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Review Article

CRISPR-controlled proteases

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With the discovery of CRISPR-controlled proteases, CRISPR–Cas has moved beyond mere nucleic acid targeting into the territory of targeted protein cleavage. Here, we review the understanding of Craspase, the best-studied member of the growing CRISPR RNA-guided protease family. We recollect the original bioinformatic prediction and early experimental characterizations; evaluate some of the mechanistic structural intricacies and emerging biotechnology; discuss open questions and unexplained mysteries; and indicate future directions for the rapidly moving field of the CRISPR proteases.

Introduction

The host–virus arms race serves as obvious facilitator for the emergence of biological complexity, as both the host and invader are challenged to evolve novel traits to outcompete each other for survival. These endless creative impacts resulted in a large repertoire of prokaryotic immune pathways aimed at impeding the propagation of invading viruses [1–3]. Intertwined with the evolution of immune systems, viruses are evolving counter-strategies geared at overcoming the obstacles of immunity [4]. So while immune interactions appear conflictual at the individual level, at a deeper level they exhibit cooperative dynamics in a continuous cycle, geared at evolutionary trajectories towards ever-increasing novelty. Analogously, the two pedals of a bicycle are not competing despite moving in opposite directions, but instead co-operate to facilitate movement of the whole in the same direction. Among the myriad bacterial immune systems — or, perhaps more aptly, facilitator systems — identified to date, CRISPR–Cas adaptive immunity stands out as one of the most extensively studied.

The CRISPR–Cas family became well-known for its anti-viral activity through guide-defined destruction of DNA [5] and RNA [6]. With the discovery of CRISPR RNA-activated proteases, proteins are added to the repertoire of cleavage targets. The best-studied CRISPR protease is Craspase, which upon target RNA activation cleaves a host-encoded protein to instigate a variety of cellular effects. Since the discovery of the predicted genomic loci containing the Craspase module, many studies looking into its biology and biotechnology have been published. Here, we distil the obvious and the obscure from these works to present an overview of Craspase. We describe early milestones (e.g. bioinformatic discovery and experimental description of gRAMP and Craspase), ponder upon research findings (e.g. the biological consequences of Craspase proteolytic activity, the biochemistry of Craspase proteolysis, the big insertion domain (BID), the impacts of biotechnology, the fusion nature of gRAMP, and the course of TPR-CHAT and gRAMP evolution), and evaluate the other members of the expanding CRISPR protease family (CalpL and SAVED-CHAT). We end with a subset of the many open questions that remain to be answered, guiding forthcoming progress in this exciting new CRISPR–Cas field.

Craspase is a CRISPR-guided protease

Bioinformatic prediction of the boundary-blurring type III-E

To keep an overview of the fast-moving CRISPR–Cas field, an updated classification of CRISPR–Cas systems is periodically published. In the edition of 2019 [7], a conspicuous new member aroused some consternation, as it was hard to classify along the conventional rules. Traditionally, new CRISPR–Cas members are grouped based on effector composition: class 1 CRISPR–Cas systems

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contain the multi-subunit effectors (e.g. Cascade in type I and Cmr/Csm in type III), whereas those in class 2 CRISPR–Cas systems comprise a single subunit (e.g. Cas9 in type II and Cas13 in type VI) [8]. The newly predicted system, found in a handful of primarily marine bacterial species, was typified by the effector candidate gRAMP (for 'giant repeat-associated mysterious protein'). Classifying as a class 2 system seemed logical, as gRAMP is encoded by a single gene. However, domain composition analysis of gRAMP showed high similarity to various subunits of the Cmr/Csm effector complexes, which appear to have fused together to constitute gRAMP. This strongly suggested an evolutionary relationship with the type III systems and the gRAMP loci were thus added to class 1 as type III-E CRISPR–Cas [7]. The gene encoding gRAMP contains Cas7-like folds, raising the possibility of RNA targeting akin to the canonical type III effector complexes [9–13], as well as a Cas11-like fold and a large insertion of unknown origin. Furthermore, the co-occurring caspase-like protease TPR-CHAT, a transcription factor and genes of unknown function were omens for the complex pathways that type III-E CRISPR–Cas turned out to encompass.

Experimental characterization of gRAMP and Craspase

In the summer of 2021, the single-unit nature of the gRAMP effector was proven [14,15]. Purified gRAMP — also referred to as Cas7–11 at this point due to the multiple Cas7-like and a Cas11-like domains in its architecture — cleaves target RNA in two guide-defined positions 6 nucleotides apart, reminiscent of the cleavage periodicity in canonical type III effectors. The guide in the gRAMP protein can be re-programmed to cleave an RNA of choice, facilitating usage for RNA knockdown in human cells. Type III-E spacer analysis revealed that some of the crRNAs are complementary to transcripts of mobile genetic element (MGE) genes, suggesting that gRAMP immunity functions through recognizing and cleaving the mRNA of those invaders. But what was the role of the co-localizing predicted protease, TPR-CHAT? Strikingly, co-expression experiments revealed that TPR-CHAT forms a stable complex with gRAMP [14] — technically making the type III-E effector multi-subunit and thus meeting the class 1 classification requirement after all — without disabling the capacity of gRAMP for binding and cleaving target RNA. The CHAT domain is part of the caspase family, containing proteases that are responsible for programmed cell death [16]. This raised the possibility that binding of RNA to the gRAMP-TPR-CHAT complex, termed Craspase (for 'CRISPR-guided caspase'), unleashes the action of the protease, presumably to instigate cell death. Around this time, a report on a homologous bacterial TPR-CHAT showed precise proteolytic activity on the host-encoded gasdermin [17]. This example hinted that Craspase similarly cuts a specific protein encoded by the host, instead of having promiscuous cell-wide activity or activity against a viral protein. However, this hypothesis could not be tested, as target proteins of Craspase were yet to be identified in the vast bacterial proteome. It turned out to be hiding in plain sight.

