The RNA- and DNA-targeting CRISPR–Cas immune systems of *Pyrococcus furiosus*

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Abstract

Using the hyperthermophile *Pyrococcus furiosus*, we have delineated several key steps in CRISPR (clustered regularly interspaced short palindromic repeats)–Cas (CRISPR-associated) invader defence pathways. *P. furiosus* has seven transcriptionally active CRISPR loci that together encode a total of 200 crRNAs (CRISPR RNAs). The 27 Cas proteins in this organism represent three distinct pathways and are primarily encoded in two large gene clusters. The Cas6 protein dices CRISPR locus transcripts to generate individual invader-targeting crRNAs. The mature crRNAs include a signature sequence element (the 5′ tag) derived from the CRISPR locus repeat sequence that is important for function. crRNAs are tailored into distinct species and integrated into three distinct crRNA–Cas protein complexes that are all candidate effector complexes. The complex formed by the Cmr [Cas module RAMP (repeat-associated mysterious proteins)] (subtype III-B) proteins cleaves complementary target RNAs and can be programmed to cleave novel target RNAs in a prokaryotic RNAi-like manner. Evidence suggests that the other two CRISPR–Cas systems in *P. furiosus*, Csa (Cas subtype Apern) (subtype I-A) and Cst (Cas subtype Tneap) (subtype I-B), target invaders at the DNA level. Studies of the CRISPR–Cas systems from *P. furiosus* are yielding fundamental knowledge of mechanisms of crRNA biogenesis and silencing for three of the diverse CRISPR–Cas pathways, and reveal that organisms such as *P. furiosus* possess an arsenal of multiple RNA-guided mechanisms to resist diverse invaders. Our knowledge of the fascinating CRISPR–Cas pathways is leading in turn to our ability to co-opt these systems for exciting new biomedical and biotechnological applications.

Keywords

clustered regularly interspaced short palindromic repeats (CRISPR); CRISPR-associated (Cas); non-coding RNA; prokaryotic immunity; *Pyrococcus furiosus*; virus

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Small RNA-guided prokaryotic immunity

To defend themselves from potentially hostile viruses and other invading mobile genetic elements, most archaea and many bacteria rely on small RNA and protein-based CRISPR (clustered regularly interspaced short palindromic repeats)–Cas (CRISPR-associated) adaptive immune systems [1–5]. CRISPR loci consist of tandem arrays of identical repeat sequences interspersed with variable 'spacer' sequences acquired from the invaders [6–9]. CRISPR loci are transcribed, and the primary CRISPR RNA transcripts undergo specific processing to generate functionally active mature crRNAs (CRISPR RNAs) [10–13]. Thus CRISPR loci capture virus and plasmid DNA sequences and produce RNAs containing the captured sequences that identify and eliminate the corresponding invaders. The protein components of the immune systems are encoded by specific modules of *cas* genes, which are typically found near the CRISPR locus [14,15]. Together, the crRNAs and Cas proteins provide RNA-directed immunity against diverse genomic invaders in a wide range of prokaryotic organisms.

Multiple distinct CRISPR–Cas systems have been identified, which differ with regard to *cas* gene content, CRISPR repeat sequences and mechanistic processes. Ten subtypes of CRISPR–Cas systems were initially identified on the basis of modules of specific *cas* genes [14,16] and named after an organism that contains only this subtype-specific *cas* gene cluster (e.g. Cse, Cas-subtype *Escherichia coli*) [14]. The CRISPR–Cas systems were recently refined and renamed to recognize categorization into three major types (I–III) (i.e. subtypes I-A to I-F, II-A and II-B, and III-A and III-B, wherein, for example, subtype I-E corresponds to the Cse system) [17]. The distribution of the diverse CRISPR–Cas modules between prokaryotic genomes is highly variable as a result of apparent lateral gene transfer [17,18].Many bacteria and a few archaea lack CRISPR–Cas systems completely. Moreover, some organisms contain a single CRISPR–Cas system, whereas other organisms, including *Pyrococcus furiosus*, harbour multiple distinct CRISPR–Cas systems.

