

## CRISPR-Cas

### Adapting to change

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1 **Title:**

2 **CRISPR-Cas: adapting to change**

3

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19

20 **Abstract:**

21 Bacteria and archaea are engaged in a constant arms race to defend against the ever-present threats  
22 of viruses and invasion by mobile genetic elements. The most flexible weapons in the prokaryotic  
23 defense arsenal are the CRISPR-Cas adaptive immune systems, which are capable of selective  
24 identification and neutralization of foreign elements. CRISPR-Cas systems rely on stored genetic  
25 memories to facilitate target recognition. Thus, to keep pace with a changing pool of hostile  
26 invaders, the CRISPR memory banks must be regularly updated by the addition of new  
27 information, through a process termed adaptation. In this review, we outline the recent advances  
28 in our understanding of the molecular mechanisms governing adaptation and highlight the  
29 diversity between systems.

30

31 **One Sentence Summary:**

32 How prokaryotes adapt their CRISPR memory to constantly-evolving invaders

33

34 **Main Text:**

35

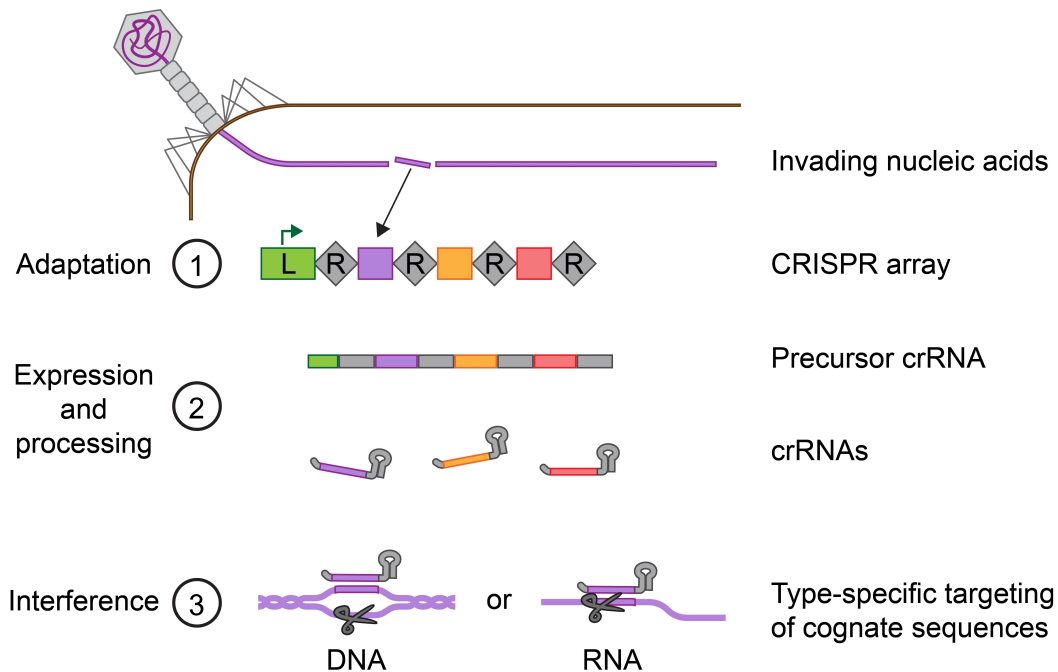
36 *Adaptive immunity in prokaryotes*

37 Bacteria and archaea are constantly threatened by phage infection and invasion by mobile genetic  
38 elements (MGEs) through conjugation and transformation. In response, a defense arsenal has  
39 evolved, including various ‘innate’ mechanisms and the CRISPR-Cas adaptive immune systems  
40 (1-3). CRISPR-Cas systems are widely distributed, present in 50% and 87% of complete bacterial  
41 and archaeal genomes, respectively, and are classified into two major classes consisting of 6 types  
42 according to their Cas proteins (4, 5). CRISPR-Cas systems function as RNA-guided nucleases  
43 that provide sequence-specific defense against invading MGEs (6, 7). Their repurposing,  
44 particularly Cas9, has stimulated a biotechnological revolution in genome editing that has resulted  
45 in breakthroughs across many biological fields (8). In native hosts, the advantage conferred by  
46 CRISPR-Cas systems over innate defenses lies in the ability to update their resistance repertoire  
47 in response to infection (termed CRISPR adaptation). Adaptation is achieved by incorporating  
48 short DNA fragments from MGEs into CRISPR arrays to form memory units termed spacers,  
49 which are subsequently transcribed and processed to CRISPR RNAs (crRNAs) (**Fig. 1**). Cas  
50 proteins associate with crRNAs to form crRNA-effector complexes, which seek and destroy  
51 invading MGEs. Thus, adaptation of CRISPR arrays is a crucial process required to ensure  
52 persistent CRISPR-Cas defense (9, 10).

53 Adaptation in nature appears widespread, highlighting the dynamic interaction between hosts and  
54 invaders (11-13). When a prokaryotic community undergoes CRISPR adaptation, individual cells  
55 acquire different, and often multiple spacers. This population diversity increases defense by  
56 limiting the reproductive success of MGE variants that evade recognition through genetic  
57 mutations (escape mutants) (14). The CRISPR polymorphisms resulting from adaptation enable  
58 differentiation of species subtypes, including economically and clinically relevant isolates, and  
59 allow tracking of pathogen outbreaks (15, 16).

60 Typically, new spacers are inserted at one end of the array in a position closest to the promoter  
61 driving CRISPR transcription – termed the leader (**Fig. 1**) (6, 17-19). This polarization of the  
62 CRISPR records provides a chronological account of the battle between phages and bacteria,  
63 analyses of which can provide insights into phage-host co-occurrences, evolution and ecology (20,  
64 21). Moreover, spacer integration at the leader end enhances defense against recently encountered  
65 MGEs, potentially due to elevated crRNA abundance (22). However, in some systems, the repeats  
66 themselves contain internal promoters, which might make leader-proximal spacer integration less  
67 important (23). CRISPR arrays typically contain 10-30 spacers, but some species contain arrays  
68 with over 500 spacers (24). Spacers that may no longer be under evolutionary selection can be lost  
69 via recombination between CRISPR repeats (11, 25).