Roughly a year after the discovery of the Craspase complex formation, a wave of insightful papers came out [18–27]. The hypothesis of RNA-activated, precise protease activity turned out to be correct: incubation of Craspase with an RNA complementary to the guide RNA leads to the destruction of Csx30, a conserved protein of unknown function, usually encoded in the type III-E operon. Detailed structural analysis revealed an α-helix in TPR-CHAT, termed 'switch helix', responsible for relaying steric interaction with the target RNA into conformational changes of the peptidase pocket. This facilitates the digestion of Csx30 into two fragments, a large N-terminal and a small C-terminal fragment, mediated by the cysteine–histidine catalytic dyad in the CHAT domain. Intriguingly, Craspase possesses internal self-control, as cleavage of the target RNA presents the off-switch for the protease. This suggested that the main function of RNase activity in Craspase could be protease regulation rather than stopping the invading MGE through interference with its mRNA, a thought borrowed from the RNA-regulated cyclic oligo adenylate (cOA) production in other type III effectors (reviewed in [6]).

At this point, the full functional pathway of Craspase — complex formation of TPR-CHAT and gRAMP to form Craspase, target RNA binding to activate the protease, Csx30 cleavage, and target RNA cleavage to deactivate the protease — was characterized (Figure 1). But the biological implications of Csx30 cleavage in viral immunity, amongst other unknowns, remained to be elucidated.

The biology, biochemistry, and biotechnology of Craspase

Biological consequences of Csx30 cleavage

In deciphering the consequences of Csx30 cleavage by Craspase, the type III-E operon turned out to hold more answers. It was found that Csx30 assembles into a complex with Csx31 and RpoE [21], two proteins that are often encoded adjacent to Csx30. In transplanted *Escherichia coli* cells, cleavage of Csx30 in the Cx30–Csx31–

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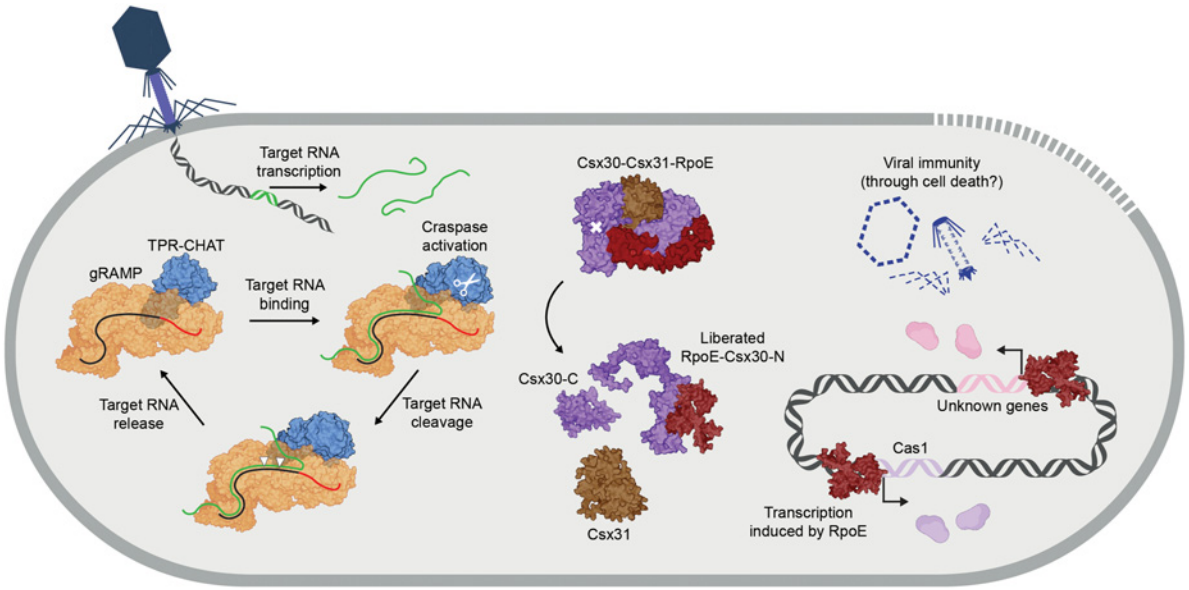

Figure 1. The life-cycle of Craspase.

Once an invading virus has progressed to transcription, Craspase will bind a viral transcript complementary to the loaded CRISPR RNA. Target RNA binding stabilizes the protease pocket of Craspase in the active conformation, allowing recognition and cleavage of Csx30 (cleavage site indicated with white cross) in the Csx30–Csx31–RpoE complex. This leads to the liberation of the N-terminal part of Csx30 bound to the transcription factor RpoE, which presumably binds to various sites in the host chromosome, including the promoter region of Cas1 and several unknown genes. Craspase activity is thought to provide viral immunity through an unknown abortive infection pathway.

RpoE complex induces cellular suicide to protect against invading viruses (Figure 1). This renders Craspase an instrument of altruism, facilitating the sacrifice of the individual for the population, a strategy not uncommon in the bacterial kingdom [6,28]. But how is the cell death phenotype accomplished, and what happens to the Cx30–Csx31–RpoE upon cleavage? The function of Csx31 is currently unknown, and the limited sequence similarity to other proteins makes its role elusive. The involvement of RpoE, annotated as a sigma factor that facilitates transcription-initiation by recruiting the RNA polymerase to promoter sites in DNA [29], is clearer. It was found that Csx30 keeps RpoE — also termed CASP-σ (for 'CRISPR-associated protease sigma factor') — in an inactive state, perhaps through sterically preventing access to the DNA [27]. Cleavage of Csx30 mediated by Craspase — also termed CASP (for 'CRISPR-associated protease') — relieves this inhibition by releasing RpoE–Csx30-N from the complex [20,27], allowing RpoE to interact with a DNA binding motif present in various locations in the native organism to potentially drive expression of the upstream genes (Figure 1). One of these genes encodes for Cas1, a key player in the acquisition of novel spacers [30], suggesting that Craspase regulates CRISPR memory formation during phage infection. Also, a putative membrane protein was found near a RpoE binding site, making membrane pore formation a possible candidate for how the proposed cell death is instigated during type III-E immunity. It is to be noted that cell death has only been observed in trans-planted *E. coli* through an unclear molecular pathway, so whether cell suicide is a true phenomenon in the native context remains to be investigated.