The CRISPR–Cas systems of *P. furiosus*

Pyrococcus furiosus (roughly translated as 'mad fireball') is a hyperthermophilic euryarchaeaon of the order Thermococcales. *P. furiosus* was initially isolated from thermal marine sediments off the coast of Vulcano island, Italy, and exhibits a remarkable optimal growth temperature of ~100°C [19]. Sequenced genomes from six distinct pyrococcal species are available and reveal interesting species-specific diversity in CRISPR–Cas genes and gene organization [20]. We chose *P. furiosus* as a model system to unlock the mysteries of CRISPR–Cas immune systems for several compelling reasons. The inherent thermal stability of the proteins and complexes greatly facilitates detailed mechanistic and structural studies with both recombinant and native complexes. The newly established genetic manipulability of *P. furiosus* [21–23] has further increased the power of *P. furiosus* as a model system and expanded the types of analysis that can be performed. Moreover, as detailed next, *P. furiosus* offers the opportunity to investigate three distinct CRISPR–Cas defence pathways.

The genome of *P. furiosus* strains DSM 3638 and COM1 include seven separate CRISPR loci (distributed throughout the genome) that together encode a total of 200 potential crRNAs (Figures 1A and 1B). All seven CRISPR loci share a common 30 bp repeat sequence interspaced by variable numbers of unique spacers (Figure 1B). The majority of spacer sequences are 37 bp in length, but range in size from 34 to 59 bp. The origins of the spacers are unknown as none map to the few currently known Thermococcales viruses or plasmids [24–27]. The *P. furiosus* repeat RNA is unstructured in solution [28], consistent with the classification of the *P. furiosus* repeat sequence in the 'unfolded archaeal cluster 6' family [29]. Adjacent to each CRISPR array is a ~500 bp leader region; these are highly conserved among the seven CRISPR loci and contain transcriptional start sites [30] and probably also regulatory elements needed for novel spacer acquisition.

The genes encoding Cas proteins are primarily organized in two large gene clusters in *P. furiosus* (Figures 1A and 1C). The first *cas* gene cluster encodes the core Cas proteins (grey), the Cmr [Cas module RAMP (repeat-associated mysterious proteins)] proteins (blue) and the Cst (Cas subtype Tneap) proteins (yellow). Csa (Cas subtype Apern) proteins (green) are encoded in the second *cas* gene cluster. The core *cas* genes are found in many organisms with different CRISPR– Cas systems and include proteins implicated in new spacer acquisition (Cas1, Cas2 and Cas4 proteins) as well as crRNA biogenesis (Cas6 protein). Together, the *P. furiosus* genome encodes proteins of three predicted effector immune complexes: Cmr (subtype III-B), Csa (subtype I-A) and Cst (subtype I-B) [14,15]. Additional copies of genes encoding predicted Cmr1, Cas6, Cas4 and three other possible Cas proteins are unlinked and scattered elsewhere in the genome [13] for a total of 27 known or predicted Cas proteins in this organism.

Expression and processing of crRNAs

By a combination of deep RNA sequencing and Northern blot analyses, we have determined that all seven *P. furiosus* CRISPR loci are transcribed and give rise to processed mature crRNA species [13,31]. We have mapped the transcription start sites and promoter elements within the CRISPR leader regions [30]. All crRNAs and Cas proteins appear to be expressed in the absence of infection [13,28,30,31], consistent with an immune system operating in 'surveillance mode'. We also found that crRNAs containing the most recently acquired invader sequences (encoded proximal to the leader) are generally more abundant [13,30,31].

Our studies revealed that *P. furiosus* Cas6 is responsible for primary processing of precursor CRISPR RNA transcripts and plays a critical role in producing individual mature crRNAs [11,28,32] (Figure 2A). We found that Cas6 is a riboendonuclease that makes a single cut within each repeat sequence of the CRISPR transcript, reducing it step-by-step to intermediate RNAs that we call 1× RNAs (Figures 1B and 2A). The 1× RNA intermediates each contain an 8-nt segment of the repeat at the 5′ end (5′-AUUGAAAG-3′) that is retained in the mature crRNAs (the 5′ tag), an invader-targeting or guide sequence, and 22 nt of repeat sequence at the 3′ end (Figure 2A). Cleavage by *P. furiosus* Cas6 produces RNAs with 5′ hydroxy and 2′–3′ cyclic phosphate chemical end groups, consistent with its divalent metal-independent cleavage activity [11,28]. Further 3′ end trimming of 1× crRNA intermediates (by unknown mechanisms) produces the various mature crRNA species

(Figures 2A and 2B). Cas6 and Cas6-like enzymes also play a critical role in producing mature crRNAs (and generates 8-nt 5′ tag sequences) in other Type I and III CRISPR–Cas systems [1,10,33–35].

