CRISPR–Cas: Complex Functional Networks and Multiple Roles beyond Adaptive Immunity

Guilhem Faure, Kira S. Makarova and Eugene V. Koonin

National Center for Biotechnology Information, *National Library of Medicine, National Institutes of Health, Bethesda, MD 20894, USA*

Correspondence to Eugene V. Koonin: *koonin@ncbi.nlm.nih.gov*
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Abstract

CRISPR–Cas is a prokaryotic adaptive immune system that functions by incorporating fragments of foreign DNA into CRISPR arrays. The arrays containing spacers derived from foreign DNA are transcribed, and the transcripts are processed to generate spacer-containing mature CRISPR-RNAs that are employed as guides to specifically recognize and cleave the DNA or RNA of the cognate parasitic genetic elements. The CRISPR–Cas systems show remarkable complexity and diversity of molecular organization and appear to be involved in various cellular functions that are distinct from, even if connected to, adaptive immunity. In this review, we discuss some of such functional links of CRISPR–Cas systems including their effect on horizontal gene transfer that can be either inhibitory or stimulatory, connections between CRISPR–Cas and DNA repair systems as well as programmed cell death and signal transduction mechanisms, and potential role of CRISPR–Cas in transposon integration and plasmid maintenance. The interplay between the primary function of CRISPR–Cas as an adaptive immunity mechanism and these other roles defines the richness of the biological effects of these systems and affects their spread among bacteria and archaea.

Introduction

CRISPR–Cas are adaptive immunity systems that are present in nearly all archaea and about one third of bacteria [1–4]. The CRISPR–Cas genomic loci consist of a CRISPR array—a battery of direct repeats with unique spacers between them—and the adjacent *cas* genes whose repertoire substantially differs among CRISPR–Cas types and subtypes.

The CRISPR–Cas immune response consists of three distinct stages:

1) Adaptation that is mediated by a distinct complex of Cas proteins that, in (almost) all cases, includes Cas1 and Cas2 and often additional Cas proteins, such as Cas4. The adaptation complex binds the target DNA and, in most cases, recognizes a short (2–4 bp) motif known as protospacer-adjacent motif (PAM). Upon PAM recognition, a portion of the target DNA (protospacer) is excised and inserted into the CRISPR array, apparently via a cut-and-paste mechanism (typically, at the beginning of the array, downstream of the leader sequence), as a spacer [5–8]. The adaptation process is what makes CRISPR–Cas systems a form of adaptive immunity, by creating molecular mementos of encounters with foreign DNAs, which are then used to protect a bacterium or archaeon against new infections with a familiar agent.
2) Expression and maturation of CRISPR (cr) RNA, when the CRISPR array is transcribed into a long precursor CRISPR (pre-cr) RNA, which is processed into small, mature crRNAs, each of which consists of a spacer and portions of the adjacent repeats. In different CRISPR–Cas systems, the pre-crRNA processing is mediated by a distinct complex of Cas proteins, a single and large Cas protein, or a non-Cas RNase, such as RNase III [9].
3) Interference when the crRNA is used as the guide to recognize sequences complementary

(or partially complementary) to the protospacer in an invading genome of a virus or plasmid, followed by cleavage and inactivation of the foreign genome by a Cas nuclease (s) [10,11].

The current classification divides the CRISPR–Cas systems into two classes that differ with respect to the composition and structure of the effector modules that are responsible for the interference and, typically, also the processing stages [2,3]. In class 1 systems (types I, III, and IV), the effector module consists of multiple Cas proteins that assemble into a heteromeric complex containing different copy numbers of Cas subunits and performs a closely coordinated series of reactions, from pre-crRNA processing to target cleavage. In class 2 systems (types II, V and VI), all activities of the effector module reside in a single, large multidomain protein, exemplified by Cas9 of type II, the programmable endonuclease that is most widely used for genome editing applications [12–14].

An implication of the functional scheme for the CRISPR–Cas that has been proposed by analogy with eukaryotic RNA interference was that, similarly to the eukaryotic counterpart, CRISPR–Cas systems, in addition to the defense function, could contribute to the regulation of gene expression [15]. Multiple subsequent observations appear to corroborate this possibility, although the full scope of non-defense functions of CRISPR–Cas systems remains unclear [16]. In this review, we focus on several non-defense activities and putative functions of CRISPR–Cas and on their multiple, intricate links to other functional systems. In particular, we address the roles of CRISPR–Cas in the control of horizontal gene transfer (HGT) and DNA repair, potential functions of "minimal" CRISPR–Cas systems encoded by transposons and plasmids, regulatory roles, and association of CRISPR–Cas with signal transduction networks.

Effects of CRISPR–Cas on HGT

HGT is a dominant process in the evolution of bacteria and archaea [17–19]. Capture of foreign genes appears to be essential in clonal populations to avoid mutational meltdown [20,21], and adaptation of prokaryotes to new environments and various forms of stress, for example, antibiotics, occurs primarily by means of HGT [22,23]. To acquire and exchange genetic material, prokaryotes employ at least three common mechanisms of HGT [24–27]: (1) transformation whereby exogenous DNA molecules are internalized by microbial cells via dedicated membrane pumps and then can integrate with the host genome through homologous or non-homologous recombination, (2) conjugation whereby a plasmid is exchanged by direct contact between two microbial cells, and (3) transduction where a segment of DNA is transported via a virus.

From the standpoint of microbial fitness, HGT is a double-edged sword. Most of the horizontally acquired DNA is likely to be deleterious to the recipient, whereas a small fraction can turn out to be beneficial or even crucial for survival [28–30]. Thus, evolution of microbial defense systems, and CRISPR–Cas in particular, involves a trade-off between full protective immunity and abrogation of (potentially) beneficial HGT.

Multiple lines of evidence indicate that CRISPR–Cas systems indeed abrogate different routes of HGT. In classic early work, Marraffini and Sontheimer [31] have shown that CRISPR interference prevents conjugative transfer of plasmids and transformation in *Staphylococcus*. More specifically, it has been found that the clinically isolated *Staphylococcus epidermidis* strain RP62a carries a CRISPR–Cas locus containing a spacer against the nickase gene that is present in all staphylococcal conjugative plasmids and encodes a nuclease essential for conjugation. Conjugation has been found to be completely inhibited in the strain carrying this spacer, and moreover, CRISPR–Cas also inhibited experimental transformation of the bacterium with a non-conjugative plasmid carrying the target gene. In contrast, closely related strains of *S. epidermidis* lack the CRISPR–Cas system and are conducive to conjugation and transformation. The ability of CRISPR–Cas systems to efficiently cleave plasmids after transformation subsequently has become the basis of CRISPR interference assays.

A seminal observation on the relationship between CRISPR–Cas and HGT was that multidrug-resistant strains of *Enterococcus faecalis* that carry antibiotic resistance loci on large conjugative plasmids typically lack functional CRISPR–Cas systems as well as a particular restriction–modification system. Indeed, it has been shown that these systems jointly inhibited conjugation by 4 orders of magnitude [32,33]. However, all *E. faecalis* strains contain an orphan CRISPR array (*i.e.*, one without adjacent *cas* genes), suggesting that the ancestral bacterium possessed CRISPR–Cas systems that have been repeatedly lost during evolution in multiple bacterial lineages. The subsequent study of the role of CRISPR–Cas in mobile genetic element (MGE) maintenance in *E. faecalis* has led to the discovery of "CRISPR tolerance" whereby bacteria can transiently maintain a MGE targeted by a CRISPR spacer albeit at a fitness cost [34]. An analogous ability to transiently accept and propagate CRISPR-targeted MGE, that is, CRISPR tolerance, has been demonstrated in several other bacteria and archaea [35–37]. The mechanism of CRISPR tolerance is not well understood but, in type II systems, seems to depend on the concentration of the effector protein Cas9 such that, at low Cas9 concentrations, the target DNA can propagate, but when Cas9 is induced to a high concentration, the target is eliminated. The tolerance phenomenon clearly requires further investigation but

potentially could represent a microbial adaptation to maintain balance between defense against foreign DNA and the HGT potential.

