-self discrimination	crRNA tag–antitag pairing	crRNA tag–antitag pairing
crRNA tag–antitag paired	RNA target cleavage, no Cas10 activation	No RNA target cleavage, no Cas13 collateral activity
crRNA tag–antitag unpaired	RNA target cleavage, Cas10 activation	RNA target cleavage, Cas13 collateral activity
Second messenger	cOA (palm domain in Cas10)	tRNA fragments[a]
DNase activity	Yes (HD domain in Cas10)	No
Secondary effector proteins[b]	NucC, Card1, Can2, Can1, Csm6, Csx1, TTHB144, TPR-CHAT, CRISPR-Lon, CRISPR-T and Csa3	Csx28
Secondary effector target	Indiscriminate RNA or DNA degradation, protease activity[a]	Indiscriminate RNA degradation and membrane depolarization
Secondary effector regulation	cOA regulation (ring nuclease, target cleavage)	Cas13 regulation (Csx27, WYL1)
Induction of dormancy	Yes	Yes

cOA, cyclic oligoadenylate; crRNA, CRISPR RNA; gRAMP, giant repeat-associated mysterious protein; HD domain, histidine–aspartate domain; HEPN domain, higher eukaryotes and prokaryotes nucleotide-binding domain. [a]Posed as a hypothesis in the literature. [b]Studied experimentally.
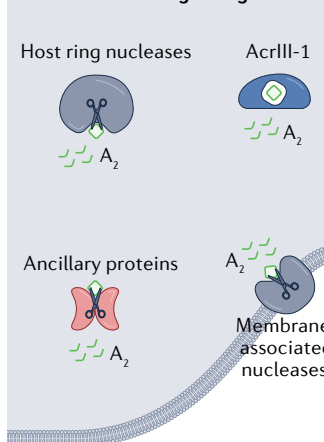
a RNA sensing and second messenger generation
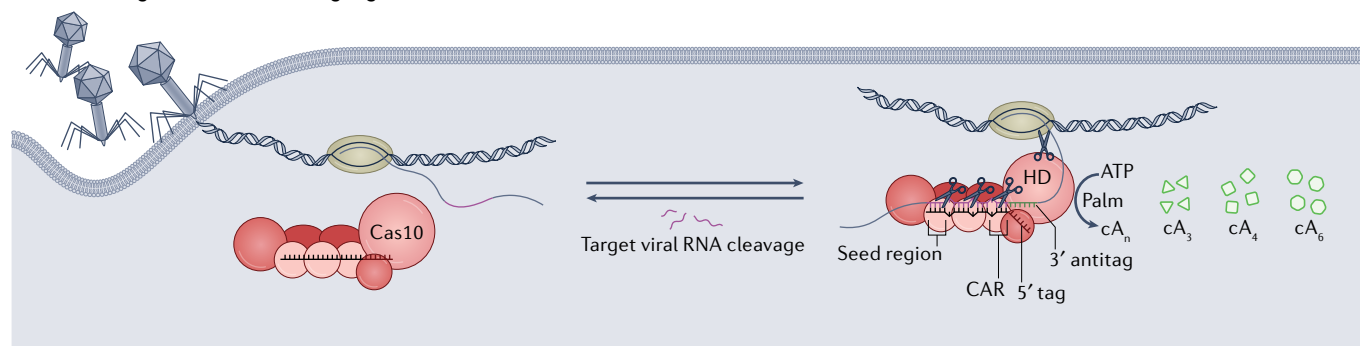
b Ancillary protein activation
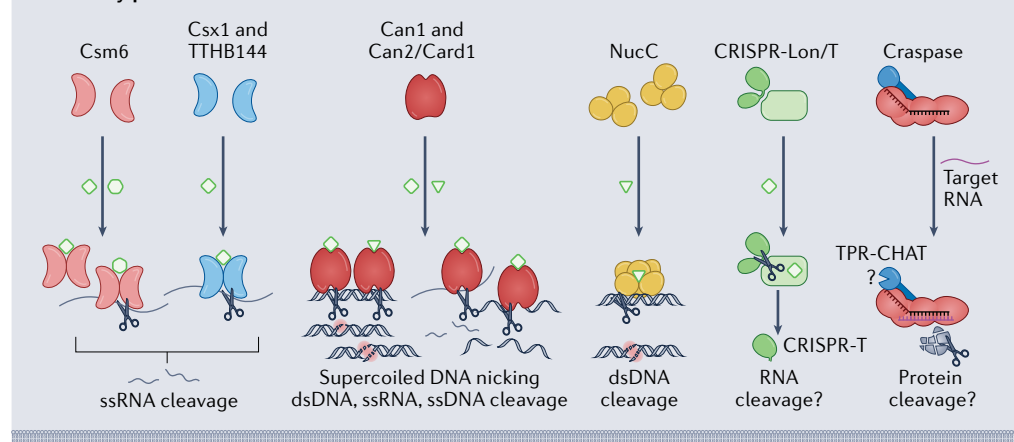
c Second messenger degradation