As liberated RpoE levels depend on Craspase activity and thus target RNA concentrations, the type III-E pathway effectively links viral load to gene expression levels (Figure 2A). This scenario is analogous to signal amplification in canonical type III CRISPR–Cas effectors, which produce cOA second messenger molecules proportionally to the intracellular target RNA levels [31]. A more speculative layer of scaling in type III-E involves differential promoter affinity for RpoE, in which strong binding of RpoE to some promoters and weak binding to others leads to high or low protein expression, respectively (Figure 2B). In this model, protein expression from strong promoters occurs readily at low doses of free RpoE, whereas significant expression from weak promoters only takes place when a large quantity of liberated RpoE is present. Genes expressed from weak promoters potentially encode the suicide executor proteins, preventing unnecessary activation during

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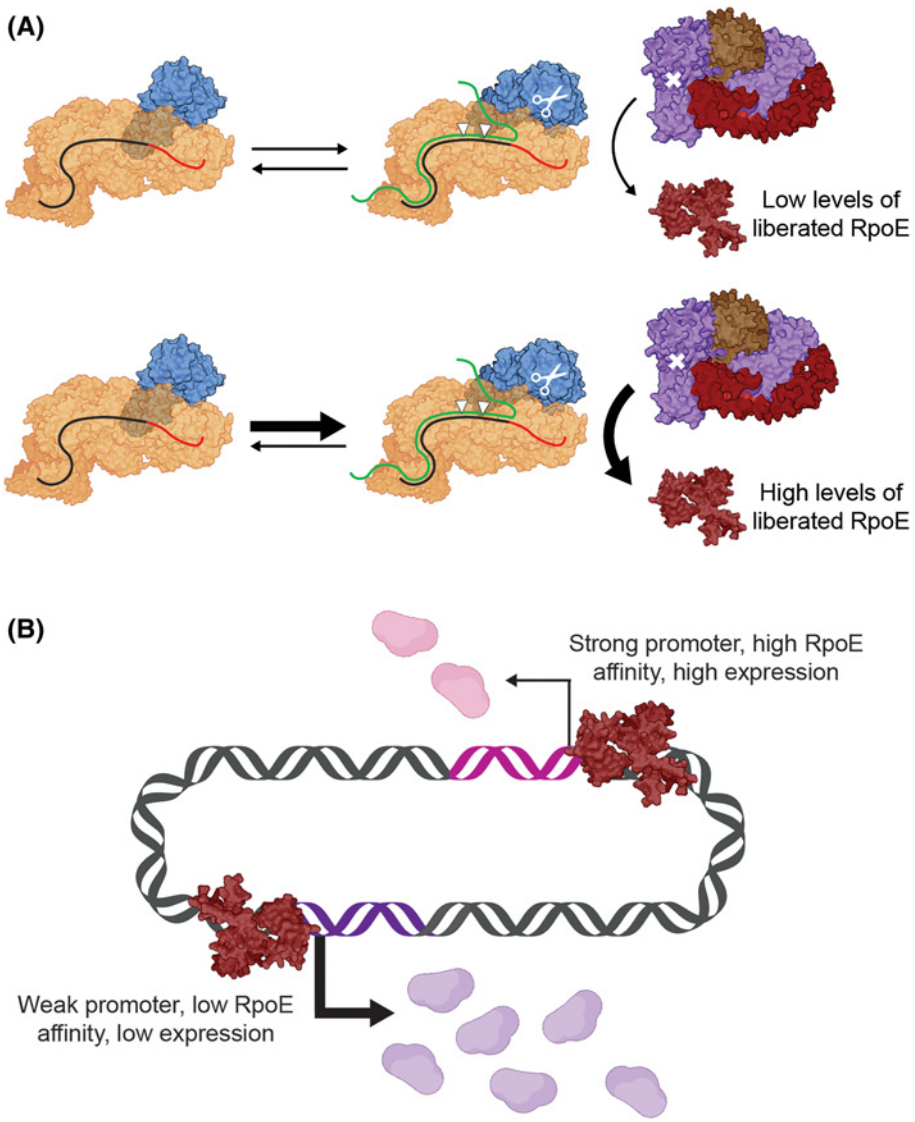

Figure 2. Potential tunability layers in the Craspase pathway.

(**A**) The protease activity of Craspase is activated upon binding a target RNA, resulting in cleavage of Csx30 (purple, cleavage site indicated with white cross) to liberate RpoE, whereupon cleavage of the bound target RNA stops the proteolytic activity and thus halts RpoE liberation. High levels of target RNA lead to quick replenishment after the release of the cleaved RNA fragments from Craspase, effectively increasing the time Craspase spends in the activated state compared with low target RNA levels. This potentially increases the Csx30 cleavage rate and consequently the levels of liberated RpoE. (**B**) Free RpoE binds promoter sites in the host chromosome, driving the expression of various genes. Differential promoter binding strengths allow for variable expression of those genes, adding a layer of intracellular tuning caused by Craspase activity.

mild infection. Proteins with less severe effects, such as Cas1, could be driven by strong promoters for the cell to benefit its enzymatic activity even at low target RNA levels. The effects of altered gene expression patterns by RpoE activity, and the intracellular consequences, are important future research directions.