Another route to tolerance could involve dependence of the CRISPR interference on the replication and/or transcription of the target DNA. The cleavage of the target foreign DNA by type III CRISPR–Cas systems appears to depend on binding of RNA transcripts of the target by a dedicated Cas RNase; accordingly, these systems seem to cleave only actively transcribed DNA [38–41]. Therefore, phage or plasmid DNA is sensitive to these CRISPR–Cas systems, whereas DNA that is acquired through transformation and is not expressed is resistant. Integrated prophages represent an intermediate case of partial tolerance that depends on the transcription from internal prophage promoters, adding to the cost of maintaining CRISPR–Cas systems [42]. Furthermore, the *Escherichia coli* subtype I-E system has been shown to inhibit both lysogenization and prophage induction as well as target integrated prophages, resulting in bacterial cell death [43]. Apart from type III, other types of CRISPR–Cas do not seem to require transcription of the targets, but the *E. coli* subtype I-E system has been shown to depend on the target replication at least, for the adaptation stage [44], and a similar requirement has been demonstrated for a subtype III-B, with an additional preference for rolling-circle replication plasmids [45]. This replication requirement could also lead to tolerance of non-replicating foreign DNA. Taken together, the data on CRISPR tolerance reveal an intricate complexity of the relationships between CRISPR–Cas systems and various forms of foreign DNA, particularly lysogenic phages, that calls for further investigation.

Extending the original seminal findings on spacers matching MGE sequences [46–48], a comprehensive analysis of CRISPR spacer matches has shown that the majority of spacers with matches are of viral origin. Among the non-viral-matching spacers, most target genes of conjugative plasmids [49]. Thus, inhibition of conjugative HGT appears to be a common activity of CRISPR–Cas systems in bacteria and archaea.

Unexpectedly, it has been found that, on the evolutionary time scale, there is no perceptible connection between the apparent activity of CRISPR–Cas systems in bacteria and archaea, as measured by the length of CRISPR arrays, and the apparent extent of HGT [50]. Thus, in the long haul, the inhibitory effect of CRISPR–Cas on HGT appears to be balanced via processes that are not yet well understood. One of these processes could be, simply, the frequent loss and regain of CRISPR–Cas loci as well as individual spacers in the course of microbial evolution [51]. A more specific mechanism is suggested by recent findings on the apparent promotion of transduction by CRISPR–Cas [52]. The links between CRISPR–Cas and transduction appear complicated. It has been shown that different CRISPR–Cas types can inhibit transduction of targeted plasmids and chromosomal DNA [52]. However, spacers against transducing phages themselves show the opposite effect of enhancing transduction by an order of magnitude or more, apparently, because abrogation of phage reproduction by CRISPR–Cas increases the survival rate of the transductants. Given the low proportion of transducing particles in phage populations, because of which CRISPR–Cas systems acquire anti-phage spacers far more frequently than spacers against transduced DNA, it appears most likely that, in nature, the stimulating effect of CRISPR–Cas immunity on transduction prevails over the inhibitory effect.

CRISPR–Cas and DNA Repair

When the gene arrays that subsequently became known as *cas* operons were first discovered by comparative genome analysis, a prediction has been made that the proteins encoded by these genes comprised a novel DNA repair system [53]. Obviously, this prediction has missed the mark as became clear once the principal, defense functions of the CRISPR–Cas systems have been discovered (see above). Nevertheless, connections between CRISPR–Cas and repair systems and mechanisms do exist at different levels and can have major effects on microbial biology. Indeed, the biochemical processes involved in repair, on the one hand, and in the CRISPR–Cas-mediated immunity, on the other hand, substantially overlap. Both functions include coordinated reactions of nucleolytic cleavage of DNA that are followed by gap filling and ligation. Some of the Cas proteins contain domains that are homologous to enzymatic domains widespread in repair systems [15,53,54]. These shared domains include helicases, such as Cas3, the signature effector protein of type I systems [55–57], and several nucleases, in particular, Cas4 [58,59] that is involved in adaptation in many type I and type II systems, and the RuvC-like nuclease, the active domain of type II and type V effectors [3,11,60].

A direct functional connection between CRISPR–Cas function and repair is essential at the adaptation stage when repair systems restore the integrity of the CRISPR array after spacer insertion [61]. In particular, the DNA polymerase activity of PolA, the key bacterial polymerase responsible for gap filling in various repair processes, is indispensable for CRISPR adaptation in the *E. coli* subtype I-E system [62]. Furthermore, it has been demonstrated that Csa3a protein, the transcriptional regulator that activates the CRISPR adaptation genes [63], additionally co-activates multiple repair genes, demonstrating the functional coordination between CRISPR–Cas and repair mechanisms [64].

Notably, knockout of the subtype I-E *cas1* gene in *E. coli* yielded a DNA repair defect phenotype, namely, an increased sensitivity to DNA damage and impaired chromosome segregation [65]. Moreover, Cas1 has been shown to physically and genetically (in the form of either positive or negative epistasis) interact with key repair enzymes such as RecB, RecC, and RuvB. The extent and mechanisms of the apparent Cas1 involvement in repair in *E. coli* remain to be thoroughly characterized, but it seems likely that this role of Cas1 is underpinned by its ability to cleave single-stranded and branched DNA molecules in stalled replication forks [65,66].

Because CRISPR–Cas and repair systems act on the same substrates, such as, for example, double-strand breaks (DSBs) in DNA molecules, an antagonistic relationship between the two types of systems might exist. Comparative genome analysis has revealed complementarity between the presence of subtype II-A CRISPR–Cas systems and the non-homologous end-joining (NHEJ) machinery in microbial genomes: among more than 5000 analyzed bacterial and archaeal genomes, only one has been found to encode both systems [67]. Given that Cas9 nucleases, the type II effectors, introduce DSB into the target DNA molecules [68,69], NHEJ could repair such breaks. This type of repair is indeed efficient in eukaryotic cells and is widely utilized for Cas9-mediated genome editing [70]. Repair of Cas9-made DSB by the NHEJ system potentially could interfere with the CRISPR-mediated immunity. However, experimentally, it has been shown that the efficiency of such repair is quite low in bacteria, and compatible with these findings, NHEJ does not inhibit CRISPR–Cas activity [67]. On the contrary, Csn2 protein, a component of the adaptation module of the type II-A systems inhibits NHEJ apparently by competing for binding DNA ends at DSB [71–74]. These results imply that NHEJ inhibition by an adaptation component of subtype II-A systems is a side effect of the requirement for binding the same structure for both CRISPR adaptation and NHEJ [67]. Apparently, because of this deleterious effect, type II-A systems are purged from the genomes of bacteria that possess NHEJ.

Seminal work of Levy and colleagues [44] shows that repair processes can act synergistically whereby a repair process supplies the substrates for spacer incorporation. Specifically, it has been shown that the adaptation component of the *E. coli* subtype I-E CRISPR–Cas system possesses high specificity toward actively replicating DNA, resulting in a strong preference for spacer acquisition from plasmid and phage genomes as opposed to the host bacterial chromosome, that is, self *versus* non-self-discrimination by the immune system. The mechanistic basis of this preference appears to be the strong bias toward utilization as protospacers of short ssDNA molecules that are produced by the RecBCD repair complex by partial degradation of stalled replication forks. Notably, the degradation of the bacterial chromosome by RecBCD is constrained by the Chi sites, unique nucleotide sequences that promote the migration of RecBCD along a DNA molecule. The Chi sites are several-fold enriched in bacterial genomes compared to genomes of plasmids and phages and therefore contribute to self *versus* non-self-discrimination by I-E CRISPR-Cas system [44]. In this case, in addition to the synergy between repair and the CRISPR–Cas function, a feature (Chi site) that protects the host chromosome from excessive degradation by a repair machinery also serendipitously enhances the CRISPR spacer acquisition specificity. Notably, it has been shown that RecBCD is required only for naïve adaptation by the *E. coli* subtype I-E CRISPR–Cas system, whereas primed adaptation depends on two distinct repair proteins, RecG and PriA [62]. Thus, different aspects of CRISPR–Cas function appear to be coupled to distinct repair pathways.