Fig. 2 | **Type III CRISPR–Cas immunity. a** | Transcription of a viral target RNA (violet) is sensed by the multiple-subunit type III CRISPR–Cas effector complex. Target recognition is initiated at the seed region in the 3′ end of the CRISPR RNA, and sufficient binding in the Cas10-activating region (CAR) with mismatching 5′ tag (red) at the 3′ antitag (green) results in Cas10 activation. Type III CRISPR–Cas displays features of a sensor system, whereby recognition and cleavage of a target transcript leads to activation and deactivation, respectively, of the Cas10 domains. The histidine–aspartate (HD) domain can cleave single-stranded DNA (ssDNA), perhaps close to the transcription bubble, whereas the palm domain uses its cyclase functionality to generate cyclic oligoadenylate (cOA) molecules (cA_n, $n = 3$-6) from adenosine triphosphate (ATP). **b** | Various ancillary proteins in type III CRISPR–Cas clusters are activated by different cOA species, or perhaps through direct interaction with a target bound type III CRISPR–Cas effector. The enzymatic activities of ancillary proteins are guided towards different forms of nucleic acid moieties and perhaps proteins: Csm6, Csx1 and TTHB144 act on single-stranded RNA (ssRNA), Can1 acts on double-stranded DNA (dsDNA) and perhaps ssRNA, Card1 acts on ssRNA and dsDNA, Can2 acts on ssRNA, ssDNA and perhaps dsDNA, NucC acts on dsDNA, CRISPR-T acts on perhaps RNA, and Craspase, in which TPR-CHAT might be activated upon target RNA binding, acts on perhaps proteins. **c** | Degradation of cOA into linear diadenylate (A_2) species through ring nuclease activity of host proteins (such as dedicated (membrane-associated) host ring nucleases and type III CRISPR–Cas ancillary proteins harbouring ring nuclease activity) or viral proteins (such as AcrIII-1) can defuse cOA molecules and thereby dampen or shut off the ancillary protein immune response in the case of infection alleviation.

to recycle the effector complex for binding of a new target (FIG. 2a).

When there is both sufficient base-pairing in the spacer portion of the crRNA (particularly in the first nucleotides at the 5′ side, also known as the Cas10-activating region[68]) and sufficient mismatches in the PFS region, the bound RNA is most likely of invader origin (FIG. 2a). Conformational changes are relayed in the complex[70], unleashing nuclease and cyclase/polymerase catalytic activities within Cas10. The Cas10 nuclease activity is exerted by a histidine–aspartate domain (HD domain) and can degrade single-stranded DNA (ssDNA)[42,67,69,77]. Whereas the exact role of Cas10 ssDNA activity has yet to be established, several hypotheses are plausible: ssDNA cleavage might promote immunity via degradation of ssDNA at or near the viral transcription bubble[77,78] (FIG. 2a), at the R-loops that arise during viral

transcription elongation[79], or in the single-stranded replication intermediates of viruses and plasmids[79]. It also has been proposed that Cas10 nuclease activity promotes host mutagenesis through the induction of host chromosomal lesions[80]. Whereas the DNA cleavage can be sufficient for viral protection in the case of abundant viral transcription[81], signal amplification is needed when transcription is limited. Here, the Cas10 cyclase/polymerase activity comes into play: two palm domains facilitate ATP binding[82,83], whereupon a GGDD motif in one of the palm domains catalyses 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 diadenylate (cA_2)) and six (cA_6) AMP units[82,84–86] (FIG. 2a). The dispersed cOA molecules in turn bind to CRISPR-associated Rossmann

Palm domain
A domain in the Cas10 subunit of various type III CRISPR–Cas effectors with structural similarity to nucleotidyl cyclases and nucleotide polymerases, catalysing the synthesis of cyclic oligoadenylate molecules from ATP.

fold (CARF) domains of proteins often found to be encoded in or near the type III CRISPR–Cas loci[82,85–88]. CARF is a nucleotide-binding domain that allosterically activates an attached effector domain upon binding the cOA nucleotide ligand, releasing its immune enzymatic activity[53,89].