70



71

72 **Fig. 1: CRISPR-Cas adaptation and defense.** A simplified schematic of CRISPR-Cas defense,  
 73 which consists of an array of Clustered Regularly Interspaced Short Palindromic Repeats  
 74 (CRISPR) and CRISPR-associated (Cas) proteins encoded by *cas* genes (omitted for clarity).  
 75 CRISPR-Cas defense consists of three defined stages 1) Adaptation, the creation of memory of  
 76 prior infections formed via the insertion of small foreign DNA sequences into the leader (L)  
 77 end of the CRISPR array, where they are stored as spacers (colored squares) between duplicated  
 78 repeats (R). 2) Expression and CRISPR-RNA (crRNA) biogenesis, the transcription and  
 79 processing of the array into small guide RNA sequences. 3) Interference, degradation of the target  
 80 foreign invader by sequence-specific binding and cleavage.

81

82 Early bioinformatic studies showed many spacers were of foreign origin, hinting that CRISPR loci  
 83 would form the memory of an immune system (15, 26-28). Subsequent confirmation of this link  
 84 between spacers and resistance to phage and MGEs was gained experimentally (6, 7, 29). Despite  
 85 the elegance of memory-directed defense, CRISPR adaptation is not without complications.  
 86 Paradoxically, the spacers required for defense must be added to CRISPRs during exposure to  
 87 MGEs (30, 31). In addition, the inadvertent acquisition of spacers from host DNA must be avoided  
 88 because this will result in cytotoxic self-targeting – akin to autoimmunity (32, 33). Recently,  
 89 significant progress has been made toward understanding the molecular mechanisms governing  
 90 how, when and why CRISPR spacers are acquired. Here, we review these studies and highlight  
 91 the insights they shed on both the function and evolution of CRISPR-Cas systems.

92

93 ***Molecular mechanism of adaptation***

94 At the forefront of adaptation are Cas1 and Cas2 proteins, which form a Cas1<sub>4</sub>-Cas2<sub>2</sub> complex (34,  
 95 35) (hereafter Cas1-Cas2) – the ‘workhorse’ of spacer integration (Fig. 2). Illustrative of their key  
 96 roles in spacer integration, the *cas1* and *cas2* genes are associated with nearly all CRISPR-Cas  
 97 systems (4). Cas1-Cas2-mediated spacer integration prefers dsDNA substrates and proceeds via a

98 mechanism resembling retroviral integration (36, 37). In addition to Cas1-Cas2, a single repeat, at  
99 least part of the leader sequence (17, 18, 22, 38), and additional host factors for repair of the  
100 insertion sites (e.g. DNA polymerase) are required (39). Spacer integration requires three main  
101 processes: 1) substrate capture 2) recognition of the CRISPR locus and 3) integration within the  
102 array.

103

#### 104 *Substrate capture*

105 During substrate capture, Cas1-Cas2 is loaded with an integration-compatible pre-spacer, which  
106 is thought to be partially duplexed DNA. In the Cas1-Cas2:pre-spacer complex, each single-  
107 stranded 3'OH end of the pre-spacer DNA extends into a single active subunit of each Cas1 dimer  
108 (40) located either side of a central Cas2 dimer (41, 42) (Fig. 2). The branch points of the splayed  
109 DNA are stabilized by a Cas1 wedge, which acts as a molecular ruler to control spacer length.  
110 Although it is likely that Cas1-Cas2 rulers exist and measure different spacer sizes in all systems,  
111 the mechanism has only been demonstrated in the *Escherichia coli* type I-E system, where two  
112 tyrosine residues bookend the core 23 nt dsDNA region (41, 42). Details of how pre-spacer  
113 substrates are produced from foreign DNA is discussed later.