Given the mechanistic similarities between CRISPR–Cas and repair systems, it seems likely that the findings outlined above do not exhaust the connections between the two types of processes. Search for further links between CRISPR-Cas systems and repair systems appears to be a promising line of study.

Involvement of CRISPR–Cas in Microbial Gene Regulation and Virulence

In the early days of CRISPR research, even before the immune function of CRISPR–Cas systems has been demonstrated experimentally, the prediction has been made, by analogy with the eukaryotic microRNAs, that, in addition to their role in defense, some CRISPR–Cas systems could regulate microbial genes via “self-specific” guide RNAs [15]. Although self-targeting spacers pose the obvious problem of autoimmunity [75], multiple cases of regulatory roles of CRISPR–Cas systems have been discovered, some of which appear to circumvent self-targeting [76]. The best characterized case seems to be the *dev* system of the myxobacterium *Myxococcus xanthus* that regulates sporulation [77]. As shown in early experiments, the expression of the *dev* operon is required for sporulation [78]. Addition of nutrients to developing *M. xanthus* cells results in proteolysis of the transcription factors MrpC and FruA that activate the transcription of the *dev* operon and, accordingly, halts the production of Dev proteins and sporulation [79,80]. Once the CRISPR–Cas systems have been characterized, it became apparent that the *dev* operon actually is a subtype I-B CRISPR–Cas locus [15,81]. The mechanism by which the *dev* operon promotes sporulation has not been fully characterized but appears to involve abrogation of expression of the small *devI* gene,

the first gene in the operon and an inhibitor of sporulation, by a complex of three proteins, DevRST, or in terms of CRISPR–Cas, Cas7–Cas5–Cas8 [82]. This three-subunit complex is actually a subcomplex of Cascade, the large, multisubunit complex that is involved in pre-crRNA processing and target recognition by type I CRISPR–Cas systems [83,84]. Because the *dev* operon does not encode Cas6, the enzyme that interacts with Cascade and cleaves pre-crRNA, crRNA appears not to be involved in regulation. However, there are indications that the DevRST complex employs a distinct antisense RNA to suppress *devI* transcription [82].

An important emerging theme appears to be the involvement of CRISPR–Cas systems in the regulation of the microbial response to envelope stress [85]. Expression of several type I CRISPR–Cas loci is induced by envelope stress that can be caused by virus infection, antibiotics, or other factors. In the only well-characterized case of CRISPR influence on the bacterial envelope, the subtype II-B CRISPR–Cas system of the intracellular pathogen *Francisella novicida* enhances the envelope integrity and confers resistance to certain antibiotics [86]. The effect is due to the down-regulation of a membrane lipoprotein (BLP) production by the Cas9 protein complexed with two small RNA species encoded in the CRISPR–Cas locus, a trans-activating (tra) crRNA and a small CRISPR–Cas associated (sca) RNA, supposedly, through hybridization of the tracrRNA with a 5′-terminal region of the lipoprotein mRNA including the start codon [76,87]. The regulation of the lipoprotein translation involves degradation of the mRNA, but neither of the two nuclease activities of Cas9 is required, suggesting a role for a distinct, yet unidentified nuclease that is not associated with the CRISPR–Cas system [87]. RNase III appears to be a good candidate for the nuclease activity involved in this regulatory circuit because the target appears to be a double-stranded structure [88]. Notably, the down-regulation of the lipoprotein production allows *F. novicida* to avoid the host immune response, and thus, the CRISPR–Cas system is a virulence determinant.

Contribution of CRISPR–Cas systems, in particular, those of type II, to virulence has been demonstrated also for *Campylobacter jejuni* [*88,89*]. The mechanisms of virulence enhancement are poorly understood. However, in the case of *Streptococcus agalacticae*, it has been shown that Cas9 down-regulates the expression of the transcription regulator RegR, the repressor of the gene for hyaluronidase, an enzyme that is essential for virulence [90]. Down-regulation of RegR involves mRNA degradation and has been speculated to depend on partial complementarity between CRISPR spacers and the *regR* gene, but there is no evidence of the responsible enzyme. Potentially, this could be a general link between CRISPR–Cas and virulence because many pathogenic bacteria produce sialidases that remove sialic acid moieties from host cell surface glycoproteins, uncovering adhesion receptors [88]. Bacterial virulence can involve Cas proteins via different mechanisms as illustrated by the finding that nuclease activity of Cas2 promotes the infectivity of *Legionella pneumophila* for amoebae [91].

Obviously, the study of regulatory functions of CRISPR–Cas systems is still in its infancy. Many more cases need to be investigated before generalizations become justified. Nevertheless, it is notable that, in two well-characterized examples, regulation of sporulation in *M. xanthus* and of lipoprotein production in *F. novicida*, CRISPR–Cas systems do not employ self-targeting spacers but rather act in a manner that is distinct from their immune function.

CRISPR–Cas, Programmed Cell Death, Dormancy, and Signal Transduction

Nearly all cellular life forms have evolved multiple, distinct yet interacting strategies of anti-pathogen defense that include resistance to parasite invasion, innate and adaptive immunity, and programmed cell death (PCD). The PCD can be viewed as the means of last resort, namely, an “altruistic” cellular suicide response to infection that is activated when immunity mechanisms allowing the cell to survive the infection fail [92–94]. Switching between immunity and PCD is thought to involve a “decision” made by the infected cell by sensing the level of genotoxic stress [95] (Fig. 1a). Although the existence of PCD in unicellular organisms might appear paradoxical, there is a growing body of evidence that such mechanisms are (nearly) ubiquitous, suggesting that kin selection enabling altruistic evolutionary strategies is important even in the evolution of unicellular life [92,93,96,97]. A notable feature of the genomic organization of immunity loci in bacteria and archaea is the common presence of genes associated with PCD, such as toxin–antitoxin (TA) and abortive-infection modules [98,99]. This colocalization of the immunity and PCD determinants suggests functional coupling between these two defense strategies that could be mediated by shared proteins that sense genotoxic stress and “predict” the outcome of infections, that is, success of failure of the immune response [95] (Fig. 1a).