Genomic neighbourhood analysis of type III CRISPR–Cas loci has revealed numerous CARF family proteins, among others nucleases, transcription factors, proteases, deaminases, nitrilases and membrane-associated proteins[87,88], suggesting a plethora of potential cOA-based defence pathways. Various nuclease-type CARF proteins have been experimentally characterized: Csm6, Csx1 and TTHB144 (REFS.[82,85,89–93]), Can1 (REF.[94]) and Card1 and Can2 (REFS.[95,96]) (FIG. 2b). Upon cA_4 or cA_6 binding to a Csm6 or Csx1 homodimer, conformational changes bring together the HEPN ('higher eukaryote and prokaryote nucleotide-binding') domains to constitute a promiscuous RNase pocket that degrades both viral and host transcripts. Similarly, TTHB144 also displayed HEPN-mediated ribonuclease activity after activation by cA_4 (REF.[97]). Viral transcript levels during some infections drastically outnumber those of the host[98–100], 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. Can1, which contains 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 and Can2 were found to possess both ssRNase activity and ssDNase activity upon activation by cA_4, presumably achieving the protective function through two separate processes: cellular dormancy due to transcript depletion and direct invading genome destruction through cleavage of ssDNA intermediates in DNA replication. Preliminary data suggest that the nucleases Can1 and Can2 respond not only to cA_4 but also to cA_3, resulting in different substrate specificities (that is, ssRNA or dsDNA) depending on the bound cOA[101]. As a single type III effector complex is capable of synthesizing multiple cOA species[102,103], encoding such multipronged ancillary nuclease or, alternatively, multiple ancillary proteins that bind different cOAs[94] might enable further fine-tuning of an appropriate immune response. Another nuclease that is activated by cOA, albeit in a CARF-independent manner, is NucC[60,104,105] (FIG. 2b). NucC was initially studied as part of the cyclic oligonucleotide-based antiphage signalling system, but some homologues of NucC are associated with type III CRISPR–Cas 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, causing cell death.

In some bacteria, proteins in association with type III systems possess a domain called 'SAVED' (for 'SMODS-associated and fused to various effector domains') instead of CARF[106]. 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[107]. A preliminary study on the CRISPR-Lon protease, which is encoded in close proximity to a type III-B CRISPR–Cas system, presents an intriguing example of this[108]. CRISPR-Lon has an integrated SAVED domain and forms a strong complex with a MazF-like protein, called 'CRISPR-T' (FIG. 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 to associate with type III systems, including nucleases, proteases, peptidases and ATPases, suggests that type III signalling goes beyond the use of cOA second messengers[87,88]. 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 multiple-subunit Craspase (CRISPR-guided caspase) complex[64] (FIG. 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 type III CRISPR–Cas clusters lacking nucleotide-sensing domains is likely to expand our knowledge of type III intermolecular communication routes beyond cOA dependency.

Although most of the type III-associated immune proteins are uncharacterized, domain function inference and experimental data suggest that ensuring an antiviral response through nonspecific, debilitating action on both the host and the virus is a common theme. These are obviously damaging measures for the host when insufficiently controlled. Therefore, tight and specific regulation of ancillary protein activation has evolved: type III CRISPR–Cas seems to be an intricately regulated signalling system, displaying properties such as signal amplification, self-regulation and tuning of signalling molecule concentrations. Recognition of just a single RNA molecule can potentially generate about 1,000 cOA molecules, which in turn enable enzymatic activation of many immune proteins[76,109]. The cOA concentration increases proportionally to the viral load, for example in the 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[76,109]. 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 by destroying excess cOA. New cOA production is stopped through cleavage of the target RNA, as this returns Cas10 to an inactive state[76]. 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[69]. Removal of excess cOA is achieved by dedicated CARF-containing ring nucleases, which cleave cOA rings into inactive linear diadenylate species[109–111] (FIG. 2c). Furthermore, some of the CARF effectors themselves have been shown to degrade

Cyclic oligonucleotide-based antiphage signalling system
A family of defence systems in bacteria and archaea related to animal cGAS–STING immunity in which a cyclase generates cyclic oligonucleotides to activate a cell death effector protein in response to viral infection.

cOA via a CARF domain[90,97], a HEPN domain[102,112,113] or fusion to a ring nuclease[114], thereby acting as an intrinsic timer to regulate their own activity[115]. Also, a family of membrane-associated nucleases was shown to possess cOA degradation capacity[116], as was the virus-encoded ring nuclease AcrIII-1 (REFS.[109,117]). The potent AcrIII-1 binds cA$_4$ with greater 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.

The wide pool of functions inherent to the type III CRISPR–Cas systems — RNA targeting, second messenger signalling and the availability of large repertoire of ancillary proteins — raises the intriguing possibility that their functionality extends beyond immune defence[87]. An example of type III cOA signalling transcending direct antiviral defence is found in the CARF-containing transcription factor Csa3 from a co-occurring type I-A system, which seems to be involved in transcriptional regulation[118–120]. Certain type III CRISPR–Cas proteins secreted by the bacterial pathogen *Mycobacterium tuberculosis* were also shown to function as virulence factors[121]. Deciphering the full range of type III CRISPR–Cas 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 (Supplementary Box 2).