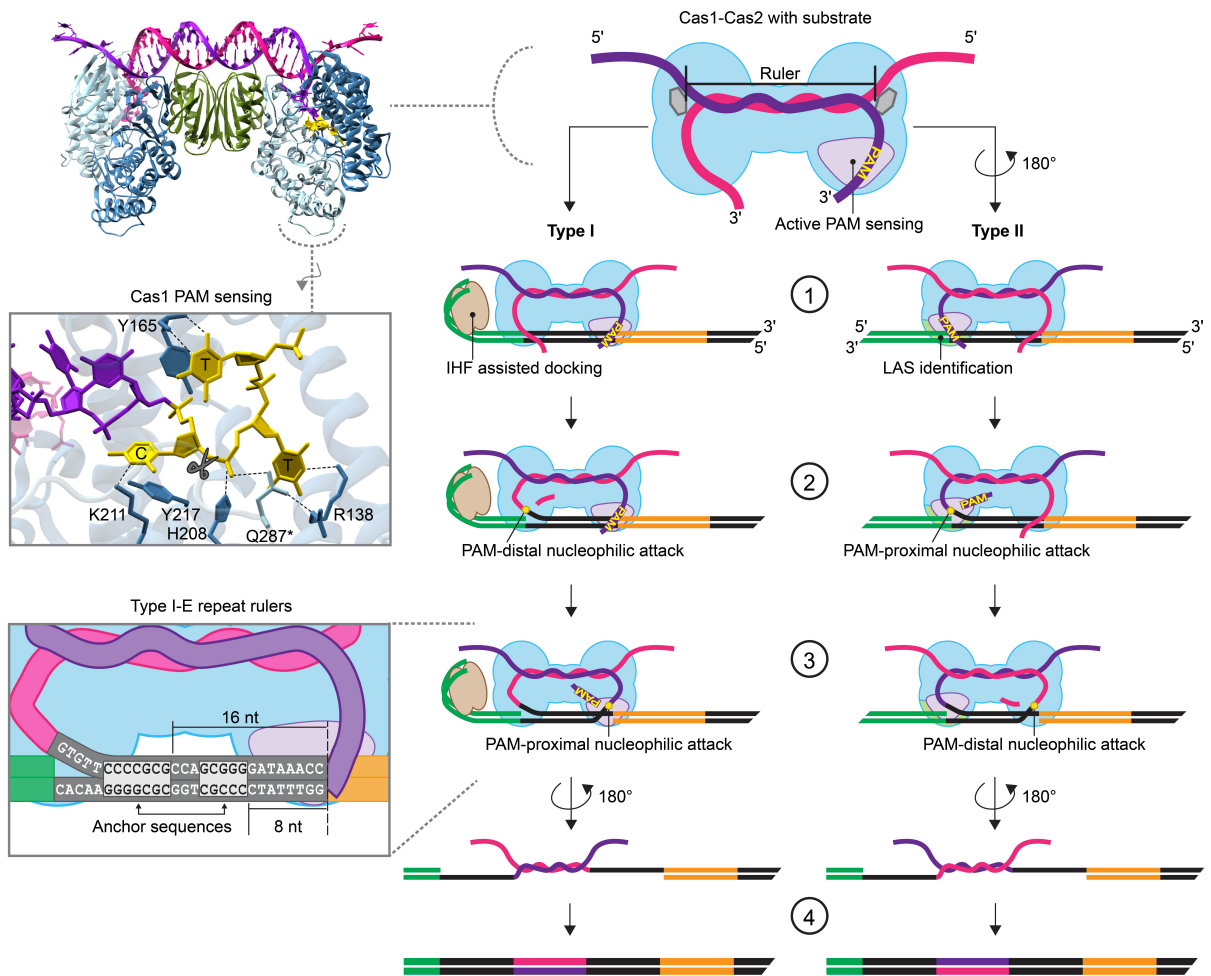
114

#### 115 *Recognition of the CRISPR locus*

116 Prior to integration, the substrate-bound Cas1-Cas2 complex must locate the CRISPR leader-  
117 repeat sequence. Adaptation complexes of several systems display intrinsic affinity for the leader-  
118 repeat region in vitro (36, 43), yet this is not always wholly sufficient to provide the specificity  
119 observed in vivo. For the type I-E system, leader-repeat recognition is assisted by the integration  
120 host factor (IHF) heterodimer, which binds in the leader (44). IHF binds DNA in a sequence-  
121 specific manner and induces ~120° DNA bending, providing a cue to accurately localize Cas1-  
122 Cas2 to the leader-repeat junction (44, 45). A conserved leader motif upstream of the IHF pivot is  
123 proposed to stabilize the Cas1-Cas2-leader-repeat interaction and increase adaptation efficiency,  
124 supporting bipartite binding of the adaptation complex to DNA sites either side of bound IHF (45).

125 IHF is absent in many prokaryotes, including archaea and gram-positive bacteria, suggesting other  
126 leader-proximal integration mechanisms exist. Indeed, type II-A Cas1-Cas2 from *Streptococcus*  
127 *pyogenes* catalyzed leader-proximal integration in vitro, at a level of precision comparable to the  
128 type I-E system with IHF (43, 44). Hence, type II-A systems may rely solely on intrinsic sequence  
129 specificity for the leader-repeat. A short leader-anchoring site (LAS) adjacent to the first repeat  
130 and ≤6 bp of this repeat were essential for adaptation (22, 38, 43) and are conserved in systems  
131 with similar repeats. Placement of an additional LAS in front of a non-leader repeat resulted in  
132 adaptation at both sites (38), whereas LAS deletion caused ectopic integration at a downstream  
133 repeat adjacent to a spacer containing a LAS-like sequence (22). Taken together, this shows  
134 specific sequences upstream of CRISPR arrays direct leader-polarized spacer integration, both via  
135 direct Cas1-Cas2 recognition and assisted by host proteins, such as IHF.

136



137

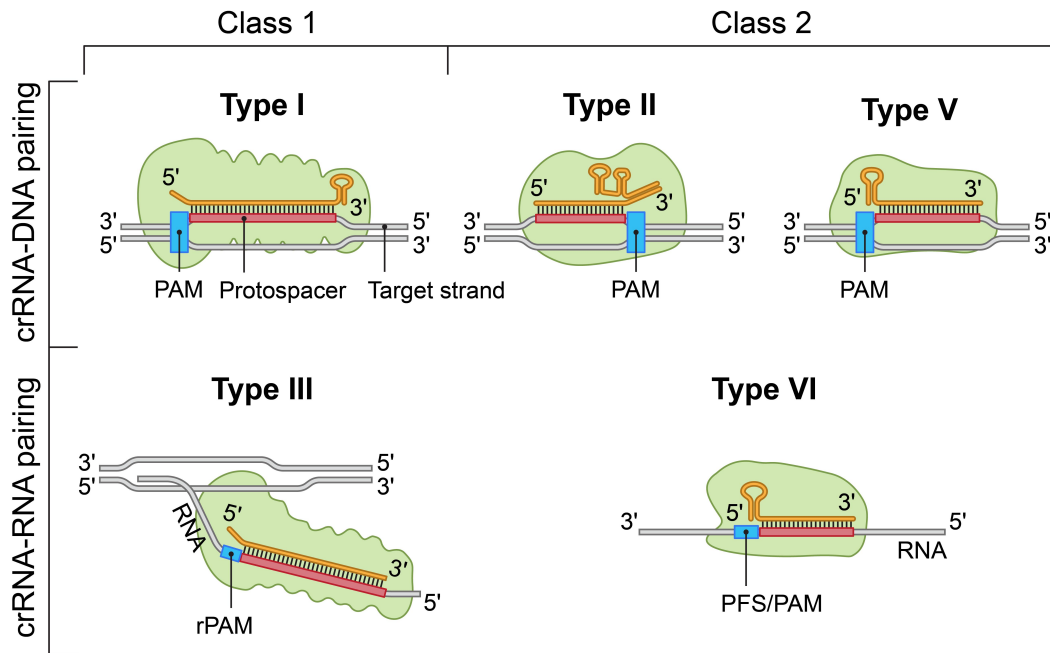
138 **Fig. 2: Cas1-Cas2-mediated spacer acquisition.** The substrate loaded Cas1-Cas2 protein  
 139 complex (*E. coli* type I-E structure shown top left; PDB 5DQZ) with the active PAM sensing  
 140 domain highlighted (light purple) and a partially duplexed DNA pre-spacer substrate (strands are  
 141 purple and pink) (41, 42). The Cas1 PAM sensing insert shows the canonical type I-E PAM (CTT),  
 142 residue-specific interactions (a residue from the non-catalytic Cas1 monomer is annotated with \*),  
 143 and site of PAM processing (scissors). The ruler mechanism determining spacer length for the type  
 144 I-E systems uses two conserved tyrosine residues (grey hexagons). Spacer integration proceeds as  
 145 follows: 1) the Cas1-Cas2:pre-spacer complex binds the leader (green) and first repeat (black). 2)  
 146 The first nucleophilic attack occurs at the leader-repeat junction and gives rise to a half-site  
 147 intermediate. 3) The second nucleophilic attack occurs at the repeat-spacer (orange)  
 148 resulting in full site integration. The type I-E repeat is magnified (lower left) to indicate the  
 149 inverted repeats within its sequence and highlight the anchoring sites of the molecular rulers that  
 150 determine the point of integration. 4) Host DNA repair enzymes fill the intergration site. For  
 151 additional details, see the text.