Apart from the associated TA, CRISPR–Cas loci encode various proteins that potentially could be involved in PCD and/or dormancy induction. A notable case in point is Cas2 protein that is a homolog of toxin RNases of the VapD family [15,100–102]. The nuclease activity of Cas2 proteins against single-stranded RNA and/or single- or double-stranded DNA has been demonstrated in several independent *in vitro* experiments [101,103–106]. The Cas2 proteins are nearly universal and essential structural

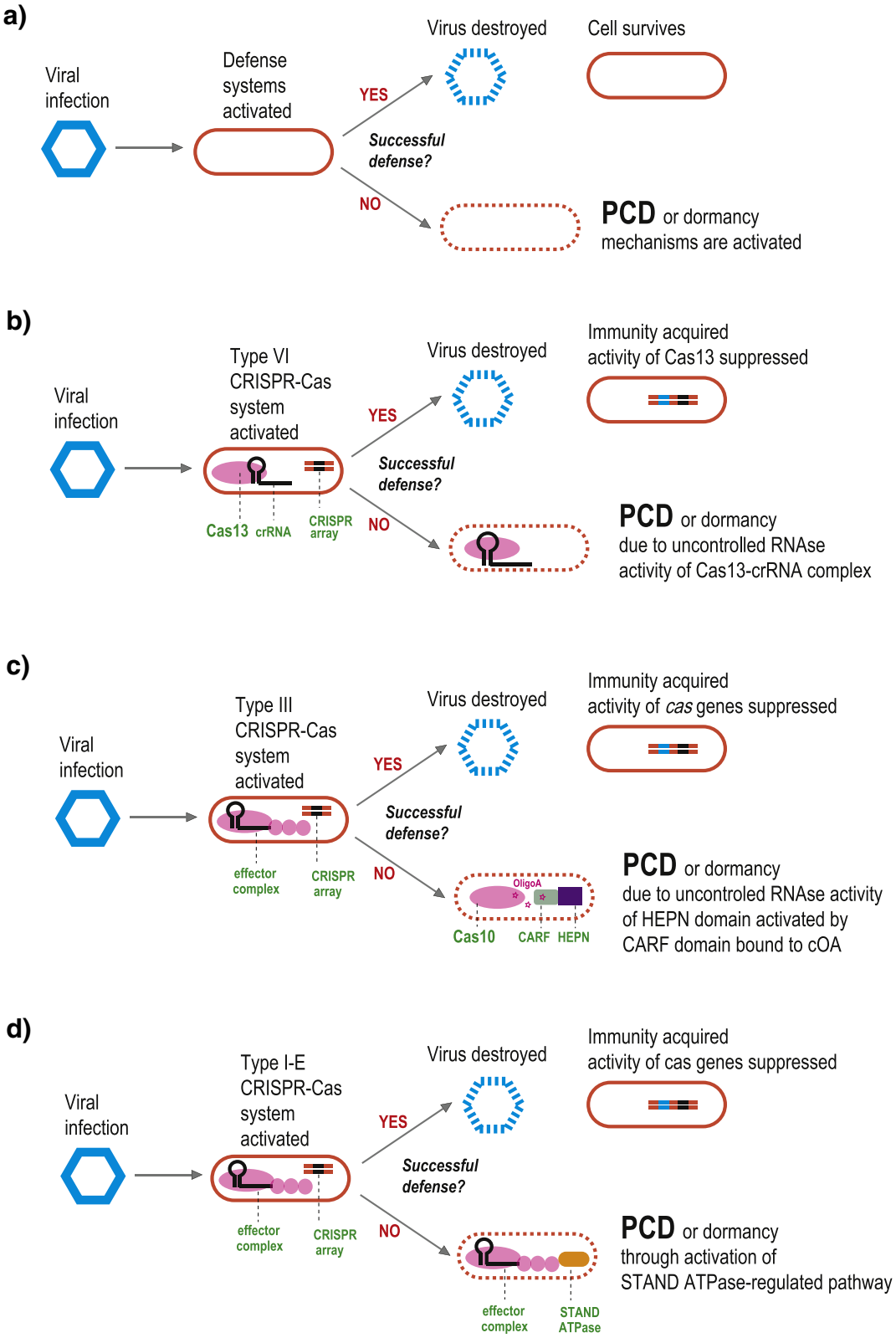

Fig. 1 (*legend on next page*)

components of the CRISPR–Cas adaptation complexes [8,107,108]. However, the role of the nuclease activity of Cas2 that, as implied by the conservation of the catalytic residues, is retained in most although not all CRISPR–Cas systems [3], in the CRISPR–Cas functions remains unclear. It cannot be ruled out that Cas2 functions as a toxin when CRISPR–Cas systems switch to the dormancy/PCD mode. Notably, it has been shown that *S. pyogenes* Cas2 assumes a catalytically inactive conformation in solution but is activated via conformation change induced by acidic pH and metal ions, a feature that is compatible with the properties expected of a toxin [104–106].

A remarkable case of an apparent CRISPR–PCD connection is type VI CRISPR–Cas systems, which are the only known variety of CRISPR–Cas that exclusively target RNA [109–114]. The type VI effector protein, Cas13, contains two higher eukaryotes and prokaryotes nucleotide-binding (HEPN) domains. Target recognition by the Cas13–crRNA complex activates promiscuous RNase activity of the HEPN domains that have been shown to decrease bacterial cell viability [109]. The current hypothesis is that the promiscuous RNase activity of Cas13 elicits dormancy or PCD, which represents ultimate integration of immunity and cellular suicide [95] (Fig. 1b).

Most of the type III CRISPR–Cas systems encode HEPN domain-containing proteins, such as Csm6 and Csx1 [3,115]. HEPN domain is the enzymatically active moiety of numerous RNases that are widespread components of TA modules and other defense systems in both prokaryotes and eukaryotes [115]. One of the most remarkable discoveries of the last few years in the CRISPR field is the demonstration that a dedicated signaling pathway leading to the activation of HEPN RNase activity is central to the function of type III CRISPR–Cas systems [116,117] (Fig. 1c). Target recognition activates the polymerase activity of Cas10 protein (the signature protein of type III CRISPR–Cas, the large subunit of the effector complex) that catalyzes the synthesis of cyclic oligoA (cOA). The produced cOA molecules bind the CRISPR-associated Rossmann fold (CARF) domain of the Csm6 protein [118], and this binding allosterically activates the promiscuous RNase activity of the second domain of Csm6, the HEPN nuclease. The role of this indiscriminate RNA degradation could be the induction of dormancy or PCD in response to infection, as a "contingency plan" in case of a failure of the immune response, in accordance with the immunity-PCD coupling hypothesis [95,98]. CARF domains showing considerable sequence and presumably structural variance are found in a great variety of accessory proteins of type III CRISPR–Cas systems [118]. Almost all CARF domains are parts of multidomain proteins in which the second domain is, most often, the HEPN RNase but in many cases, is either a distinct nuclease or a DNA-binding helix-turn-helix (HTH) domain [118], suggesting that there exist multiple branches of the cOA–Cas10 signaling pathway. Strikingly, it has been demonstrated that a distinct group of CARF domain proteins are nucleases that cleave cOA, thus reversing the activation of the HEPN domain [119] and exerting tight control over the activation of indiscriminate RNA cleavage [120].

Taken together, all these findings suggest that the cOA–Cas10 signaling pathway that is built into most of the type III CRISPR–Cas system is likely to function via diverse mechanisms that remain to be investigated. The RNase activity induced by this pathway appears to be essential for some forms of immunity, in particular, that against conjugative plasmids, but not for others, such as that against bacteriophages, and the requirements also differ among type III from different bacteria and archaea [121–123]. Given the extensive evidence of the role of HEPN superfamily nucleases as dormancy- or PCD-inducing toxins [115,124,125], an attractive hypothesis is that those CARF domain proteins that contain nuclease domains elicit dormancy or PCD as a last resort form of defense when immunity fails [95,98,126]. Those CARF domains containing an HTH domain can be predicted to function in a different manner, conceivably, by regulating the expression of genes the identity of which remains to be determined. It should be noted, however, that the cOA–Cas10 pathway does not appear to be essential for the function of the type III CRISPR–Cas systems because a minority of these, although complete in all other respects and, by inference, functional, lack CARF domain proteins and/or contain an enzymatically inactivated Cas10 protein as inferred from the replacement of the essential catalytic amino acid residues in the Palm domain [3]. A comprehensive characterization of the CRISPR-linked CARF domain proteins appears to be an important research program for years to come that should elucidate poorly understood functions of CRISPR–Cas systems and, in particular, their links to signal transduction pathways.

Toxin properties of Cas proteins have not been assessed systematically, but anecdotal evidence of the CRISPR–PCD connection seems to be accumulating.