Craspase proteolytic biochemistry

It was hypothesized that the biochemical cause for Craspase protease activation lies in the decreased distance between the catalytic histidine and the cysteine upon target RNA binding, as structural analysis showed a

reduction from ~7 Å to ~3 Å [18,20]. The reduced distance should allow for deprotonation of the cysteine by the histidine, followed by nucleophilic attack on the substrate and rupture of the scissile bond (Figure 3A). Although this explanatory model is widespread in the literature on cysteine proteases [32], also an alternative biochemical scenario has been proposed [33]. As fitting of the target protein substrate into the active pocket of caspase requires the cysteine and histidine residues to be separated beyond the hydrogen bonding distance, deprotonation of the cysteine by the histidine was considered unlikely. Instead, molecular density functional calculations suggested that deprotonation of the cysteine directly by the substrate is the most plausible atomistic description (Figure 3B). This mode of proteolytic biochemistry was determined based on human legumain and caspases, so it remains to be investigated whether it is true for Craspase. It is noteworthy that the distance between the catalytic histidine and cysteine in inactive TPR-CHAT is similar to the distance between these residues in inactive human separase [20], hinting at a potential convergence in activation principles. Solving cryogenic electron microscopy structures of Craspase in interaction with the target protein Csx30 will likely decipher the atomic details at the protease interface, revealing the extent to which Craspase proteolysis works akin to human caspases.

The big insertion domain in gRAMP

The most conspicuous structural divergence in gRAMP compared with ancestral multi-subunit effector domains took place in the C-terminal Cas7-like domain (Cas7.4), which gained a BID protruding from its architecture. The structure of BID could not be easily resolved [18,20,34], suggesting that it is flexibly probing the exterior surrounding gRAMP. The floppy and protruding characteristics make it plausible that the BID acts as a sponge to localize nucleic acids close to the effector for up-regulation of target recognition (Figure 4), akin to the strategy suggested for WYL1 in the type VI CRISPR–Cas effector Cas13 [35,36]. Furthermore, the likely close ancestor of gRAMP, the type III-Dv complex, also contains an insertion in its Cas7 domain and was shown to interact with the 3′ end of the crRNA, where the seed region for target RNA binding was hypothesized to reside [37,38]. Similarly, the BID in gRAMP is architecturally positioned at the 3′ end of the crRNA where the initial target recognition may take place, suggesting that sponge-like localization of nucleic acids at this region could immediately facilitate target RNA binding. Although a seed region for target RNA binding could be residing in the crRNA portion bound by the BID of gRAMP, the fact that target RNAs lacking complementarity to this crRNA region are being cleaved efficiently [18] shows that this is not an absolute requirement.

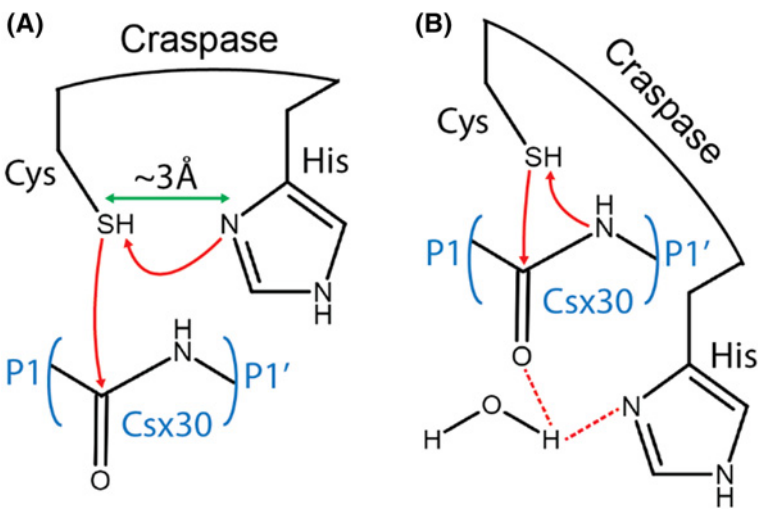

Figure 3. Two models for the proteolytic biochemistry of Craspase.

(**A**) Close distance between the cysteine and histidine of the catalytic dyad in Craspase allows for deprotonation of the cysteine by the histidine, facilitating a nucleophilic attack at the scissile bond between P1 and P1′ amino acid positions of the target protein Csx30. This model is adopted from the classical biochemistry described for cysteine proteases. (**B**) Alternatively, similar to human legumain and caspases, deprotonation of the cysteine could occur directly by the substrate, followed by the nucleophilic attack.

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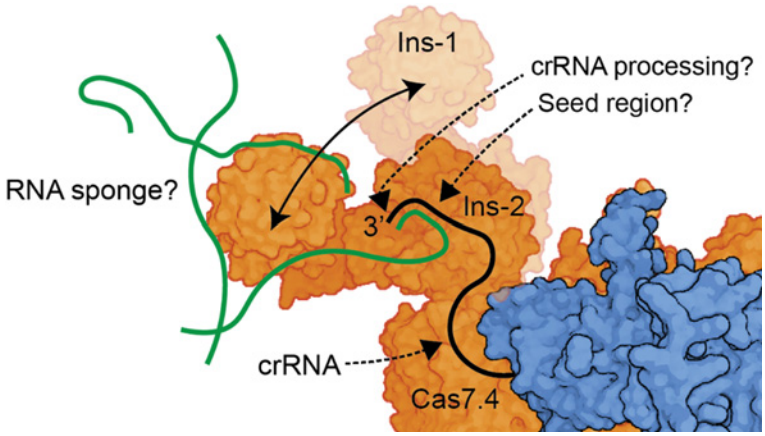

Figure 4. The big insertion domain in gRAMP.