In a series of biochemical and structural studies, we determined how Cas6 specifically recognizes and cleaves precursor crRNAs [11,28,32]. Cas6 recognizes the upstream region of the repeat sequence (nucleotides 2–9) and cleaves at a distal downstream site (between nucleotides 22 and 23; 8 nt upstream of the 5′ end of the guide sequence) using an active site composed of a triad of amino acids (tyrosine, histidine and lysine) that resemble the catalytic triad found in tRNA intron-splicing endonuclease [11,28,36]. Our data also indicate that the distance between the binding site and cleavage site, as well as the identity of the nucleotide at the cleavage site, is critical forCas6 action [11,28,32]. A co-crystal structure of *P. furiosus* Cas6 bound with a non-hydrolysable CRISPR repeat RNA revealed that nucleotides 2–9 contact the protein along the positively charged central cleft on the opposite face of the protein to where cleavage occurs, suggesting that the repeat RNA wraps around through the central cleft to the putative active site [32]. *In vivo*, Cas6 stably associates with its cleavage product (1× crRNA intermediates), but is not associated with mature crRNAs (that have undergone 3′ trimming, resulting in loss of the identified Cas6-binding site) [28].

The Cmr RNA-targeting defence system

We have isolated and characterized the Cmr effector complex from *P. furiosus* strains DSM 3638 and COM1 (Figure 3). We identified two crRNA species and six Cmr protein subunits associated with native complexes by RNA deep sequencing and MS [13]. The complexes specifically cleave complementary RNAs *in vitro*. The two crRNAs associated with the Cmr complex (45 and 39 nt in length) share the common 8-nt 5′ tag (produced by Cas6 cleavage), but have guide sequences (complementary to the target RNA) of 37 and 31 nt respectively, regardless of the length of the guide sequence encoded by the CRISPR spacer [13,30,31]. We reconstituted functional complexes using the recombinant Cmr proteins and synthetic crRNAs. Mutational analysis showed that the 5′ tag is critical for the function of the Cmr crRNP (CRISPR ribonucleoprotein), probably as a Cmr protein recognition site [30]. All six Cmr proteins are important for efficient target RNA cleavage (C.R. Hale, R.M. Terns and M.P. Terns, unpublished work).

The structural organization of the *P. furiosus* Cmr complex and roles of the individual Cmr proteins and crRNA subunits in the target RNA cleavage reaction are currently under investigation. X-ray structures have been obtained for *P. furiosus* Cmr2 [37,38], the Cmr2–Cmr3 heterodimer [39,40] and Cmr5 [41]. Cmr2 belongs to the Cas10 family of Type III CRISPR–Cas system signature proteins [17]. Cmr2 is the largest of the Cmr proteins and is composed of several domains including an N-terminal permutated histidine–aspartate (HD) superfamily hydrolase domain, zinc-finger domain, ferredoxin-like fold (cyclase/polymerase domains) and a highly conserved nucleotide/metal-binding domain [37,38]. Cmr2 was a lead candidate for the catalytic component in the Cmr complex cleavage, but mutational analysis ruled out a role for both the HD domain and the nucleotide/metal-binding domain of Cmr2 in target RNA cleavage [37]. Cmr5 is the smallest subunit of the complex, is mostly α-helical in nature [41] and associates physically with Cmr4 ([41], and C.R. Hale, R.M. Terns

and M.P. Terns, unpublished work). Cmr1, Cmr3, Cmr4 and Cmr6 are predicted RNA-binding proteins as they contain one or two predicted ferredoxin-fold-like domains that are common to many Cas proteins and resemble RRMs (RNA-recognition motifs) found in RNA-binding proteins from all three domains of life [14,15].