Fig. 1. Coupling between CRISPR–Cas immunity and PCD/dormancy induction. (a) A general scheme of the "decision" made by the infected cell on immunity *versus* altruistic suicide depending on the success or failure of defense systems in controlling an infection. (b) Proposed scheme of PCD or dormancy induction in bacteria and archaea encoding type III CRISPR–Cas systems. (c) Proposed scheme of PCD or dormancy induction in bacteria encoding type VI CRISPR–Cas systems. (d) Proposed scheme of PCD or dormancy induction of in bacteria and archaea encoding subtype I-E system.

Thus, Csa5, the small subunit of the pre-crRNA-processing Cascade complex of subtype I-A systems [127] of the archaeon *Sulfolobus solfataricus*, is toxic to the archaeal cells when expressed in a CRISPR–Cas-deficient strain, although the mechanism of toxicity remains unknown [128]. Notably, Csa5 expression is up-regulated in rudivirus SIRV2 infection, suggesting that this protein might indeed promote suicide of infected cells [128].

In addition to these biochemical observations, comparative genomic analyses provide further indications of a potential functional link between CRISPR–Cas and PCD. In particular, many CRISPR–Cas loci, in addition to *cas* genes, encode type II TA systems [15,98,99]. Recently, it has been shown that, in multiple strains of the bacterium *Clostridium difficile*, CRISPR arrays co-localize with type I TA modules [129]. All these observations are in line with the hypothesis on coupling between CRISPR-mediated immunity and PCD [95,98].

A degenerate variant of subtype I-E systems lacking Cas1, Cas2 and Cas3, and accordingly confidently predicted not to be competent in either adaptation or target cleavage encodes an NTPase of the STAND superfamily [130] that, in addition to the P-loop NTPase domain, contains an array of tetratricopeptide repeats [131]. The presence of such repetitive regions is typical of STAND NTPases, which are involved in various signal transduction networks that remain poorly characterized in prokaryotes but, in eukaryotes, primarily contribute to various forms of PCD [97,130]. Phylogenetic analysis of the STAND NTPase family clearly indicates that the CRISPR-associated proteins form a strongly supported clade which implies a long-term, functionally important link between these NTPases and CRISPR–Cas [131]. In many loci, the gene adjacent to the NTPase gene encodes a small membrane protein, suggesting that these CRISPR–Cas systems, apparently, like many other ones, are membrane associated. Thus, the STAND ATPases can be predicted to connect this variant of subtype I-E to membrane-associated signal transduction networks, possibly involving PCD (Fig. 1d).

Taken together, all these findings present a substantial body of indirect evidence of the proposed CRISPR–PCD connection. Nonetheless, direct experimental validation of the immunity–suicide coupling hypothesis is still missing. Notably, mathematical modeling of the coevolution of viruses with defense systems, including those that cause PCD, indicates that coupling can evolve primarily in cases when immunity and PCD mechanisms share components [132]. The accumulating data seem to fulfill this prediction as demonstrated by the emerging roles of Cas10 and Csa5 in PCD, in addition to their established functions in the CRISPR-mediated immunity.

The cOA–Cas10 pathway that connects Cas10 and Csm6 (or other CARF domain proteins) in type III CRISPR–Cas systems is the only well-characterized case of signal transduction involved in CRISPR–Cas function (Figs. 1b and 2). However, comprehensive analysis of CRISPR-linked proteins leaves little doubt that many other signal transduction pathways contribute to the functions of different groups of CRISPR–Cas systems, mostly, those of type III. In general, CRISPR–Cas loci are highly variable in terms of gene composition and contain numerous genes, many of which could represent random, functionally irrelevant cargo [99,133]. Recently, in two independent studies, computational strategies have been developed to predict genes that are functionally linked to CRISPR–Cas [131,134]. These analyses led to the identification of several dozen proteins that can be considered strong candidates for a functional association with CRISPR–Cas. Analysis of the domain composition and predicted activities of the proteins encoded by these genes revealed two major, overlapping functional themes, signal transduction and membrane association. Below we briefly discuss some of the most notable examples.

The most abundant of the membrane-associated proteins identified in type III systems is CorA, a member of a family of divalent cation channels that is widespread in bacteria and archaea and is the primary route for electrophoretic Mg^{2+} uptake [135] (Fig. 3a). The CorA protein is encoded in numerous subtype III-B loci with diverse genomic architectures, in many of which the *corA* gene is adjacent or fused to a gene encoding a DHH family nuclease [136]. Some of these loci also include a gene encoding a predicted nuclease (RNase) of the NYN family [137]. The CorA protein forms a pentamer that consists of two transmembrane helices contributed by each subunit and a bulky cytosolic part [138,139]. The evolutionarily conserved association of CorA with subtype III-B CRISPR–Cas implies a functional link between the CRISPR-mediated immunity and membrane processes. It appears most likely that CorA was recruited for membrane tethering of the respective CRISPR–Cas systems, and the connection with two distinct nucleases suggests that CorA might facilitate additional, either specific or non-specific, DNA and RNA during the CRISPR response. However, more precise understanding of the role of CorA in the function of type III CRISPR–Cas systems awaits experimental investigation.

In the course of the systematic search for CRISPR-linked genes [131], it has been found that many type III systems of subtypes A, B, and D contain genes encoding proteins containing a highly diverged variant of the CARF domain (independently described previously as SAVED domains [140]) and two predicted transmembrane helices. Some of these proteins contain an additional domain that is a distinct version of the Lon family [141] proteases (Fig. 3b). According to the membrane topology prediction, the CARF domain of these proteins is located in the cytosol whereas the Lon domain is extracellular. The