The big insertion domain (BID), dissected into insertion-1 (ins-1) and insertion-2 (ins-2) is protruding from Cas7.4 and flexibly probing the environment, perhaps to act as RNA sponge to localize potential target RNAs close to the suspected seed region. The BID in gRAMP from *Candidatus* 'Scalindua brodae' has been suggested to also play a role in crRNA processing. TPR-CHAT is visualized in blue and gRAMP is visualized in orange.

An analysis of Craspase from *Desulfonema magnum* dissected the BID in two parts, termed 'insertion-1' and 'insertion-2' [23]. Insertion-1 has direct interactions with the crRNA, whereas insertion-2 is protruding from the protein without crRNA contact. One could speculate that insertion-1 of the BID is involved in crRNA recognition, serving as an anchor for ribonucleoprotein formation. Insertion-2 might possess the proposed sponge functionality, equipping the BID with a double role in both crRNA recognition and RNA localization. A possible additional BID function is slowing down target RNA turnover, as it was observed that target RNA with complementarity extending into the crRNA portion bound by BID are cleaved less efficient compared with shorter ones [18]. Perhaps the BID physically prevents target RNA displacement, slowing down target RNA binding kinetics and thus reducing cleavage turnover.

In Craspase from *Desulfonema ishimotonii*, the BID is dispensable without detectable loss in RNA targeting capacity [15], indicating that it has adopted a role beyond RNase functionality on target RNA. However, removal of the BID in Craspase from *Candidatus* 'Scalindua brodae' distinctly reduces cleavage activity, which may be due to involvement in pre-crRNA processing [21]. Functional differences between orthologues Craspases, such as the role of the BID, accommodation of pre-crRNA processing, and regulatory mechanisms of TPR-CHAT (see section On the origin of gRAMP), perhaps reflect a need for subcategorization within the type III-E CRISPR–Cas effectors.

Craspase in biotechnology

It was quickly realized that the precise proteolytic action, strict RNA-dependent activation requirements, and self-regulatory capacity make Craspase a strong candidate for biotechnological applications. Craspase self-deactivation is prevented by mutating the RNase pockets in gRAMP, yielding a variant whose protease stays active (i.e. Stay-On Craspase) after target RNA binding [18]. This could provide distinct benefits for the usage of Craspase where RNA recognition and protease activity are desired over RNA cleavage, such as in molecular diagnostics. RNA detection tools using Craspase were achieved in multiple ways, all exploiting the principle of coupling RNA recognition and subsequent protease activity to a fluorescence readout. One application involves a protein element recognized by cells for targeted protein degradation, called a degron, linked to the fluorescent protein via Csx30 [26]. The fluorescent protein remains intact and thus fluorescent only when freed from the degron, which is achieved through Csx30 processing by activated Craspase. In another application, the RpoE promoter sequence was used to drive the expression of a fluorescent protein only when Craspase was activated to cleave Csx30 for release of RpoE [27]. A third application involves a recombinase capable of activating an engineered promoter, which was cloned to drive the expression of a fluorescent protein [27]. Tethering of the recombinase to the eukaryotic membrane through a part of Csx30 allowed nuclear localization and subsequent fluorescent protein expression only after Csx30 cleavage by Craspase.

Nucleic acid manipulation and diagnostics using Craspase add to the existing RNA targeting CRISPR–Cas toolbox (reviewed in [6]), but the unique biotechnological promise lays in its potential for precise protein editing. Currently, available proteases are either promiscuous, uncontrollable, or both, limitations that Craspase overcomes. Craspase is currently specific for the native target protein Csx30 or derivatives thereof, but in the future, Craspase may be engineered to cleave a protein of interest. This opens up a range of new biotechnological avenues, such as cancer therapeutics. Here, Craspase may be evolved to cleave a vital protein in human cells to selectively kill tumour cells, discriminating cancerous from normal cells through precise recognition of oncogenic transcripts. Current targeted therapeutics are based on the inhibition of oncogenic proteins [39], requiring laborious development of novel molecules for each oncogenic variant. Recognition of RNA by Craspase instead provides the benefit of easy reprogramming upon cancer mutagenesis, only requiring loading of Craspase with a guide cognate to the new oncogenic transcript.

Evolutionary perspectives on Craspase

On the origin of gRAMP

The clear evolutionary relation between the domains in gRAMP and multi-subunit type III CRISPR–Cas effector complexes strongly suggests that one evolved from the other [7]. The relative abundance of multi-subunit effectors compared with relatively rare gRAMP points at an evolutionary trajectory from multi-subunit to gRAMP as the most parsimonious reconstruction. Likely representing an intermediate step on the evolutionary march from completely multi-subunit to fully fused type III systems is the type III-Dv effector, which is composed of a mix of free subunits (Cas10, Csx19, and Cas7 with an insertion) and fused subunits (Cas7–Cas7 and Cas7–Cas5–Cas11) [37].