To date, the Cmr effector complex is the only CRISPR–Cas complex known to target RNA (rather than DNA) and is found in ~70% of archaea and ~30% of bacteria with CRISPR–Cas systems, suggesting that it plays an important role in the war against invaders [42]. In recent work, we demonstrated that the Cmr complex can be directed to cleave a desired target RNA *in vitro* (in a prokaryotic RNAi-like manner) by programming it with an appropriate crRNA [30], indicating that the site-specific cleavage function of crRNA–Cmr complexes could be exploited for gene knockdown.

Csa and Cst DNA-targeting defence systems

We have also isolated native *P. furiosus* crRNP complexes containing Cst and Csa proteins and distinct profiles of crRNAs (S. Majumdar, R.M. Terns and M.P. Terns, unpublished work) (Figure 3).We identified the Cas proteins by MS and the crRNAs by deep sequencing and Northern blot analysis. The crRNAs associated with the Cst and Csa proteins all include the 8-nt 5′ repeat sequence tag and possess various 3′ ends that often include a portion of the downstream repeat. Using plasmid challenge assays similar to those used to study the function of the Csa and Cst systems in *Thermococcus kodakarensis in vivo* [43], we have obtained evidence that CRISPR–Cas-mediated defences also target DNA in *P. furiosus*, presumably via the action of the Csa and/or Cst effector complexes (J.R. Elmore, R.M. Terns and M.P. Terns, unpublished work).

Conclusion

Studies of the CRISPR–Cas systems from *P. furiosus* are yielding fundamental information about mechanisms of crRNA biogenesis and silencing for three of the diverse CRISPR–Cas pathways (Figure 3). These studies reveal that organisms such as *P. furiosus* possess multiple RNA-guided mechanisms (including both RNA- and DNA-targeting systems) that provide layered protection against diverse invaders. Future studies are required to obtain a detailed understanding of how each of the individual RNA- and DNA-targeting systems function and are regulated as well as to determine the extent to which these coexisting systems influence the activity of one another. Detailed knowledge of the CRISPR–Cas pathways is also expected to lead to exciting new biomedical and biotechnological applications.

Acknowledgments

Abbreviations used

Cas	CRISPR-associated
Cmr	Cas module RAMP (repeat-associated mysterious proteins)
CRISPR	clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
crRNP	CRISPR ribonucleoprotein
Csa	Cas subtype Apern
Cse	Cas-subtype *Escherichia coli*
Cst	Cas subtype Tneap