152

153 *Integration into the CRISPR array*

154 In almost all types of CRISPR-Cas systems, the presence of a short sequence motif in the target  
155 nucleic acid adjacent to where the crRNA basepairs is essential for interference (the target-strand  
156 that the crRNA pairs to is known as the protospacer) (**Fig. 3**) (46). This sequence motif is termed  
157 a protospacer adjacent motif (PAM) and is a key feature for spacer selection during adaptation (17,  
158 27, 47, 48). Acquisition of interference-proficient spacers requires processing of the pre-spacer  
159 substrate at a specific position relative to a PAM and also integration into the CRISPR array in the  
160 correct orientation. The active site of each Cas1 monomer contains a PAM sensing domain (41,  
161 42) and the presence of a PAM within the pre-spacer substrate ensures integration in the  
162 appropriate orientation (49-51). Accordingly, PAM proximal processing, resulting in complete or  
163 partial (in the case of type I-E) removal of the PAM, is likely to occur after Cas1-Cas2 orients and  
164 docks at the leader-repeat. In contrast, if complete processing occurred before docking to the  
165 CRISPR locus, then the PAM directionality cue would be lost. Cas1-mediated processing of the  
166 pre-spacer creates two 3'OH ends required for nucleophilic attack on each strand of the leader-  
167 proximal repeat (36, 37, 52). The initial nucleophilic attack most likely occurs at the leader-repeat  
168 junction and forms a half-site intermediate, then a second attack at the existing repeat-spacer  
169 junction generates the full-site integration product (**Fig. 2**). The precise order of the pre-spacer  
170 processing and integration steps remains to be fully determined, yet considerable progress toward  
171 elucidating the reaction mechanisms has been made.

172 Following the first nucleophilic attack, Cas1-Cas2 employs molecular rulers that harness the  
173 intrinsic sequence-specificity of the complex to define the site of the second attack and ensure  
174 accurate repeat length duplication. CRISPR repeats are often semi-palindromic, containing two  
175 short inverted repeat (IR) elements, but the location of these can vary (53). In type I-B and I-E  
176 systems, the IRs occur close to the center of the repeat (**Fig. 2**) and are important for adaptation  
177 (54, 55). In the type I-E system, both IRs act as anchors for the Cas1-Cas2 complex, positioning  
178 the active site for the second attack at the repeat-spacer boundary (54). However, in the type I-B  
179 system from *Haloarcula hispanica*, only the first IR was essential for integration, and thus a single  
180 molecular ruler directed by an anchor between the IRs was proposed (55). In contrast, in the type  
181 II-A systems of *Streptococcus thermophilus* and *S. pyogenes* the IRs are located distally within the  
182 repeats, suggesting these short sequences may directly position the nucleophilic attacks without  
183 molecular rulers (38, 43). Although further work is required to determine how the spacer  
184 integration events are directed in different CRISPR-Cas systems, it seems likely the conserved  
185 leader-repeat regions at the beginning of CRISPR arrays maintain recognizable sequences to  
186 ensure Cas1-Cas2 localizes appropriately and spacer insertion and repeat duplication is of the  
187 correct length.



188  
189

190 **Fig. 3: Target interactions and the PAMs of different CRISPR-Cas types.** DNA targets are  
 191 recognized by the crRNA-effector complexes of types I, II and V, resulting in formation of an R-  
 192 loop with the non-target strand displaced. The target strand contains the protospacer (red), which  
 193 is complementary to the spacer (crRNA, orange) sequence. The protospacer adjacent motif (PAM,  
 194 blue) is located at either the 3' end of the protospacer (type I and type V) or the 5' end (type II).  
 195 The PAM assignment is consistent with target-centric nomenclature (46). Type III and VI  
 196 recognize RNA targets, with type III exhibiting transcription-dependent DNA targeting. Some type  
 197 III systems require an RNA-based PAM (rPAM). Type VI systems exhibit a protospacer flanking  
 198 sequence (PFS) specificity, which is analogous to a PAM.

