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Self vs. non-self discrimination during CRISPR RNA-directed immunity

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Abstract

All immune systems must distinguish self from non-self to repel invaders without inducing autoimmunity. Clustered, regularly interspaced, short palindromic repeat (CRISPR) loci protect bacteria and archaea from invasion by phage and plasmid DNA through a genetic interference pathway^{1–9}. CRISPR loci are present in ~40% and ~90% of sequenced bacterial and archaeal genomes respectively¹⁰ and evolve rapidly, acquiring new spacer sequences to adapt to highly dynamic viral populations^{1, 11–13}. Immunity requires a sequence match between the invasive DNA and the spacers that lie between CRISPR repeats^{1–9}. Each cluster is genetically linked to a subset of the *cas* (CRISPR-associated) genes^{14–16} that collectively encode >40 families of proteins involved in adaptation and interference. CRISPR loci encode small CRISPR RNAs (crRNAs) that contain a full spacer flanked by partial repeat sequences^{2, 17–19}. CrRNA spacers are thought to identify targets by direct Watson-Crick pairing with invasive “protospacer” DNA^{2, 3}, but how they avoid targeting the spacer DNA within the encoding CRISPR locus itself is unknown. Here we have defined the mechanism of CRISPR self/non-self discrimination. In *Staphylococcus epidermidis*, target/crRNA mismatches at specific positions outside of the spacer sequence license foreign DNA for interference, whereas extended pairing between crRNA and CRISPR DNA repeats prevents autoimmunity. Hence, this CRISPR system uses the base-pairing potential of crRNAs not only to specify a target but also to spare the bacterial chromosome from interference. Differential complementarity outside of the spacer sequence is a built-in feature of all CRISPR systems, suggesting that this mechanism is a broadly applicable solution to the self/non-self dilemma that confronts all immune pathways.

S. epidermidis strain RP62a (ref. ²⁰) contains a CRISPR locus that includes a spacer (*spc1*) that is identical to a region of the *nickase* (*nes*) gene found in nearly all sequenced staphylococcal conjugative plasmids (Fig. 1a and Supplementary Fig. 1a), including those

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that confer antibiotic resistance in methicillin- and vancomycin-resistant *Staphylococcus aureus* strains^{21–23}. The *S. epidermidis* CRISPR system limits conjugation between staphylococci by an *spc1*-directed interference pathway³. As in other species^{2, 7, 17–19, 24, 25} the *S. epidermidis* CRISPR locus is transcribed and processed into crRNAs³, and northern analysis indicates that most *spc1*-containing crRNAs are ~49 nucleotides long (Supplementary Fig. 1b). During CRISPR interference in *S. epidermidis*, target specificity appears to be achieved by direct pairing of the *spc1* crRNA with the plasmid DNA³. DNA targeting most likely underlies CRISPR interference in many other bacterial and archaeal species as well^{2, 26}. Since a sequence match also exists between the crRNA and the CRISPR locus DNA that encodes it, a central issue in CRISPR interference is therefore how the crRNA identifies bona fide targets without attacking the CRISPR locus in the host chromosome.

We previously demonstrated that CRISPR interference can prevent transformation of pC194-based plasmids that contain a target sequence³. To test whether CRISPR spacers have an intrinsic ability to evade interference, we cloned the repeat/spacer sequences of the RP62a CRISPR locus, along with ~200 base pairs (bp) from either side of the repeats and spacers, into pC194 (Fig. 1a). The resulting plasmid, pCRISPR(wt), which contains three possible interference targets (*spc1*, *spc2* and *spc3*), was transformed into wild-type and *crispr* cells. Unlike the *nes* protospacer-containing plasmid, pCRISPR(wt) transformation efficiency was similar in both strains [Fig. 1a; Supplementary Table 1 shows the transformation efficiency values for all plasmids here described]. These results indicate that the potential targets present in the CRISPR locus are specifically exempted from CRISPR interference.

We hypothesized that differences between flanking regions of spacers and targets – i.e., the presence or absence of repeats – could provide the basis for self/non-self discrimination. To test this, we replaced 15 bp from either side of the *nes* target with the corresponding *spc1*-flanking repeat sequences. The resulting plasmids [pNes(5'DR,15) and pNes(3'DR,15)] were tested for CRISPR interference by transformation into wild-type and *crispr* cells (Fig. 1b and Supplementary Fig. 2a,b). Only pNes(5'DR,15) escaped interference, indicating that repeat sequences upstream of a target (i.e., adjacent to the 5' end of the crRNA spacer sequence) can protect that target. Similar experiments further narrowed the protective region to the eight bp closest to the target (Fig. 1b and Supplementary Fig. 2b). This region contains five mutations in the *nes* upstream sequence, since the 5'-AGA-3' sequence from position –5 (i.e., 5 bps upstream of the start of the *nes* protospacer) to –3 are shared with *spc1* 5' flank (Supplementary Fig. 2a). Each of these five mutations was individually tested for its effect on CRISPR interference (Fig. 1c). Only the guanosine-to-adenosine change at position –2 (G-2A) conferred protection. These results demonstrate that repeat sequences upstream of spacers prevent CRISPR interference, and point to position –2 as an important determinant of this effect.