What evolutionary benefits could have attributed to the emergence of subunit fusions in type III systems? One rationale is that the use of multi-subunit effectors comes with the risk of producing more subunits than required for the desired stoichiometry of the complex [40]. Fusing the subunits together into a single gene mitigates the unnecessary energy expended during subunit overproduction. Moreover, multi-subunit effectors are restrained in the functional landscape they can explore, as structural morphing of the subunits required for functional change could hinder the proper assembly and function of the complex. Multi-subunit type III effectors are usually assembled from multiple Cas7 proteins that are encoded by the same gene and are thus phenotypically identical. Fusion of subunits frees the effector from the restriction of subunit assembly to allow for structural differentiation of the domains, as has become apparent from the idiosyncratic shapes each of the Cas7-like domains in the gRAMP display [18] (Figure 5). Such structural diversification allows for change of function, which could be evolutionary advantageous. An example of this may be found in the number of RNA cleavages that are made by the type III effector complexes. Generally, each Cas7 subunit in canonical type III effectors cleaves the target RNA once, constraining the effector to make as much RNA cleavages as there are Cas7 proteins in the complex [6]. In gRAMP, two of the four Cas7-like domains apparently lost their RNA endonuclease activity towards target RNA. As target RNA cleavage serves as the off-switch for protease activity in Craspase, this reduction in number of cleavage sites may have fine-tuned the controllability of the protease. Interestingly, there are also multi-subunit effector complexes in which some Cas7 subunits do not cleave the target RNA [41]. It was suggested that the missed cleavage sites are not due to enzymatic incompetency, but rather due to a non-cleavable structural architecture of the crRNA:target RNA duplex at the Cas7 interface.

Another example of how the gained degrees of freedom through subunit fusion resulted in expanded functionality is the observed flexibility of the Cas11-like domain in gRAMP from *D. magnum* [23]. Docking of TPR-CHAT onto gRAMP during the formation of Craspase promotes the rigid conformation of Cas11, mediated by a flexible motif in gRAMP termed 'insertion finger'. Stabilized Cas11 results in reduced binding and cleavage of target RNA in Craspase compared with the stand-alone gRAMP, suggesting a role for TPR-CHAT in regulating RNase activity. In Craspase from both *Candidatus* 'S. brodae' [18] as well as *D. ishimotonii* [15], a similar reduction in RNA accessibility between gRAMP and Craspase was observed. It was hypothesized that the interaction with TPR-CHAT stabilizes the 'gating loop', a long linker from Cas11 to Cas7.2 that forces target binding to initiate at the 3′ end of the crRNA [18]. Since the gating loop is displaced by incoming target RNA, the stabilized gating loop in Craspase effectively increases the conformational energy barrier for target RNA binding, thus lowering RNA accessibility. So it seems that TPR-CHAT regulates gRAMP in multiple ways, induced by physical interaction with the insertion finger and gating loop (Figure 6). It makes for interesting speculation whether gRAMP has a cellular role as a stand-alone protein, one that requires

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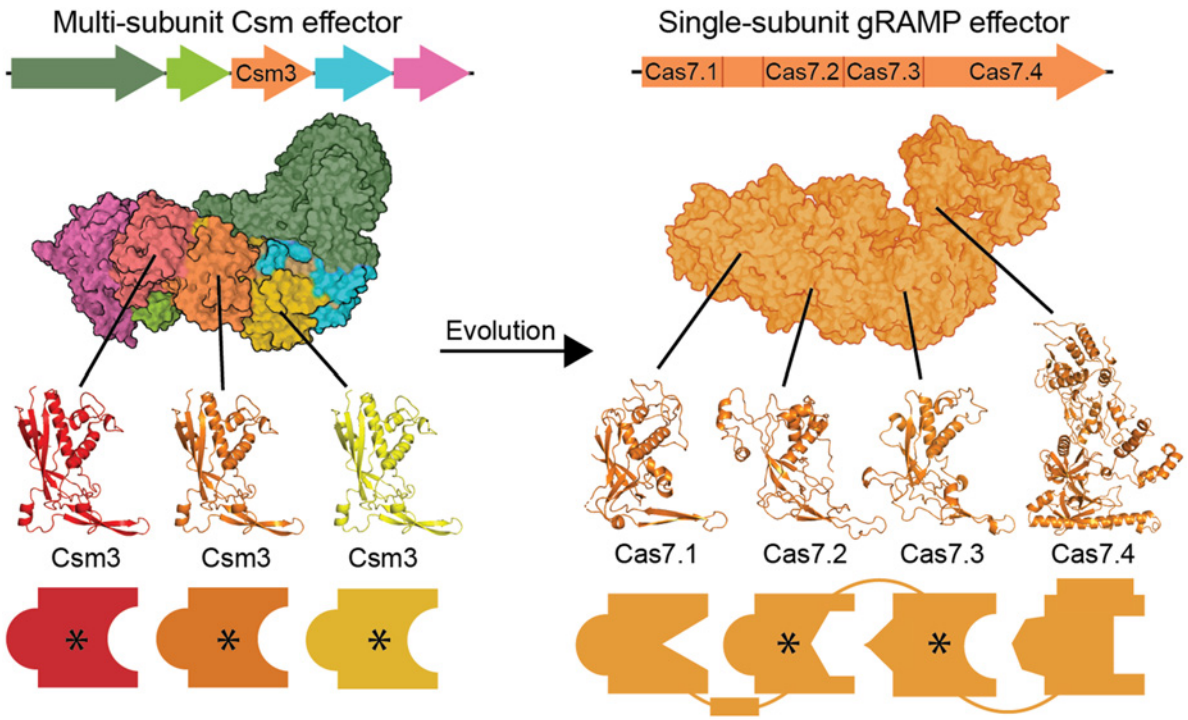

Figure 5. Evolution of gRAMP from the multi-subunit type III effector.