199

## 200 ***Production of spacers from foreign DNA***

### 201 *Naïve adaptation*

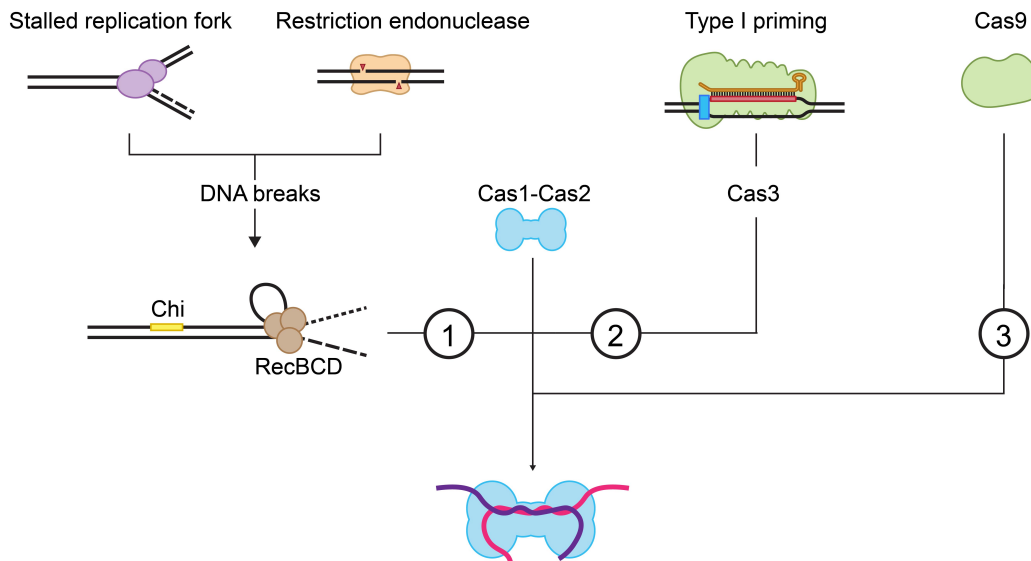
202 Acquisition of spacers from MGEs that are not already catalogued in host CRISPRs is termed  
 203 naïve adaptation (56) (**Fig. 4**). To facilitate naïve adaptation, pre-spacer substrates are generated  
 204 from foreign material and loaded onto Cas1-Cas2. Currently, the main known source of these  
 205 precursors is the host RecBCD complex (57). Stalled replication forks that occur during DNA  
 206 replication can result in double strand breaks (DSBs), which are repaired via RecBCD-mediated  
 207 unwinding and degradation of the dsDNA ends back to the nearest Chi sites (58). During this  
 208 process, RecBCD produces ssDNA fragments that are proposed to anneal, forming substrates  
 209 suitable for use by Cas1-Cas2 (57). Loading of substrates into Cas1-Cas2 is likely enhanced by  
 210 interaction between Cas1 and RecBCD (59), positioning the adaptation machinery adjacent to the  
 211 site of substrate generation. The increased number of active origins of replication and the paucity  
 212 of Chi sites on MGEs, versus the host chromosome, biases naïve adaptation toward foreign DNA.  
 213 Furthermore, RecBCD recognizes unprotected dsDNA ends, which are commonly present in



214 phage genomes upon injection or prior to packaging, thereby providing an additional phage-  
215 specific source of naïve adaptation substrates (57, 60).

216 Despite the clear role of RecBCD in substrate generation, naïve adaptation also occurs in its  
217 absence, albeit with reduced bias toward foreign DNA (57). Events other than DSBs might also  
218 stimulate naïve adaptation, such as R-loops that prime plasmid replication (61), lagging ends of  
219 incoming conjugative elements (62), and even CRISPR-Cas mediated spacer integration events  
220 themselves (51, 57). Furthermore, it is unknown whether all CRISPR-Cas systems display an  
221 intrinsic adaptation bias towards foreign DNA. Complicating results, spacer acquisition from the  
222 host genome in native systems could be underestimated because the resulting self-targeting means  
223 these genotypes are typically lethal (32, 33, 51, 63). For example, in the *S. thermophilus* type II-A  
224 system, adaptation appears biased toward MGEs, yet nuclease-deficient Cas9 (dCas9) failed to  
225 discriminate between acquisition from host versus foreign DNA (63) and it is unknown whether  
226 the adaptation was reliant on DNA break repair. Further studies in a range of host systems are  
227 required to clarify how diverse CRISPR-Cas systems balance the requirement for naïve adaptation  
228 from MGEs against the risk of self-acquisition events.

229



230

231 **Fig. 4: Cas1-Cas2 substrate production pathways.** 1) Naïve generation of substrates by  
232 RecBCD activity on DNA ends resulting from DSBs from stalled replication forks, innate defenses  
233 such as restriction endonuclease activity or from the ends of phage genomes (not shown). 2)  
234 Primed substrate production in type I systems. 3) Cas9-dependent spacer selection in type II  
235 systems. For details, see the text.

236

### 237 *crRNA-directed adaptation (Priming)*

238 Mutations in the target PAM or protospacer sequences can abrogate immunity, allowing MGEs to  
239 escape CRISPR-Cas defenses (47, 64, 65). Furthermore, the immunological effectiveness of  
240 individual spacers varies: often several target-specific spacers are required to both mount an  
241 effective defense (66, 67) and prevent proliferation of MGE escape mutants (13, 14). Thus,  
242 CRISPR-Cas systems need to adapt faster than the foreign element can evade targeting. Indeed,

243 type I systems have evolved a mechanism known as primed adaptation (priming) to facilitate rapid  
244 CRISPR adaptation (68, 69), even against highly divergent invaders (65) (**Fig. 4**). In contrast to  
245 naïve adaptation, priming utilizes target recognition by crRNAs from pre-existing spacers to direct  
246 spacer acquisition toward invaders whose proliferation exceeds the existing defense capabilities.  
247 This often occurs with MGE escape mutants, but also when the CRISPR-Cas expression level is  
248 insufficient to provide immunity – even with spacers perfectly targeting the MGE (65, 68-72).

249 Priming begins with target recognition by crRNA-effector complexes. Therefore, factors that  
250 influence target recognition (i.e. the formation and stability of the R-loop – see **Fig. 3**), including  
251 PAM sensing and crRNA:target complementarity, affect the efficiency of primed adaptation (64,  
252 65, 67, 73-80). Furthermore, these same factors influence conformational rearrangements in the  
253 target-bound crRNA-effector complex, coalescing to favor either interference or priming (67, 74,  
254 75, 78, 81). In type I-E systems, the Cas8e (Cse1) subunit of Cascade can adopt one of two  
255 conformational modes (78, 81), which may promote either direct or Cas1-Cas2-stimulated  
256 recruitment of the effector Cas3 nuclease (74, 75, 81).