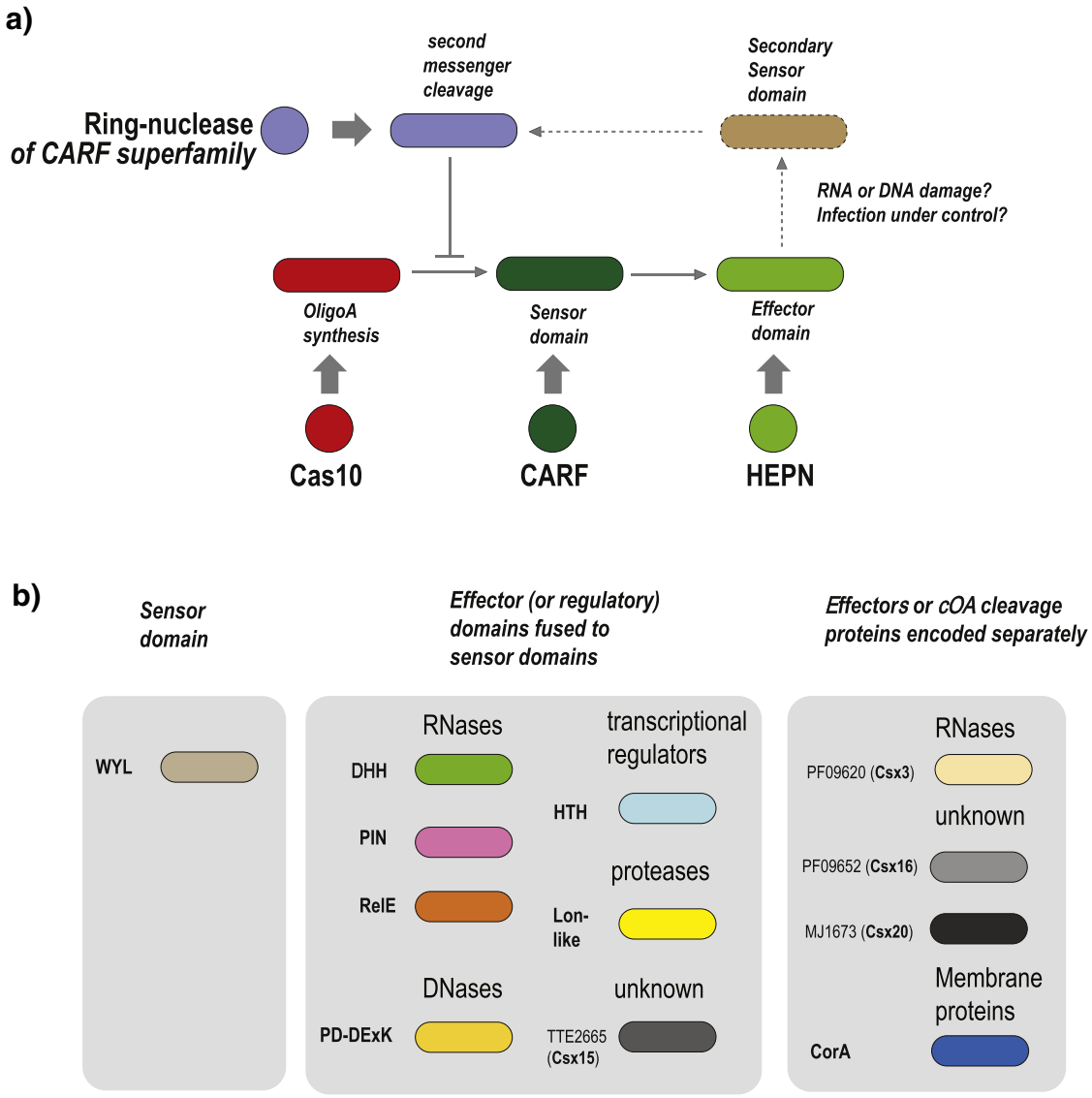

Fig. 2. The cOA–Cas10 signaling pathway in type III CRISPR–Cas systems. (a) General scheme of second-messenger signaling pathways. Actual protein families that have been experimentally shown to contribute to this pathway are shown by circles of the same color as the oval shapes representing the main functions in the pathway. (b) The most common predicted components of the cOA-mediated signal transduction pathway in type III CRISPR–Cas systems, apart from the experimentally characterized ones shown in panel a. Functionally uncharacterized components are shown by shades of gray and denoted by Pfam identifiers or by locus-tag of a representative protein. Gene names shown in parentheses follow the current nomenclature of *cas* genes [3]. Abbreviations: cOA, cyclic oligoadenylates; CARF, CRISPR-associated Rossmann fold; WYL, predicted ligand-binding domain associated with many CRISPR–Cas systems (named after the respective amino acids that are partly conserved in the family); HEPN, PIN, RelE, ribonucleases of the respective families; HTH, helix-turn-helix DNA-binding domain; HD, PD-DExK, nuclease (or phosphatases) of the respective superfamilies; CorA, divalent cation channel.

respective CRISPR–Cas loci encode no CARF domain proteins with recognizable effector but encompass Cas10 proteins that are predicted to be active nucleotide polymerases. Thus, the membrane-bound CARFs, most likely, recognize signaling cOA molecules produced by Cas10. However, the identity of the effector that is activated by cOA in this case remains elusive. The Lon family protease could play this role in the fusion proteins, but the specific mechanism remains uncertain. In the systems

that lack the fusion, the effector could be recruited *in trans*.

Other predicted membrane proteins are conserved in multiple type III CRISPR–Cas loci but contain no identifiable soluble domains that would allow for specific functional prediction. Such proteins can be predicted to tether the CRISPR–Cas machinery in the membrane (Fig. 3c). A membrane association of at least some CRISPR–Cas system is consistent with the previous reports on the activation of the

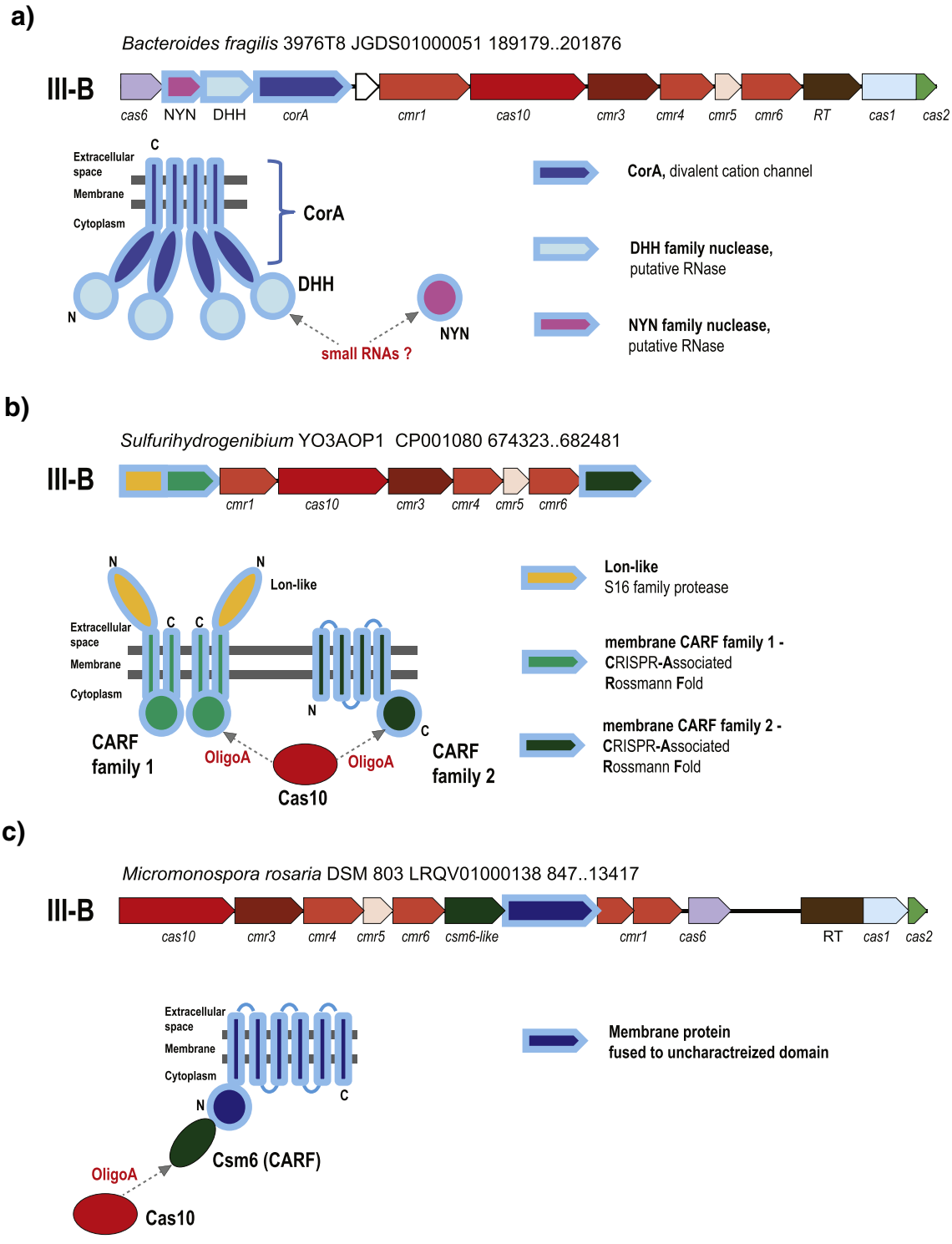

Fig. 3 (*legend on next page*)

E. coli subtype I-E CRISPR–Cas system by envelope stress [142] and on enhancement of bacterial envelope integrity by subtype II-B CRISPR–Cas system of *F. novicida* [86].

Minimalist CRISPR–Cas Systems in Transposons and Plasmids: RNA-Guided Integration?