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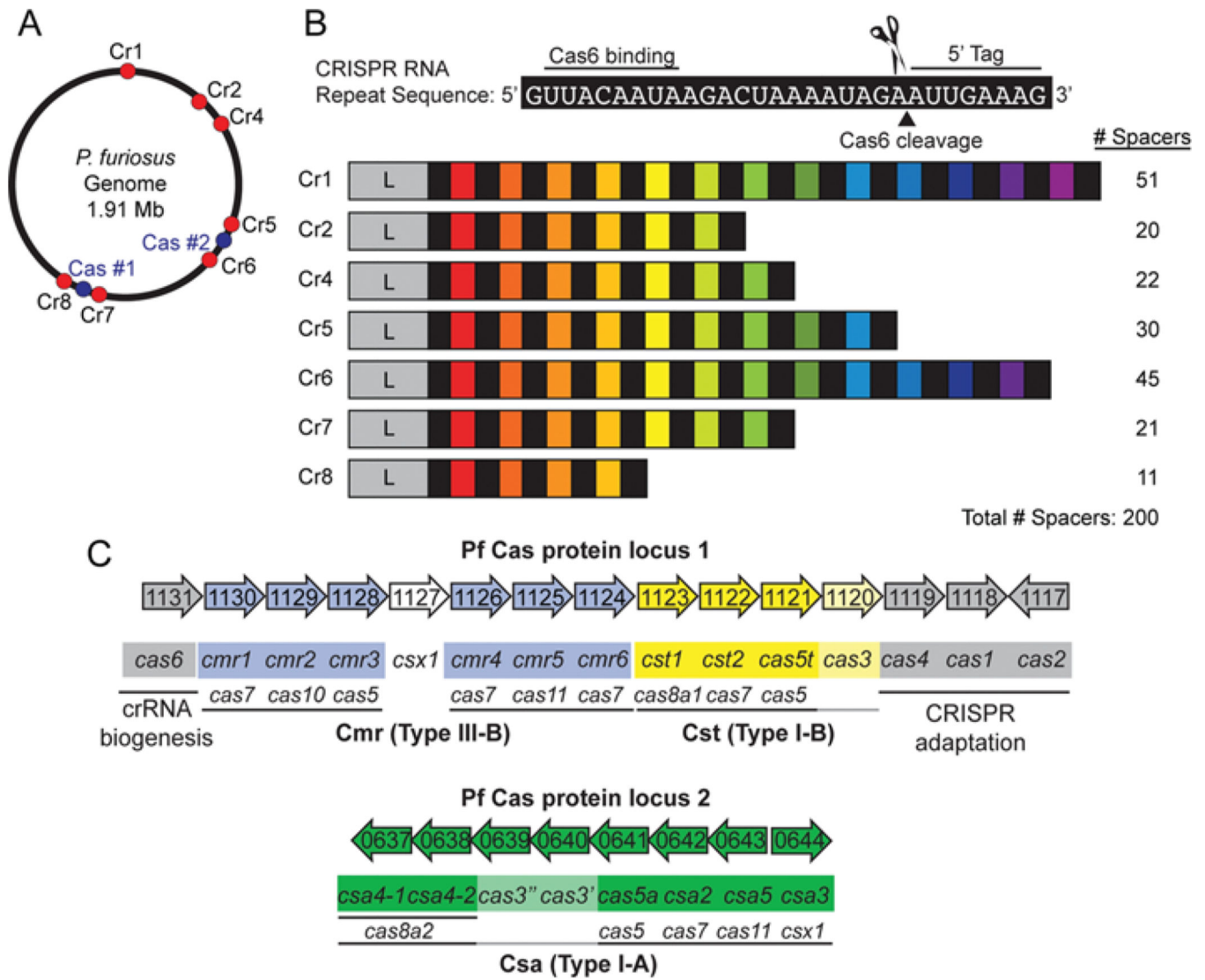
Figure 1. *P. furiosus* CRISPR–Cas systems