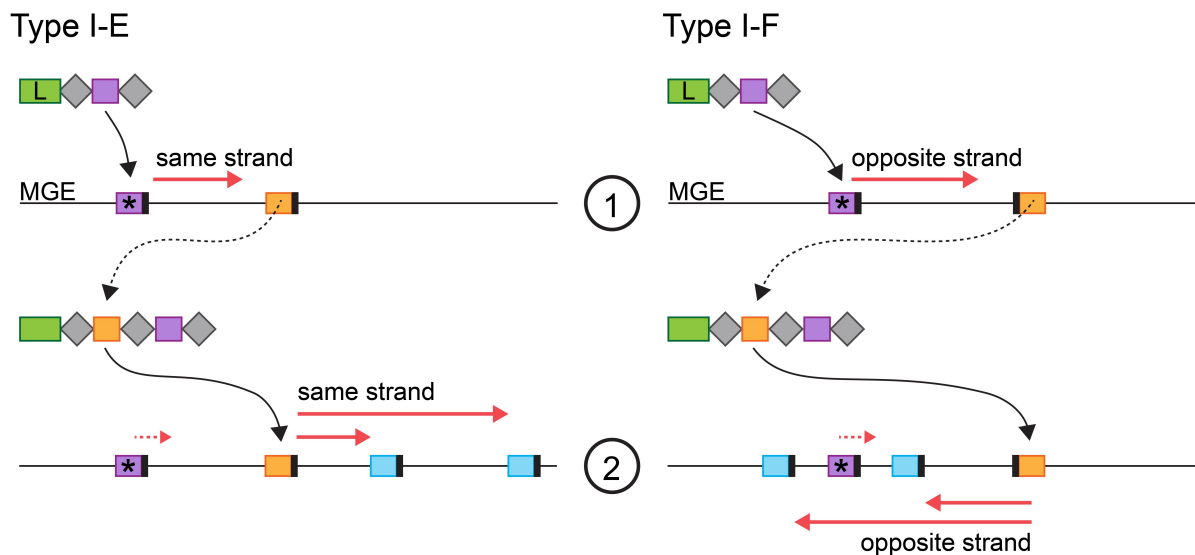
257 Cas3, found in all type I systems, exhibits 3' to 5' helicase and endonuclease activity that nicks,  
258 unwinds and degrades target DNA (82-85). In vitro activity of the type I-E Cas3 produces ssDNA  
259 fragments of ~30-100 nucleotides that are enriched for PAMs in their 3' ends, which anneal to  
260 provide partially duplexed pre-spacer substrates (73). The spatial positioning of Cas1-Cas2 during  
261 primed substrate generation has not been clearly established, although Cas1-Cas2-facilitated  
262 recruitment of Cas3 would imply the adaptation machinery is localized close to the site of substrate  
263 production (74, 81). In support of this, Cas3 in type I-F systems is fused to the C-terminus of Cas2  
264 and forms a Cas1-Cas2-3 complex (35) that couples the adaptation machinery directly to the source  
265 of substrate generation during primed adaptation (51, 86).

266 Despite different crRNA-effector:target interactions favoring distinct Cas3 recruitment modes,  
267 primed adaptation can occur from both escape mutants and interference-proficient targets (51, 68,  
268 69, 87). When target copy-number influences are excluded for type I-E and type I-F systems,  
269 interference-proficient targets promote stronger spacer acquisition than escape targets (51, 87).  
270 This provides a positive feedback loop, reinforcing immunity against recurrent threats even in the  
271 absence of escapees (51, 69). However, because target interference rapidly destroys the invader,  
272 more spacer acquisition is provoked by escape mutants where replication of the MGE outpaces its  
273 destruction. Over time, the prolonged presence of the invader, combined with the priming-centric  
274 target recognition mode, results in higher net production of pre-spacer substrates from escape  
275 mutants (51, 72, 73, 87).

276 Because priming initiates with site-specific target recognition (i.e. targeting a 'priming'  
277 protospacer), Cas1-Cas2 compatible substrates are subsequently produced from MGEs with  
278 locational biases (**Fig. 5**). Mapping the MGE sequence positions and strands targeted by newly  
279 acquired spacers (i.e. their corresponding protospacers) revealed subtype-specific patterns and has  
280 provided much of our insight into the priming mechanisms (50, 51, 68, 69, 86, 88, 89). In type I-  
281 E systems, new protospacers map to the same strand (50, 69) as the priming protospacer (**Fig. 5**).  
282 For type I-B priming, Cas3 is predicted to load onto either strand at the priming protospacer,  
283 resulting in a bidirectional distribution of new protospacers (88). For type I-F priming, the first  
284 new protospacer typically maps to the strand opposite the priming protospacer, in a direction  
285 consistent with Cas3 loading and helicase activity on the non-target strand. Furthermore, once the  
286 first spacer is acquired, two targets in the MGE will be recognized and substrate production can  
287 be driven from both locations (51, 86) (**Fig. 5**). However, in a head-to-head contest interference-

288 proficient targets dominate, thus, subsequent spacers (i.e. the second and beyond) generally result  
 289 from targeting by the first new spacer and are typically located back towards the original priming  
 290 protospacer(51) (**Fig. 5**). The dominance of the first new spacer also holds true for type I-E (69,  
 291 87) and likely all other systems that display priming. However, these are generalized models and  
 292 many questions remain unresolved, such as the mechanisms resulting in strand selection and why  
 293 some spacer sequences are more highly acquired from MGEs than others. Further analyses of  
 294 priming in different systems, particularly the order of new spacers acquired, will greatly inform  
 295 our understanding of primed Cas1-Cas2 substrate production.