The operon for the multi-subunit Csm effector (PDB 6IFN) generally harbours one gene encoding for the RNase Csm3 (Cas7) subunit, which provides multiple copies in the effector complex upon assembly with the other subunits. Structural (indicated with a puzzle piece) or enzymatic (indicated with an asterisk) changes to Csm3 would affect all protein copies in the complex, potentially hampering complex assembly and functional divergence, respectively. This restrains the possible evolutionary explorations that multi-subunit complexes can undertake. In contrast, the Cas7-like domains are fused together in gRAMP, alleviating the need for correct translation levels of the subunits, subunit assembly, and enzymatic homogeneity among the domains. Correspondingly, the Cas7-like domains evolved to take on vastly differing morphologies, most notable Cas7.4 with the big insertion domain. Moreover, contrary to most multi-subunit effectors, not all Cas7-like domains in gRAMP contain an RNase pocket that is active on target RNA.

increased activity against target RNA compared with when gRAMP interacts with TPR-CHAT. Curiously, the insertion finger is not present in *Candidatus* 'S. brodae', suggesting that the reduced RNA binding and cleavage activity in its Craspase can be ascribed mainly to the rigidity of the gating loop.

The evolutionary relationship between TPR-CHAT and eukaryotic separase

The CHAT domain in TPR-CHAT exhibits a significant degree of structural similarity to the eukaryotic separases, and both CHAT and separase contain the highly conserved histidine–cysteine catalytic dyad [20,23]. This suggests a close evolutionary relationship between CHAT and separase. An elegant hypothesis for linking CHAT to the evolution of separase can be traced back to the formation of the early eukaryote. Eukaryogenesis is widely believed to originate from the endosymbiotic fusion of an archaeal and bacterial cell, with the latter eventually evolving into the mitochondrion [42,43]. During this process, the bacterial endosymbiont might have encoded CHAT-like domains, perhaps to kill the host cell once it became unhospitable [16,44]. Through the process of gene migration from the DNA of the endosymbiont to the developing eukaryotic chromosome, the early eukaryote may have adopted these proteins for other purposes [45]. In this scenario, the acquisition of CHAT was followed by functional repurposing for chromosome-related processes, as separases now serve an essential role in eukaryotic chromosome segregation [46]. In the bacterial evolutionary lines, CHAT evolved to have functions in various pathways, such as pyroptosis [17], cell division [47] and, as seems the case for Craspase, viral immunity.

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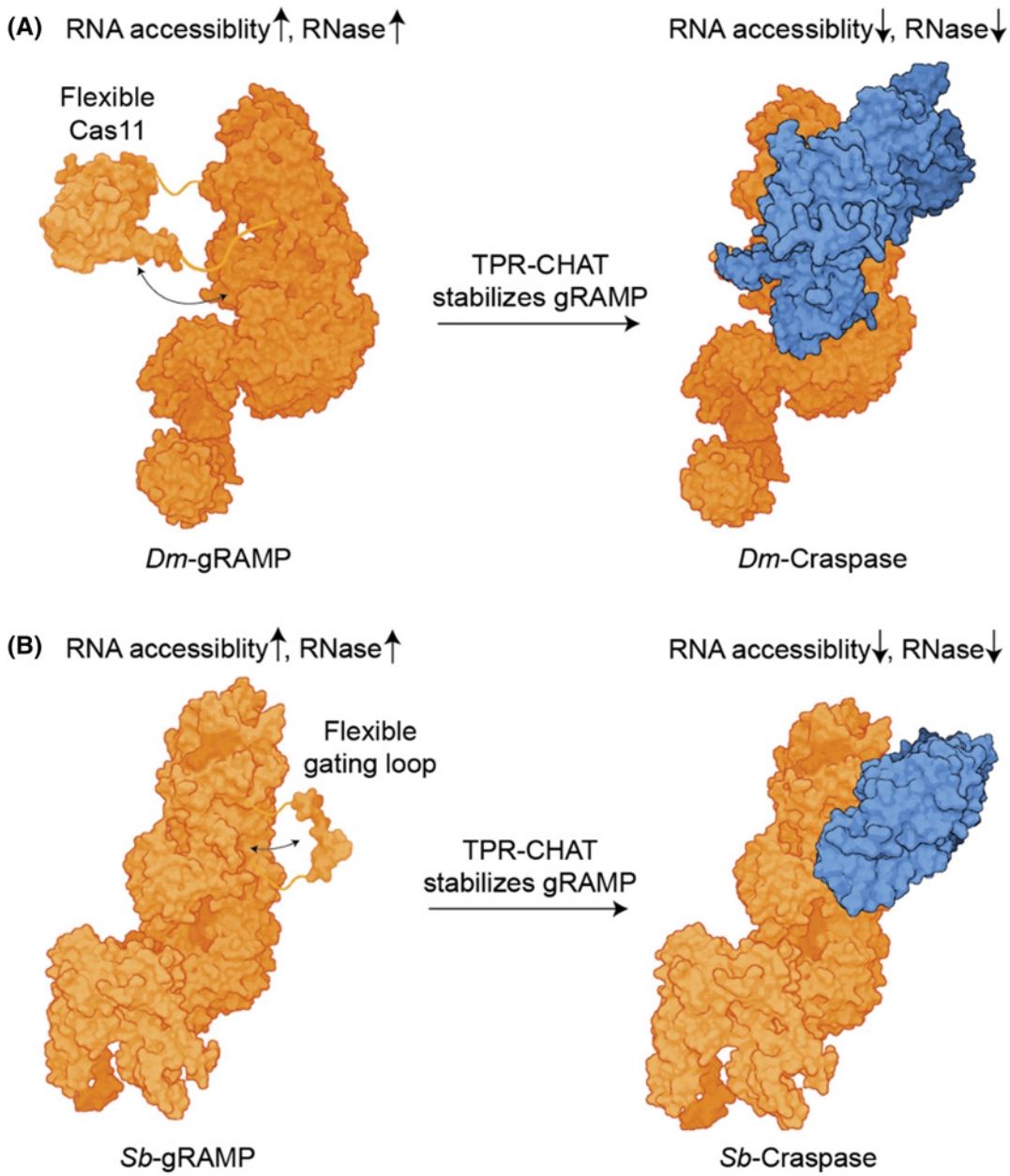

Figure 6. gRAMP regulation by TPR-CHAT.