Type VI CRISPR–Cas. In type VI CRISPR–Cas, the functionalities for crRNA processing, invader recognition and immune response are contained in a single effector protein: Cas13 (REFS.[16,122–124]). Until now, six different type VI subtypes have been identified: types VI-A to VI-D, Cas13X and Cas13Y[53,125]. The Cas13 proteins across subtypes are distantly related, sharing only 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[124]; newer insights showed that type VI spacer sequences match the genome of DNA viruses, indicating binding of viral transcripts[126,127]. Cas13 has the remarkable capacity of reaching femtomolar sensitivity in finding a target RNA in a population of non-target RNAs[128]. 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 bystander RNA[122,129,130], leading to inhibition of the invading DNA virus[28,130–134]. Unloaded Cas13 is inactive, indicating the presence of an autoinhibited 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 centre segment of the crRNA[123,124,129,135] (FIG. 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' (FIG. 3a), because imperfect base-pairing in this

portion prevents HEPN-nuclease activation[129]. Extensive base-pairing between the repeat-derived crRNA portion and the PFS also blocks the formation of the HEPN catalytic pocket, which is likely to prevent autoimmunity[40,136]. Bound target RNA is cleaved by Cas13 only when there is sufficient base-pairing in the switch region and non-complementarity between the 5′ tag and the PFS (FIG. 3a). Although genomes of viruses that escaped Cas13 targeting were found to contain deletions of tens to hundreds of bases[28,134], one would imagine that strict matching requirements in the seed and switch regions enable viruses to also 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 (REF.[137]). This leads to RNA cleavage outside the target RNA-binding region (FIG. 3a), with different homologues of Cas13 displaying differing ribonucleotide cleavage preferences[138,139]. 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[130]. Extensive host transcript depletion interferes with vital cellular pathways, whereupon cells enter dormancy. This state of hibernation is maintained as long as the active virus continues to produce target RNA. Inhibition of target transcription (signifying the defeat of the virus through, for example, viral DNA elimination by coexisting restriction–modification systems[140]) was found to reverse dormancy, implying that cells stay alive during the process[130]. Direct cleavage of the target RNA seems to be less important for the antiviral response, as preactivation of Cas13 with non-viral RNA is sufficient to clear a viral infection. This indicates that Cas13, once activated, can provide immunity against co-infecting viruses whose transcripts cannot be directly recognized. Indeed, cross-protection was shown to be a feature of Cas13 targeting, establishing broad and nonspecific immunity[130].

Although the biotechnological potential of Cas13 in RNA-based applications has gained substantial attention (Supplementary Box 2), the study of its physiological functionality has received relatively little attention. Recent studies, however, increased the degree of our understanding. A preliminary study challenged the paradigm of strict nonspecific RNA targeting by Cas13, as it shows that Cas13a has a bias towards cleavage of specific RNA molecules, most substantially tRNAs[141] (FIG. 3b). Massive tRNA cleavage results in compromised translation and dormant behaviour of the cell, limiting the success of the virus in the cell population. Interestingly, the preliminary data also suggest that in addition to Cas13-mediated tRNA cleavage, certain mRNAs and 16S ribosomal RNA are also cleaved upon Cas13 activation, although not by Cas13 itself. Instead, presumably RNases
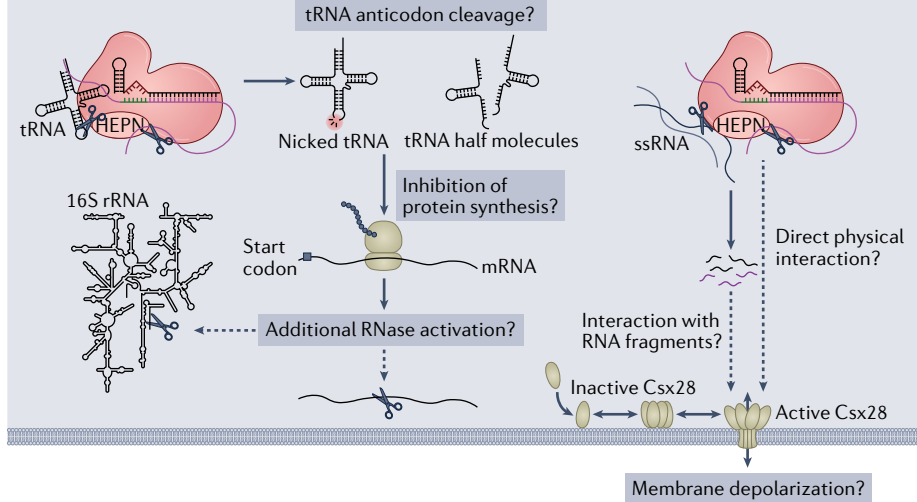