Apart from their typical presence in archaeal and bacterial chromosomes, CRISPR–Cas systems or the constituents have been increasingly detected in viruses, transposons, and plasmids [143]. The virus-encoded ones are full-capacity CRISPR–Cas variants that appear to function as antidefense devices by targeting the host defense components [144,145]. In contrast, transposons and plasmids harbor minimalist CRISPR–Cas variants that are predicted to be incapable of target cleavage [143] (Fig. 4). The most common of such systems is a minimal version of subtype I-F systems that is encoded by a large family of Tn7 transposons; smaller groups of Tn7-like transposons carry minimal type I-B systems [146]. Phylogenetic analyses of both the Tn7 genes and Cas7, the most highly conserved protein in these minimalist CRISPR–Cas systems, show that the Tn7–CRISPR association evolved through a single event of a subtype I-F locus capture by a transposon and two additional events of acquisition of type I-B systems. The transposon-encoded CRISPR–Cas variants lack both the adaptation module and the Cas3 protein that is required for target cleavage and accordingly cannot be competent neither in adaptation nor in interference. These systems consist of the Cas6 and Cas7 proteins, along with a Cas8–Cas5 fusion in subtype I-F and in subtype I-B. Thus, they encompass all the subunits of the pre-crRNA processing complex and thus are predicted to be capable of generating mature crRNAs and recognizing the target DNA (Fig. 4). The short CRISPR arrays associated with the Tn7-encoded CRISPR–Cas systems contain spacers targeting plasmids and bacteriophages that share hosts with the corresponding transposons. Currently, there are no experimental data on the activity of the transposon-encoded CRISPR–Cas systems. Nevertheless, taken together, the observations outlined above suggest the intriguing possibility that these minimalist systems facilitate insertion of transposons into mobile elements by generating R-loops at the target sites. In evolutionary terms, the transposon-encoded CRISPR–Cas systems clearly are derived forms that evolved from the respective complete systems.

A variety of minimalist CRISPR–Cas systems comprises type IV. Type IV loci are typically found on plasmids and, in some cases, in prophages of diverse bacteria and, similarly to the transposon-encoded type I systems, consist of Cas7, Cas5, Cas8 genes and sometimes Cas6 along with an additional gene, which in different type IV variants encodes either a DinG family DNA helicase or an uncharacterized small protein [3] (Fig. 4). Type IV systems are only rarely associated with CRISPR arrays and accordingly can be predicted to utilize *in trans* arrays present on the same plasmid away from the *cas* genes or on the host chromosome. The functions of this CRISPR–Cas type remain obscure, but given their preferential localization on plasmids, it appears likely that these minimalist variants facilitate maintenance and/or mobility of plasmids via as-yet unknown mechanisms. Many subtype IV-B loci that are typically located on plasmids or predicted prophages [131] (Fig. 4) encode a putative enzyme of the CysH family, which belongs to the adenosine 5′-phosphosulfate (PAPS) reductase family [147]. Some of these loci also encode a predicted enzyme of the ADP-ribosyltransferase family [148] (Fig. 4). Similarly to the subtype I-E systems discussed above as well as “minimal” I-F systems encoded by Tn7-like transposons [146], type IV systems lack nucleases that could cleave the target DNA and therefore can be predicted to perform non-defense functions similarly to transposon-encoded CRISPR–Cas systems [146]. Analogously to the case of the STAND NTPases, the CRISPR-associated CysH homologs comprise a well-supported clade in the phylogenetic tree of the CysH protein family [131] (Fig. 4). As with other predicted CRISPR accessory genes, the CysH-like enzyme and the associated proteins might play a role in a signal transduction

Fig. 3. Examples of membrane proteins identified in CRISPR–Cas loci and proposed signal transduction pathways involving these proteins. (a) CorA, divalent cation membrane channel encoded in type III-B CRISPR–Cas loci along with two distinct nucleases. (b) Membrane-associated CARF domain-containing proteins. (c) Uncharacterized membrane protein family in diverse type III loci. For each locus, species name, genome accession number, and the respective nucleotide coordinates and CRISPR–Cas system subtype are indicated. The genes in a representative locus are shown by block arrows that indicate the transcription direction. The scale of an arrow is roughly proportional to the respective gene length. Homologous genes and domains are color coded. Models of the membrane topology of the predicted CRISPR-linked membrane proteins protein are shown, with N- and C-termini indicated [131]. Hypothetical interactions of the identified CRISPR-linked proteins with CRISPR–Cas system components are also depicted (see text). The *cas* gene names follow the current nomenclature [3]. Abbreviations and other gene names: RT, reverse transcriptase; DHH, DHH family nuclease; NYN, NYN family nuclease; Lon, Lon family protease; CARF, CRISPR-associated Rossmann fold domain.

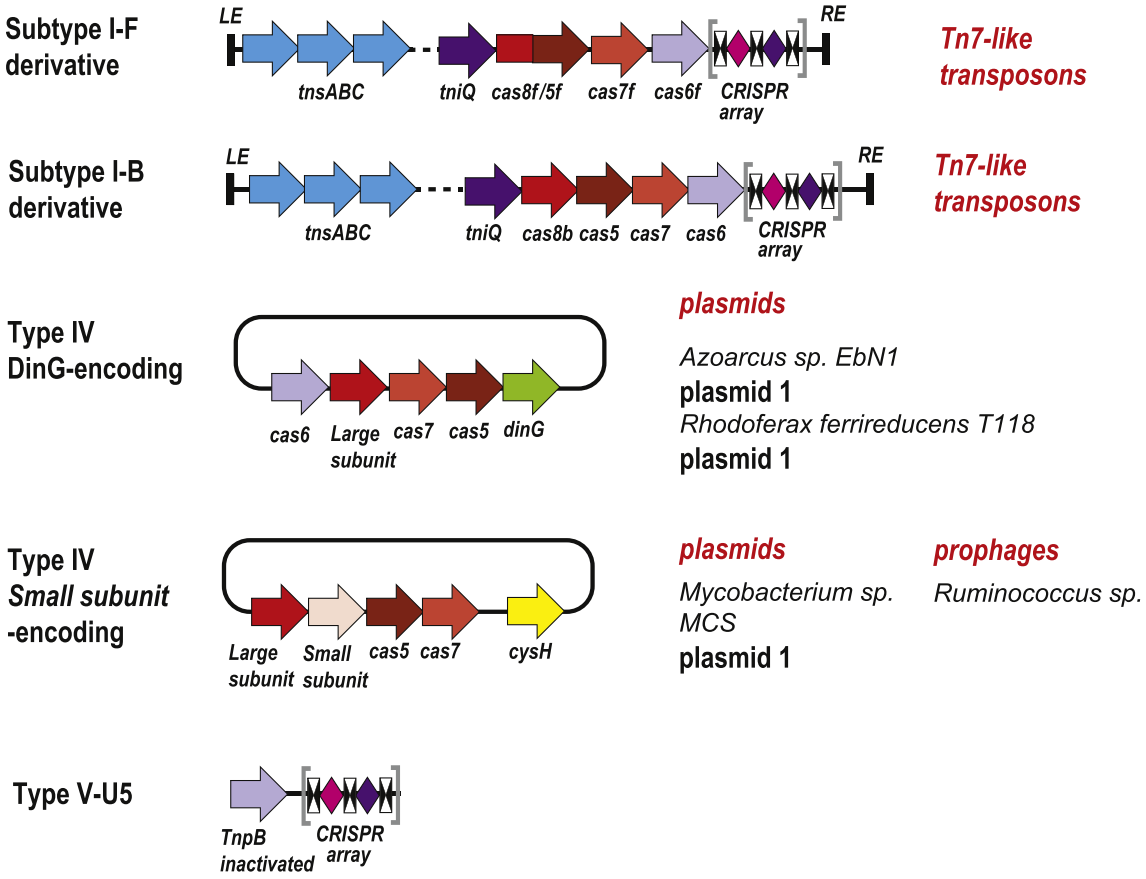

Fig. 4. Minimalist CRISPR–Cas loci in transposons and plasmids. The genes are shown by block arrows (not to scale).

pathway connecting CRISPR–Cas with cellular regulatory networks and perhaps stabilizing the prophages and plasmids in the host bacteria.