The flexible Cas11-like domain in gRAMP (orange) from *Desulfonema magnum* (*Dm*-gRAMP) (**A**) and a flexible long linker, named gating loop, in gRAMP from *Candidatus* 'Scalindua brodae' (*Sb*-gRAMP) (**B**) are stabilized through interaction with the respective TPR-CHAT (blue). This increase in conformational rigidity reduces target RNA accessibility and cleavage in Craspase compared with gRAMP.

The emerging theme of CRISPR-associated proteases

With the discovery of Craspase, CRISPR–Cas effectors have moved beyond the era of nucleic acids targeting into the domain of protein targeting to instigate anti-viral cascades. Anti-viral defence through the deployment of proteases has been described before, such as the protease-mediated cleavage of antitoxin molecules in toxin–antitoxin systems to initiate abortive infection [48]. However, the integration of proteases with CRISPR guidance represents a remarkable advancement: the ability to accurately trigger protease through recognition of a

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specific nucleic acid sequence. Next to Craspase, the CRISPR protease family contains two other experimentally described members (Table 1): CalpL (for 'CRISPR-associated Lon') and SAVED-CHAT.

A main difference with Craspase is that CalpL and SAVED-CHAT are not physically associated with the type III effector but instead are triggered by cOA second messenger signalling [49,50]. CalpL forms a tripartite complex with CalpT and CalpS and, upon binding of cA_4 molecules, cleaves CalpT to liberate CalpS. CalpS is a predicted sigma factor and was found to physically interact with the RNA polymerase, leading to the hypothesis that its transcriptional functioning is unleashed upon release from the complex. SAVED-CHAT, upon binding of cA_3 molecules to the SAVED domain, forms long filaments that lead to protease activation of the CHAT domain. Activated SAVED-CHAT in turn cleaves and activates Prokaryotic Caspase (PCaspase), a protease with specificity towards a range of proteins. One of the target proteins of PCaspase is a putative sigma factor, suggestive of involvement in transcriptional regulation.

The prospective transcriptional pathways in both CalpL and SAVED-CHAT provide a convergent evolutionary scenario to the induced transcriptional response in the Craspase system, thus revealing a common theme among CRISPR-associated proteases: the ability to couple nucleic acid detection with transcriptional regulation. Future exploration will unveil the extent of this phenomenon and the potential involvement of CRISPR-associated proteases in different molecular pathways.

Conclusions and outlook

Craspase research came a long way from its bioinformatic birth to insights into its physiological role and biotechnological potential. Ongoing efforts will undoubtedly address open questions regarding Craspase biology, biochemistry, and biotechnology in the near future. For example, what are the downstream effects of the genes transcribed by liberated RpoE? What is the role of Csx31? How are spacers acquired in type III-E systems? What are the structural and sequential code details of the interaction between Craspase and Csx30–Csx31–RpoE? How are target RNA binding and cleavage temporally related to protease activity in Craspase? What are the limits and off-targets of RNA and protein knockdown in cells? Are there CRISPR-controlled proteases that are activated by DNA, or can they be created by directed evolution? Do anti-CRISPRs exist for Craspase? Do gRAMP or Craspase variants exist with characteristics different from the currently described ones? Similar questions can be asked for the other CRISPR-controlled proteases, ensuring interesting proteolytic times ahead.

Table 1. Key characteristics of the CRISPR-associated protease family members

CRISPR-associated protease	Craspase	CalpL	SAVED-CHAT
CRISPR–Cas subtype	III-E	III-B	III-B
CRISPR–Cas effector	gRAMP	Cmr	Cmr
Protease	TPR-CHAT	SAVED-Lon	SAVED-CHAT
Nucleic acid trigger for activation	RNA	RNA	RNA
Communication between CRISPR–Cas effector and protease	Physical association	Second messengers (cA_4)	Second messengers (cA_3)
Oligomerization of the protease before activation	No	Yes	Yes
Protease catalytic dyad	Cys-His dyad	Ser-Lys dyad	Cys-His dyad
Target protein	Csx30	CalpT	PCaspase (which subsequently cleaves a range of proteins)
Anti-viral effect	Abortive infection	Unknown	Abortive infection
Effect of protease activity on associated transcription factor	Liberation of RpoE/CASP-σ (predicted sigma factor)	Liberation of CalpS (predicted sigma factor)	Cleavage of PCc-σ (predicted sigma factor)

Perspectives

- The specific and targeted cleavage of proteins by the CRISPR-controlled proteases is an emerging theme in CRISPR–Cas. The CRISPR-controlled proteases are involved in novel and complex anti-viral pathways, and are promising candidates for biotechnological applications involving molecular diagnostics and targeted protein cleavage.

- The best-studied member of the CRISPR-controlled protease family is Craspase. Upon target RNA binding, the protease activity of Craspase is activated to cleave host-encoded Csx30. This results in the liberation of RpoE to facilitate a transcription regulatory response and seems to initiate a dormancy phenotype. Target RNA cleavage by Craspase shuts off the protease activity, equipping the complex with self-regulatory capacity.

- The transcriptional changes and their downstream effects evoked by Craspase activity, the details of the induced abortive infection pathway, and the extent of Craspase usability for biotechnology are immediate questions to be addressed. Furthermore, the continuous deciphering of the other CRISPR-controlled proteases such as CalpL, SAVED-CHAT, and potentially undiscovered ones, will undoubtedly yield interesting insights in the near future.

Competing Interests

Sam P. B. van Beljouw and Stan J. J. Brouns are inventors on patent applications submitted by Delft University of Technology that covers uses of gRAMP and Craspase.

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Abbreviations

BID, big insertion domain; cOA, cyclic oligo adenylate; MGE, mobile genetic element.

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