(**A**) Distribution of the CRISPR loci (Cr1, Cr2, Cr4–Cr8; red) and two major *cas* gene clusters (Cas; blue) throughout the *P. furiosus* genome. (**B**) CRISPRs comprise leader regions (L) preceding series of repeat (black) and spacer (coloured) elements. The actual numbers of spacers are indicated. The consensus crRNA repeat sequence found in the seven CRISPR loci is shown above with the Cas6-binding and cleavage sites, and the 8-nt 5′ tag crRNA sequence indicated. (**C**) Genome organization of predicted *P. furiosus* (Pf) *cas* genes. Subtype III-B Cmr (blue), subtype I-B Cst (yellow), subtype I-A Csa (green) and core *cas* genes (grey) are indicated, and recent *cas* gene superfamily designations [15] are indicated below [14]. The known or predicted general function of the core *cas* genes is indicated.
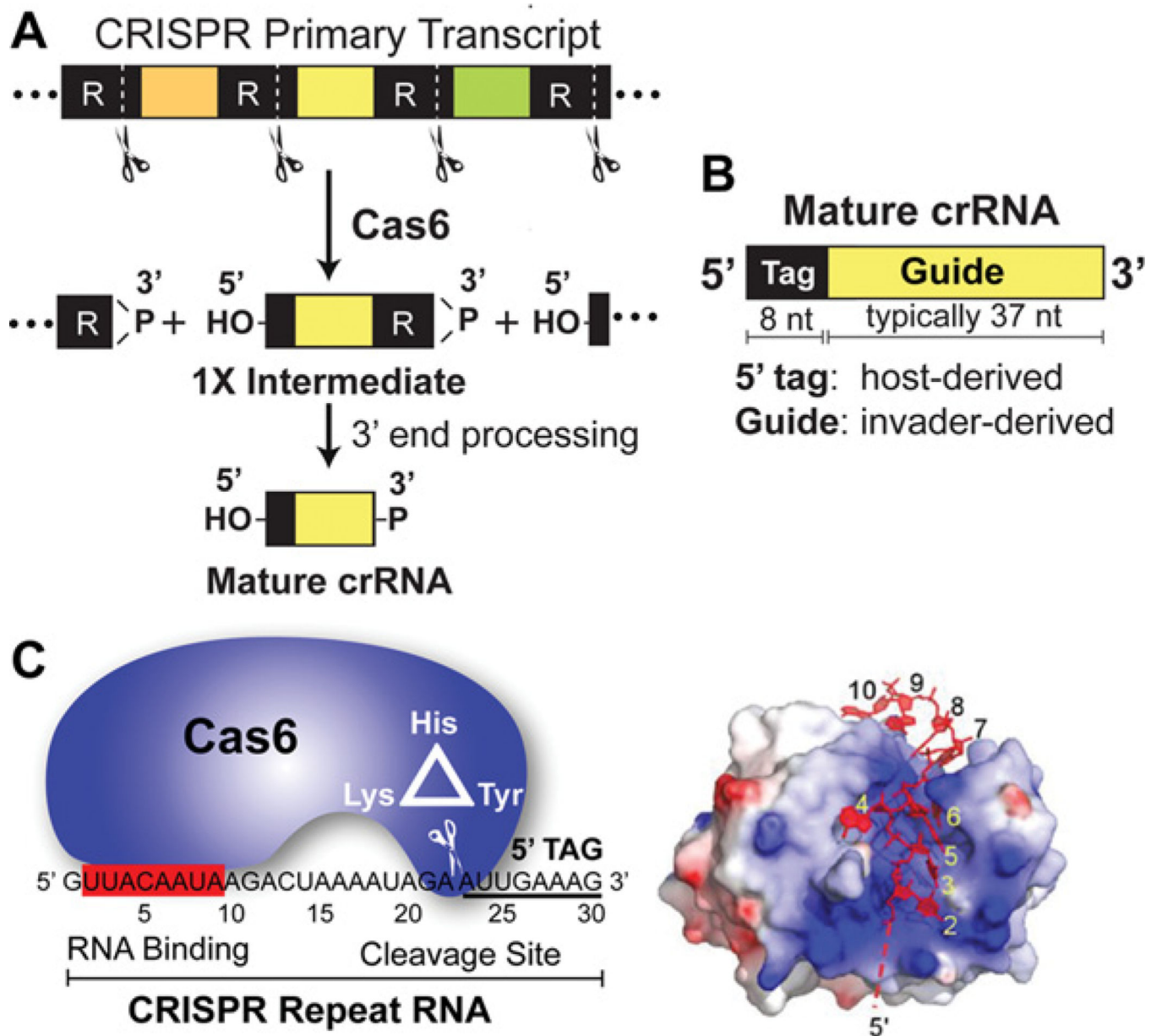
Figure 2. Cas6 and crRNA biogenesis