296



297

298

299 **Fig. 5: Primed adaptation from a multi-copy MGE by type I-E and I-F CRISPR-Cas systems.**  
 300 1) An existing spacer (purple) with homology to an MGE sequence that has escaped interference  
 301 (the ‘priming’ protospacer denoted with an asterisk) directs target recognition – the PAM adjacent  
 302 to the protospacer is shown in black (PAMs at the right or left of protospacers indicate the strand  
 303 each protospacer is on). The crRNA-effector complex recruits Cas3 and the 3’ to 5’ helicase  
 304 activity (illustrated by the red arrow) results in the acquisition of a new spacer that maps to a  
 305 protospacer (orange) from a site distal to the initial priming location. 2) The new interference-  
 306 proficient spacer directs targeting of the MGE and recruitment of Cas3. Hence, subsequent spacers  
 307 (mapping to blue protospacers) typically originate from Cas3 activity (red arrows) beginning at  
 308 this location. See text for details.

309

### 310 *Cas protein-assisted production of spacers*

311 Given the apparent advantages conferred by priming in type I systems, mechanisms to utilize  
 312 existing spacers to direct adaptation are likely to exist in other CRISPR-Cas types. For example,  
 313 DNA breaks induced by interference activity of class 2 CRISPR-Cas effector complexes could  
 314 trigger host DNA repair mechanisms (e.g. RecBCD), thereby providing substrates for Cas1-Cas2.  
 315 In agreement with a generalized DNA break-stimulated adaptation model, restriction enzyme  
 316 activity stimulated RecBCD-facilitated adaptation (57). This may also partially account for the  
 317 enhanced adaptation observed during phage infection of a host possessing an innate defense

318 restriction-modification system (31), but whether this was RecBCD-dependent is unknown. For  
319 CRISPR-Cas-induced DNA breaks, spacer acquisition would be preceded by target recognition,  
320 hence the resulting adaptation could be considered related to ‘priming’ (90). Although direct  
321 evidence to support this concept is lacking, adaptation in type II-A systems requires Cas1-Cas2,  
322 Cas9, a tracrRNA and Csn2 (63, 90). In support of a role for Cas9 in substrate generation, the  
323 PAM-sensing domain of Cas9 enhances the acquisition of spacers with compatible PAMs (90).  
324 However, Cas9 nuclease activity is dispensable (63) and existing spacers are not strictly necessary  
325 (90), suggesting that PAM interactions of Cas9 could be sufficient to select appropriate new  
326 spacers. Some Cas9 variants can also function with non-CRISPR RNAs and tracrRNA (91), raising  
327 the possibility that host or MGE-derived RNAs might direct promiscuous Cas9 activity, resulting  
328 in DNA breaks, or replication fork stalling and trigger spacer integration.

329

### 330 ***Roles of accessory Cas proteins in adaptation***

331 Although Cas1 and Cas2 play a central role in adaptation, type-specific variations in *cas* gene  
332 clusters occur. In many systems, Cas1-Cas2 is assisted by accessory Cas proteins, which are often  
333 mutually exclusive and type-specific (4). For example, in the *S. thermophilus* type II-A system,  
334 deletion of *csn2* impaired the acquisition of spacers from invading phages (6). Csn2 assembles into  
335 ring-shaped homo-tetramers with a calcium-stabilized central channel (92, 93) that binds  
336 cooperatively to the free ends of linear dsDNA and can translocate by rotation-coupled movement  
337 (94, 95). Given that substrate-loaded type II-A Cas1-Cas2 is capable of full-site spacer integration  
338 in vitro (43), Csn2 is likely to play an earlier role in either pre-spacer substrate production,  
339 selection or processing. Potentially, Csn2 binding to the free ends of dsDNA provides a cue to  
340 direct nucleases necessary for substrate generation (94).

341 Cas4, another ring-forming accessory protein, is found in type I, II-B and V systems (4).  
342 Confirming its role in adaptation, Cas4 is necessary for type I-B priming in *H. hispanica* (88) and  
343 interacts with a Cas1-2 fusion protein in the *Thermoproteus tenax* type I-A system (96). Fusions  
344 between Cas4 and Cas1 are found in several systems, supporting a functional association with  
345 adaptation. Cas4 contains a RecB-like domain and four conserved cysteine residues, which are  
346 presumably involved in the coordination of an iron-sulfur cluster (97). However, Cas4 proteins  
347 appear to be functionally diverse with some possessing uni- or bi-directional exonuclease activity  
348 (97, 98), while others exhibit ssDNA endonuclease activity and unwinding activity on dsDNA  
349 (98). Due to its nuclease activity, Cas4 is hypothesized to trim pre-spacer substrates and aid  
350 adaptation by generating 3’ overhangs in the duplex pre-spacer substrate.

351 To provide immunity, type III systems require spacers complementary to RNA transcribed from  
352 MGEs (Fig. 3) (99, 100). Some bacterial type III systems contain fusions of Cas1 with reverse  
353 transcriptase domains (RTs), which provide a mechanism to integrate spacers from RNA substrates  
354 (101). The RT-Cas1 fusion from *M. mediterranea* can integrate RNA precursors into an array,  
355 which are subsequently reverse transcribed to generate DNA spacers (101). However, integration  
356 of DNA-derived spacers also occurs, indicating that the RNA derived-spacer route is not exclusive  
357 (101). Hence, the integrase activity of RT-Cas1-Cas2 is extended by the reverse transcriptase  
358 activity, enabling enhanced build-up of immunity against highly transcribed DNA MGEs and  
359 potentially from RNA-based invaders.

360 Despite evidence that accessory Cas proteins are involved in spacer acquisition, their roles mostly  
361 remain elusive. Furthermore, other host proteins may also be required for pre-spacer substrate

362 production. For example, RecG is required for efficient primed adaptation in type I-E and I-F  
363 systems, but its precise role remains speculative (39, 102). Additionally, it remains enigmatic why  
364 some CRISPR-Cas systems appear to require accessory proteins, whilst closely related types do  
365 not. For example, type II-C systems lack *cas4* or *csn2* that assist in type II-A and II-B adaptation,  
366 respectively. These type-specific differences exemplify the diversity that has arisen during  
367 evolution of CRISPR-Cas systems.