Restriction–modification systems

Genetic compositions encoding nucleases and methylases in which specific methylation patterns generated by the methylases prevent or facilitate cleavage by the nucleases, presenting a means for discriminating self from non-self.
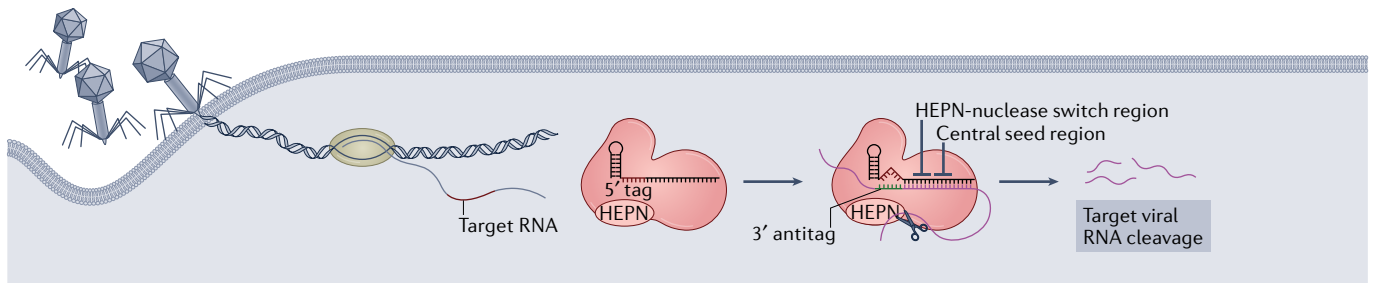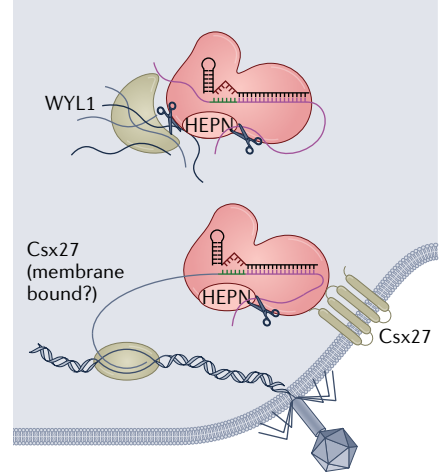
Fig. 3 | **Type VI CRISPR–Cas immunity. a** | Target RNA recognition by Cas13, the effector protein in type VI CRISPR–Cas systems, occurs in the central seed region and leads to the activation of a RNase pocket formed by HEPN domains ('higher eukaryotes and prokaryotes nucleotide-binding domains') when the HEPN-nuclease switch region matches the corresponding target sequence and the 5′ tag (red) mismatches the 3′ antitag (green). The activated RNase catalytic site is capable of degrading bound target RNA as well as bystander RNA. **b** | A preliminary study suggests that activated Cas13 variants can cleave tRNA molecules in the anticodon loop, which can result in ribosome stalling and subsequent activation of additional RNases to establish a dormant phenotype, hampering viral propagation. Other preliminary work suggests that target bound Cas13 can activate the ancillary protein Csx28, perhaps by target RNA degradation products or through physical interaction, which might form pores in the membrane to instigate membrane depolarization. **c** | Csx27 and WYL1 are believed to regulate Cas13 activity, although exact functionalities have not been 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. rRNA, ribosomal RNA.

Membrane depolarization
The process by which the basal electrochemical potential across cellular membranes changes from a negative state to a positive state.

present in the cell are activated by ribosome stalling as a consequence of tRNA inactivation, leading to the observed additional RNA cleavage (FIG. 3b). It is interesting to speculate on the possibility that Cas13-generated tRNA fragments can function as signalling molecules, akin to cOA second messengers in type III, for activation of downstream pathways[141], as broadly observed in eukaryotic systems[142]. Cas13-induced downstream protein activation has recently been shown for the type VI accessory protein Csx28 (REF.[127]), which, on the basis of preliminary data, forms a membrane pore to enhance antiviral defence through membrane depolarization[133] (FIG. 3b). This indicates that Cas13 has the capacity to also act as a signal relay, besides directly interfering with the viral life cycle 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–Cas 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 by up to five orders of magnitude[127,137]. 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[137,143] (FIG. 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[126,144,145]. Alternatively, because WYL1 possesses affinity for ssRNA, it is hypothesized that WYL1 acts as an RNA sponge that upregulates Cas13d cleavage by confining RNA close to its active pocket (FIG. 3c). For both Csx27 and WYL1, the exact mechanistic functioning as well as the biological implications are still unclear.

Toxin–antitoxin systems
Genetic compositions encoding at least a toxin and a counteracting antitoxin whereby the toxin component becomes active when the antitoxin is absent.

Kin selection
A theory of natural selection describing behaviour that favours the fitness of closely related kin over the fitness of the individual enacting the behaviour.