(**A**) Cas6 cleaves within the repeat regions of the CRISPR transcript to liberate the individual embedded crRNAs. The products of Cas6 cleavage (1× crRNAs) undergo 3′ end trimming. (**B**) The architecture of a mature crRNA. This abundant crRNA form includes the 5′ repeat-derived tag and complete guide sequence. Other mature crRNAs in *P. furiosus* contain some repeat sequences at their 3′ ends or slightly less than full-length guide sequences (not depicted). (**C**) Cas6 is an endoribonuclease critical for crRNA biogenesis. Cas6 interacts with the 5′ region of the CRISPR repeat RNA (red box) and cleaves at a distal site using an active site composed of three key amino acids (indicated). The Cas6-binding region in the crRNA interacts with the positively charged cleft of Cas6.
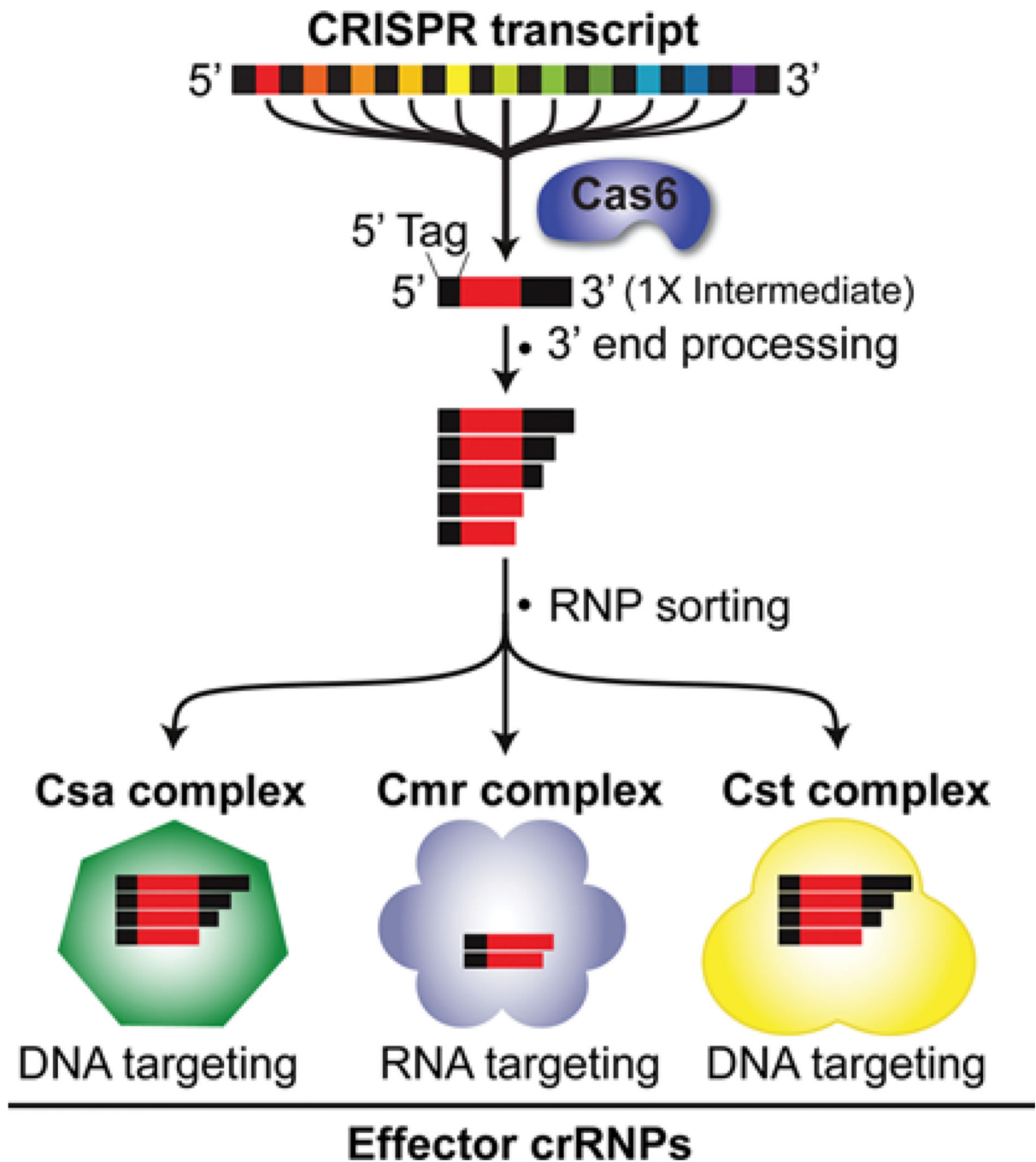
Figure 3. crRNAs are loaded into three distinct crRNP immune effector complexes in *P. furiosus*

Following release by Cas6 and further 3′ end trimming, crRNAs of specific size formed from all seven CRISPR loci become incorporated into the three distinct crRNP complexes containing Cmr, Csa or Cst Cas proteins. There is evidence that Cmr crRNPs cleave target RNAs, whereas Csa and Cst complexes cleave invader DNA.