368

### 369 ***Evolution of adaptation***

370 The expanding knowledge of spacer integration has led to a promising theory for the evolutionary  
371 origin of CRISPR-Cas systems (103). Casposons are transposon-like elements typified by the  
372 presence of Cas1 homologs, casposases, which catalyze site-specific DNA integration and result  
373 in the duplication of repeat sites analogous to CRISPR adaptation (104, 105). It is proposed that  
374 ancestral innate defenses gained DNA integration functionality from casposases, seeding the  
375 genesis of prokaryotic adaptive immunity (106). The innate ancestor remains to be determined,  
376 but is likely to be a nuclease-based system. Co-occurrence of casposon-derived terminal inverted  
377 repeats and casposases in the absence of full casposons might represent an intermediate of the  
378 CRISPR signature repeat-spacer-repeat structures (107). However, the evolutionary journey from  
379 the innate immunity-casposase hybrid to full adaptive immunity remains unclear. Nevertheless,  
380 comparative genomics indicate that all known CRISPR-Cas systems evolved from a single  
381 ancestor (4, 5).

382 The more compact class 2 CRISPR-Cas systems likely evolved from class 1 ancestors, through  
383 acquisition of genes encoding new single-subunit effector proteins and loss of additional *cas* genes  
384 (5). Evolution of CRISPR-Cas types would have required stringent co-evolution of the adaptation  
385 machinery, leader-repeat sequences (108), crRNA processing mechanisms and effector complex  
386 function. However, despite the subsequent divergence of CRISPR-Cas systems into several types,  
387 Cas1-Cas2 remains the workhorse of spacer acquisition, central to the success of CRISPR-Cas  
388 systems (4, 5). As long as spacers can be acquired from MGEs, unique effector machineries  
389 capable of utilizing the information stored in CRISPRs will continue to evolve.

390 Mechanisms to generate Cas1-Cas2 compatible substrates, such as primed adaptation might have  
391 arisen because naïve acquisition is an inefficient and undirected process, potentially leading to  
392 high rates of lethal self-targeting spacers. However, despite the apparent advantages of primed  
393 adaptation, it was recently reported that promiscuous binding of crRNA-effector complexes to the  
394 host genome results in a basal level of self-priming, the extent of which is likely underrepresented  
395 due to the lethality of such events (51). Host *cas* gene regulation mechanisms have arisen to  
396 balance the likelihood of self-acquisition events against the requirement to adapt to new threats,  
397 for example, when the risk of phage infection or HGT is high (109, 110). Alternatively, it has been  
398 proposed that selective acquisition of self-targeting spacers could provide benefits such as  
399 invoking altruistic cell death (111), rapid genome evolution (33), regulation of host processes (112,  
400 113), or even preventing the uptake of other CRISPR-Cas systems (114).

401

### 402 **Outlook**

403 The past four years has seen rapid progress to understand the adaptation phase of CRISPR-Cas  
404 immunity. Despite this progress, many facets of CRISPR adaptation require further attention.

405 Synergy between innate defense systems and adaptation is relatively unexplored, but two roles can  
406 be envisioned; DNA breaks (57) stimulating generation of substrates for spacer acquisition (**Fig.**  
407 **4**) or stalling of infection to ‘buy time’ for adaptation (31, 115, 116). Analogously, it remains to  
408 be determined whether interference by CRISPR-Cas systems other than type I can also stimulate  
409 primed adaptation. If not, the benefits of priming might provide an explanation for why type I  
410 systems are more prevalent than other types.

411 It is also unclear why many CRISPR-Cas systems have multiple arrays used by a single set of Cas  
412 proteins, rather than a solo array. Given that Cas1-Cas2 is directed to leader-repeat junctions  
413 during integration, multiple arrays might provide additional integration sites, increasing adaptation  
414 efficiency. In addition, parallel CRISPR arrays should increase crRNA production from recently  
415 acquired spacers (i.e. due to polarization) (22). Whereas some strains have multiple CRISPR arrays  
416 belonging to the same type, other hosts have several types of CRISPR-Cas systems simultaneously  
417 (117). The benefits of harboring multiple CRISPR-Cas systems are not entirely clear, but can result  
418 in spacers used by different system to extend targeting to both RNA and DNA (118). From an  
419 adaptation perspective, multiple systems might enable a wider PAM repertoire to be sampled  
420 during spacer selection. Additional systems in a single host could also be a response to defy phage-  
421 and MGE-encoded anti-CRISPR proteins, which can inhibit both interference and primed  
422 adaptation (119-121), or may allow some systems to function in defense, while others perform  
423 non-canonical roles in gene regulation (113).

424 While Cas effector nucleases (e.g. Cas9) have been harnessed for many biotechnological  
425 applications, the use of repurposed CRISPR-Cas adaptation machinery has yet to be widely  
426 exploited. The sequence-specific integrase activity holds promise in synthetic biology, such as for  
427 the insertion of specific sequences (or barcodes) to mark and track cells in a population. In *E. coli*  
428 the feasibility of such an approach is evident (49), but transition to eukaryotic systems will provide  
429 the greatest utility where lineage tracking and cell fate could be followed, as has been performed  
430 with Cas9 (122). The elements required for leader-specific integration must be carefully  
431 considered for the introduction of CRISPR-Cas adaptation into eukaryotic cells, as unintended  
432 ectopic integrations could be problematic given the larger eukaryotic sequence space. Ultimately,  
433 our understanding of adaptation in prokaryotes may lead to applications where entire CRISPR  
434 systems are transplanted into eukaryotic cells to prevent viral invaders. As we begin to comprehend  
435 adaptation in more detail the opportunities to repurpose other parts of these remarkable prokaryotic  
436 immune systems is increasingly becoming reality.

437

438 **References and Notes:**

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