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.

Dormancy as an immune strategy

Bacteria can go into a physiological state of low metabolism known as dormancy, which facilitates survival in unfavourable conditions. Dormancy enables the utilization of energy and resources to sustain vital processes, repair damage and prevent further damage, rather than to grow and propagate[146]. When conditions become more favourable, the cell can re-emerge and continue regular metabolism. In scenarios of sustained stress, the cell accumulates so much damage that cell death is inevitable, which 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 and the presence of toxic compounds or parasitic invaders[147].

Many immune systems, including type III and type VI CRISPR–Cas, use dormancy as a strategy to halt viral invasion[31]. The broad activities (for example, DNase, RNase, membrane depolarization or proteolysis) responsible for dormancy induction in type III and type VI are known or predicted, but the exact cellular pathways involved in generating the dormancy phenotype are often obscure (TABLE 2). Especially the exact downstream effects of global RNA degradation are still to be investigated in detail. Preliminary data suggest that Cas13 exhibits collateral RNase activity against primarily tRNAs, presumably resulting in dormancy through ribosome stalling[141]; for collateral RNase activity in type III CRISPR–Cas, it is not well known whether there is a bias towards degradation of certain transcripts (for example, mRNA, ribosomal RNA, tRNA or 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[148], but there are 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[104,105].

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 probably synergistic scenarios to prevent viral success (FIG. 4): interference with the viral life cycle buys time for the already present DNA-targeting enzymes to destroy the foreign genomes[149,150]; and viruses are trapped in the cytosol of the dormant cell, preventing it from completing its life cycle and spreading to neighbouring cells, implying a kin-selection strategy[149,150]. The exact processes required for exiting dormancy after viral clearance have not been 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[151].

Buying time. The events that happen after viral infection, including viral genome replication and protein synthesis, occur rapidly[152,153]. Moreover, cells can be infected with multiple viruses at the same time[154], and the DNA target sequence can be in a genomic region that is expressed late in the infection cycle[30]. This could quickly lead to accumulation of viral DNA and a cellular state in which the viral genome copy number outruns the DNA-targeting systems[155]. 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 (FIG. 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 them during a CRISPR–Cas defence. Although this effect was not studied directly in infected cells, growth-inhibited bacteria were shown to have increased spacer acquisition[156]. Kinetic studies on the relations between viral replication and the activity of co-occurring DNA-targeting and RNA-targeting systems could shed experimental light on the buying time concept, which remains mostly hypothetical to this day.

Kin protection. At first glance, unicellular programmed cell death seems paradoxical. Whereas immune systems often confer benefits on 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 being released and thus effectively reduces the viral epidemic (FIG. 4). This decreases the chance for the infection to spread to neighbouring cells,

Table 2 | **Experimentally described type III and type VI CRISPR–Cas able or expected to cause dormancy phenotypes**

Effector protein	CRISPR–Cas type	Target	Cellular pathway	Phenotype
NucC	III	dsDNA	Host chromosome degradation	Cell death
Card1 and Can2	III	ssRNA, ssDNA, dsDNA	Unknown	Dormancy
Can1	III	dsDNA, ssRNA	Replication fork destabilization through DNA nicking[a]	Dormancy
Csm6, Csx1 and TTHB144	III	RNA	Unknown	Dormancy
TPR-CHAT	III	Protein[a]	Unknown	Unknown
CRISPR-Lon	III	RNA[a]	Unknown	Unknown
Cas13	VI	RNA	Ribosome stalling[a]	Dormancy
Csx28	VI	Cell membrane	Perturbed membrane integrity	Dormancy or cell death*

dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; ssRNA, single-stranded RNA.
[a]Posed as a hypothesis in the literature.

which are often closely related kin, and thus likely also susceptible to the infecting virus[157]. Additionally, the sacrificed cell may leak valuable cellular resources into the population[158], perhaps even cOA signalling molecules to prime defence in neighbouring cells. So whereas the individual cell does not benefit from suicide, protection of kin makes the maintenance of suicide genes and pathways evolutionarily advantageous[159]. Moreover, co-infecting viruses that are resistant to other forms of defence are also taken down in the process, further highlighting why acting on the host and the virus simultaneously instead of the invader only can be beneficial[130,151].