A distinct variety of "minimal" CRISPR–Cas systems is subtype V-U, which contains small (predicted) effector proteins closely related to transposon-encoded TnpB and is thought to be a "newborn" CRISPR–Cas variant [60]. Among the five distinct subgroups within subtype V-U varieties, one, V-U5, contains a TnpB homolog that appears to be inactivated as inferred from the replacement of the catalytic amino acid residues in the RuvC-like nuclease domain [60] (Fig. 4). Accordingly, this system can be predicted to perform functions that do not involve target cleavage.

Functional and Evolutionary Implications: The Unknown Biology of CRISPR–Cas

In this article, we discuss a variety of functional connections of the CRISPR–Cas systems, beyond their primary role as an adaptive immunity mechanism. These "non-canonical" facets of CRISPR–Cas include their effect on HGT, apparent links with PCD, dormancy induction, repair and signal transduction pathways, and recruitment of CRISPR–Cas for various functions by MGE. Some of these phenomena involve defense-related functions that, however, substantially differ from the "canonical" adaptive immunity, as in the case of PCD, whereas others have to do with "normal" cellular functions as in the case of gene expression regulation. A notable emerging theme is the apparent reductive evolution of CRISPR–Cas systems, in particular those recruited by transposons, plasmids, and phages, that lose their interference capacity but apparently retain the target recognition mechanism. In stark contrast to the molecular mechanisms of adaptive immunity that have been explored in exquisite molecular details, the direct evidence on the non-canonical functional links is extremely scarce. It seems fair to submit that, of the multiple facets of CRISPR–Cas biology, we have working understanding of only a single major function, even if the "mainstream" one. In particular, the connections between CRISPR–Cas with HGT and PCD that are likely to define the actual role of CRISPR–Cas in the microbial world as well as

the evolutionary dynamics of the CRISPR–Cas loci still remain poorly investigated and supported only by limited, largely, circumstantial evidence. Thus, notwithstanding the explosive rise of CRISPR research during the last decade, the study of these systems in their native environment remains a goal for the future.

In addition to the functional implications, the discovery of signal transduction connections of CRISPR–Cas systems suggests trends in CRISPR–Cas evolution and even might provide clues for the origin of these systems. Reductive evolution, whereby a CRISPR–Cas system loses the interference capacity and is re-purposed for (still poorly understood) functions that do not involve target cleavage, apparently occurred on many independent occasions. Another notable trend is the recruitment of signal transduction proteins, such as, for example, STAND NTPases, and membrane proteins, such as CorA, that are predicted to connect CRISPR–Cas systems to functional networks distinct from adaptive immunity. Such recruitment may or may not be accompanied by loss of the interference capacity.

The discovery of the cOA–Cas10 signaling pathway that is built in the type III CRISPR–Cas systems suggests the possibility that CRISPR–Cas systems originally evolved from a signal transduction mechanism that triggered dormancy or PCD in response to stress [140,149] (Fig. 5). Under this scenario, a polymerase-cyclase homologous to Cas10 and a CARF-HEPN protein homologous to Csm6 comprise the ancestral core of CRISPR–Cas. A putative minimal signaling system consisting of a Cas10 homolog has been identified in several bacterial genomes, although the identity of the effector protein and the signal sensed by this system remain obscure [140]. The origin of CRISPR–Cas from this type of stress-response systems is compatible with the previously proposed model, in which type III systems are considered ancestral and the RAMP superfamily proteins (Cas5, Cas6, and Cas7) are postulated to have evolved from the Palm domain of Cas10, with which they share the core RNA recognition motif fold [150]. Definitive tests for deep evolution scenarios are hard to come up with, but further characterization of the signal transduction functionalities of the CRISPR–Cas systems combined with comparative genomic search for potential evolutionary intermediates might shed light on the origin of microbial adaptive immunity.

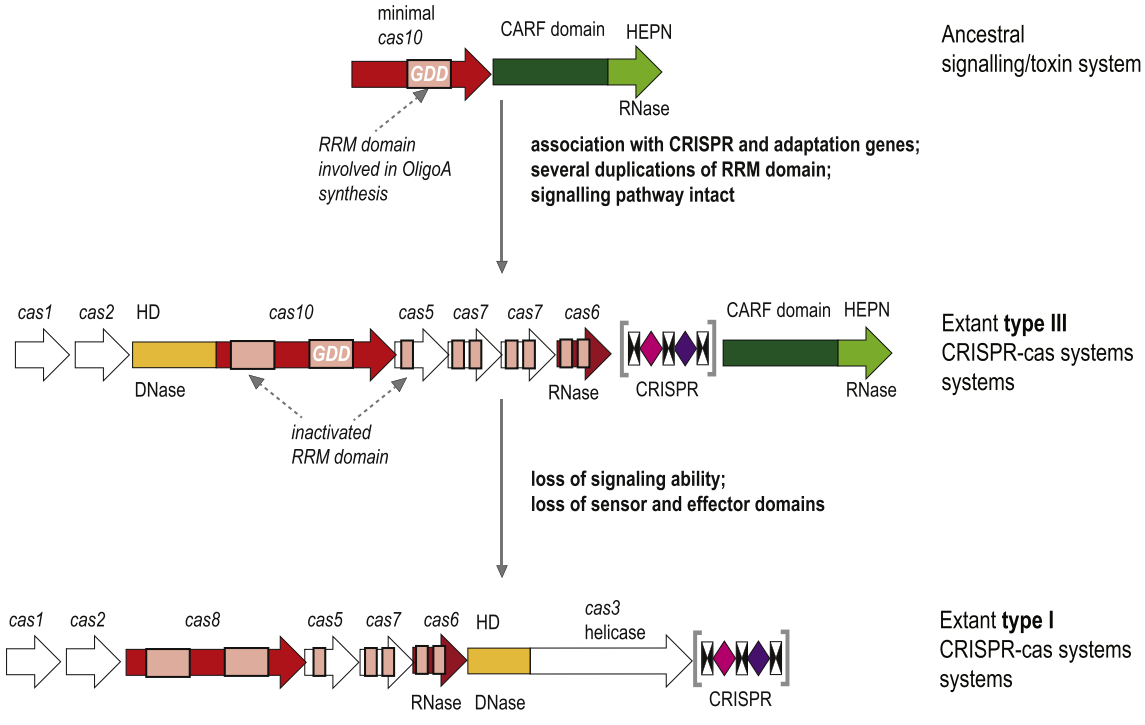

Fig. 5. Proposed scenario of the origin of CRISPR–Cas systems from a stress-induced signal transduction module. RRM, RNA recognition motif, the domain that is shared by numerous enzymes and RNA-binding protein including the Palm domain-containing polymerases and cyclases, such as Cas10, and Cas5, Cas6, and Cas7 proteins that jointly comprise the RAMP (Repeat_Associated Mysterious Protein) superfamily [15,100,150]. The GDD signature that is part of the catalytic center of the Palm domain-containing polymerases and cyclases is shown in the Cas10 box to emphasize the enzymatic activity of this version of the RRM domain.

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programmed cell death;
DNA repair;
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transposable elements

Abbreviations used:
HGT, horizontal gene transfer; MGE, mobile genetic element; DSB, double-strand break; NHEJ, non-homologous end-joining; PCD, programmed cell death; TA, toxin–antitoxin; HEPN, higher eukaryotes and prokaryotes nucleotide-binding; CARF, CRISPR-associated Rossmann fold; PAM, protospacer-adjacent motif.

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