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 host genome by DNA-targeting systems, distinguishing features have evolved to discern self DNA and non-self DNA. Two principles for discriminating self and non-self (from here on 'self/non-self discrimination') are well described: mask self and damage non-self; and recognize non-self and damage non-self. The first principle is used in type II restriction–modification systems, whereby the own genome is masked by methylation sites[160]. This prevents accessibility of the restriction enzymes, such that only the unmethylated viral genomes are detected and cleaved. The second principle is found in DNA-targeting CRISPR–Cas systems, which act only on genetic elements that carry a PAM next to the target[161]. The combination of a PAM and the 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 the host and the virus, without strictly discriminating self from non-self. Due to intrinsic differences between the lifestyles of the host and the virus, such as the capacity to enter dormancy 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, and outlive non-self. As opposed to pointing directly at non-self before the immune response, this principle acts without actively seeking out who is who and effectively differentiates only in hindsight which was self (that is, the biological unit that survived) and which was non-self (that is, the biological unit that perished). We therefore term it 'blindfolded self/non-self discrimination' (FIG. 5a). For example, the activity of various RNA-targeting CRISPR–Cas proteins (such as Cas13, Csm6 and Card1) is guided towards RNA of both host and virus. The virus is programmed for fast intracellular replication, so depleted RNA levels (for example, tRNA, ribosomal RNA and mRNA levels) and resulting protein scarcity (for example, transcription and translation machinery, and structural proteins) can lead to logistical problems in the viral life cycle. By 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 neutralization, 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, presumably resulting in the collapse of replication forks in both the virus and the host[94]. This is detrimental to the virus, where many replication forks are present due to its fast replication cycle. Conversely, the host prevents catastrophic damage by entering a dormant state,

Immunity through DNA targeting

Immunity through RNA targeting
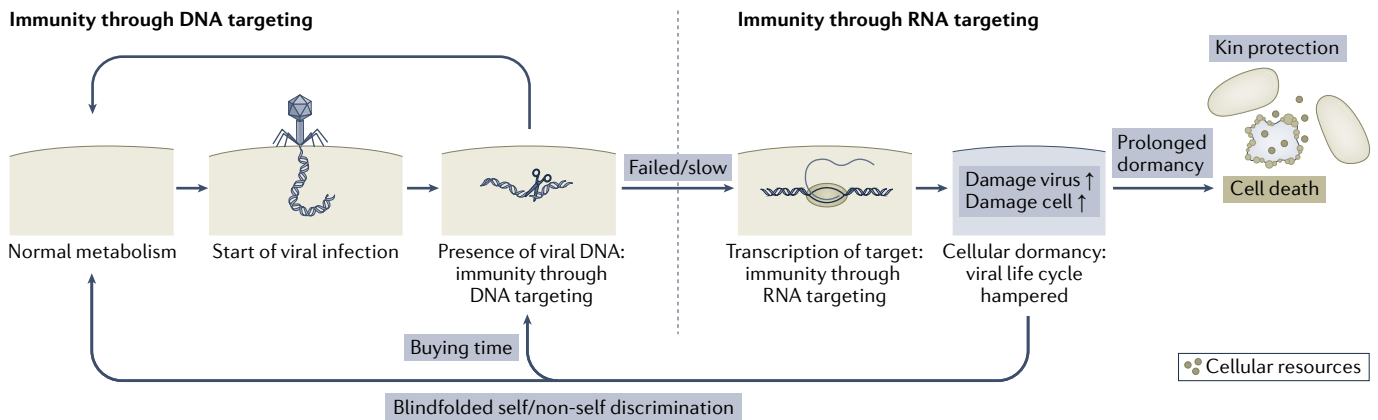
Fig. 4 | **Model of the dormancy strategy used by type III and type VI CRISPR–Cas systems.** Infection by a virus is signified by the presence of viral DNA inside the cell. DNA-targeting systems provide the first line of immunity by immediately attacking the invader genomes, usually through genomic DNA cleavage. When the anti-DNA response is unsuccessful (for example, viruses circumventing DNA-targeting mechanisms or viral genome replication outrunning genome cleavage), the viral life cycle progresses to transcription. Target transcripts are recognized by type III and type VI CRISPR–Cas effector proteins, typically leading to an immune response that involves global damage to both the host and the virus. The resulting cellular dormancy and hampered progression of the viral life cycle 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 inflicted self-damage. Alternatively, as the fast viral processes are more severely affected than 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 FIG. 5). In the 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 into the environment to provide nutritional aid to neighbouring cells.

a Blindfolded self/non-self discrimination

b Escaping blindfolded self/non-self discrimination?
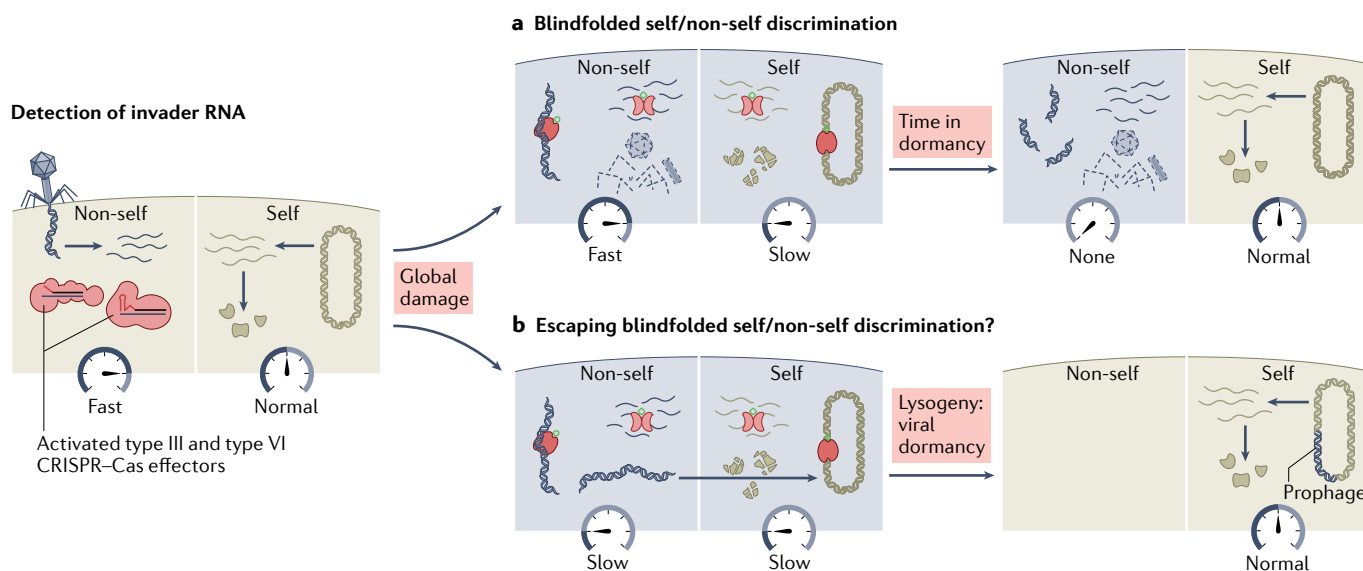
Fig. 5 | **Blindfolded self/non-self discrimination.** Upon viral infection, unsuccessful immediate clearance of the virus leads to progression of the viral life cycle to transcription (the rate of metabolism is indicated by the speedometers at the bottom of the cell). Target transcripts are recognized by the type III and type VI CRISPR–Cas effector proteins that typically initiate a global immune response. **a** | The global immune response includes damage to both the host and the virus, as exemplified by the activity of the RNase Csm6 (pink) and the single-stranded DNA nickase Can1 (red), affecting the processes of replication, transcription and translation. To sustain this stress, the host enters a state of dormancy in which metabolism is slowed down; by contrast, the viruses will pursue their fast intracellular life cycle and eventually collapse due to the lack of cellular means. In this way, self is discriminated from non-self as if wearing a blindfold; that is, 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 intracellular life cycle, temperate viruses have the capability of integrating into the host genome as a prophage during the process of lysogeny. This can be seen as a form of viral dormancy, slowing the viral processes down such that they cannot be 'detected' by blindfolded self/non-self discrimination.

ensuring few replication forks. Upon clearance of the virus, the host can repair broken DNA and continue normal metabolism. Thus, autoimmunity — a phenomenon that is generally thought to be avoided — is used for the benefit of the cell 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 (FIG. 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 a counter strategy, one might expect lysogenic viruses to be over-represented in bacteria that use dormancy as an immune response.

Conclusions and outlook

CRISPR–Cas immune strategies have far exceeded the 'simple' paradigm of cleaving invader nucleic acids. Type III and type VI CRISPR–Cas systems provide immunity through sensing invader transcripts, whereupon a plethora of broad and often rigorous responses are initiated. The mechanisms include collateral RNA degradation, own chromosome destruction, replication fork collapse, tRNA inactivation and membrane depolarization. But although the general workings of type III and type VI CRISPR–Cas are understood, many of the details remain obscure, and various open questions are still to be answered. For example, under what circumstances does type III and type VI immunity lead to cell death? Which cellular pathways generate the dormancy phenotype during type III and type VI immunity? To which extent is cellular dormancy induced by viral infection reversible, and which processes are responsible? Do cOA signalling molecules leak into the environment upon suicide induced by the type III immune response, and are they capable of priming defence in neighbouring cells? Are there other systems in the cell that are activated by the cOA produced during the type III immune response? How is the cooperation between DNA-targeting immune systems 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 what extent, is Cas13 activity regulated by ancillary proteins? How does signal relay occur in type VI? What is the effect of viral RNA modifications (for example, modified nucleosides or 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, in what ways do bacteria and archaea use innate immune systems that act on RNA? Such unanswered questions in the field may guide future research priorities. As bacteria and archaea have a long history with virus outbreaks, numerous surprises in RNA-targeting CRISPR–Cas immunity undoubtedly await discovery.

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