Short (2–4 bp), conserved sequences called “CRISPR motifs”^{12, 24, 25} or “protospacer adjacent motifs” (PAMs)²⁷ have been found near protospacers in other CRISPR systems, and mutations in these motifs can compromise interference^{12, 25}. A CRISPR motif has not been defined in *S. epidermidis* protospacers, of which only two have been specified³. To test

whether G-2 is part of a conserved motif important for interference, we tested plasmids carrying the mutations G-2C and G-2T. Surprisingly, unlike the G-2A mutation, C and T transversions had no effect on transformation efficiency (Fig. 1c). This result was corroborated in a conjugation assay³ using pG0400 G-2A or G-2C mutants (Supplementary Fig. 3) and excludes the possibility that a G at position -2 is simply a crucial CRISPR motif residue. Instead, this observation suggests that only an A at position -2 – i.e., the nucleotide present in the repeats – allows protection, and that any deviation from this nucleotide enables interference.

In light of this observation, we considered the complementarity of crRNAs with target protospacers and with CRISPR sequences: although the spacer region of a crRNA can pair with target DNA (Fig. 2a) and CRISPR DNA alike, only the CRISPR DNA will be fully complementary with the CRISPR repeat sequences at the crRNA termini. We therefore hypothesized that specific base pairs in the crRNA/DNA heteroduplex *outside* of the spacer enable protection, thereby providing a mechanism to avoid autoimmunity. To explore this possibility we introduced compensatory mutations in the crRNA (Fig. 2a, b) by changing sequences upstream of *spc1* in pCRISPR(wt), which can complement the interference deficiency of the *crispr* strain. The wild-type *spc1* crRNA contains an adenosine at position -2, so we generated the A-2G, A-2C and A-2T mutants. We then tested each for interference with conjugation of wild-type pG0400 as well as its G-2A and G-2C mutant derivatives. In agreement with our hypothesis, the *nes* target was protected from interference only when pairing was possible at position -2: rA-dT, rG-dC, and rC-dG Watson-Crick appositions each resulted in evasion of the CRISPR system by the conjugative plasmid (Fig. 2b). All crRNA mutants were functional and therefore correctly processed², since all pCRISPR plasmids were able to restore interference in *crispr* cells with pG0400 derivatives that were mismatched at position -2 (Fig. 2b). Interestingly, a rU-dG wobble apposition also protected the conjugative plasmid from interference while a rG-dT wobble apposition did not, despite the greater stability of rG-dT pairs in an otherwise Watson-Crick-paired heteroduplex²⁸. These results provide strong evidence that crRNA/target noncomplementarity at position -2 is important for interference. Further mutagenesis of the -5 to -2 sequence of *spc1* crRNA indicated that the minimal complementarity required for protection involves positions -4, -3 and -2 (Fig. 2c and Supplementary Fig. 4). Altogether these results indicate that protection of the *nes* target during conjugation in *S. epidermidis* requires complementarity between crRNA and target upstream flanking sequences at these positions, and strongly suggest that the mechanism of protection requires base-pair formation in this region (Fig. 4a). Furthermore, the base pairing implied by our compensatory analyses reinforces our earlier conclusion³ that the target of the *S. epidermidis* *spc1* crRNA is the antisense strand of the *nes* DNA locus, not the sense-oriented mRNA.

If base pairs at positions -4, -3 and -2 confer protection on an otherwise susceptible target, then abolition of base pairing in the same region should confer susceptibility on an otherwise protected CRISPR locus. Deletion analyses in pCRISPR (Fig. 3a and Supplementary Fig. 5a) demonstrated that sequences immediately upstream of spacers prevent autoimmunity. We then tested the effect of substitutions on the direct repeat upstream of *spc1* (*DR1*) on CRISPR protection (Fig. 3b, c). To avoid the expression of

mutant crRNAs that would complicate our analyses, we generated pDR1(wt), a deletion variant of pCRISPR(wt) that lacks the 200 bp preceding the CRISPR locus. This plasmid is unable to produce functional crRNAs (data not shown) but is protected from interference in wild-type cells (Fig. 3b,c). In contrast, plasmid pDR1(AGAA→TCTG), which contains mutations at positions -5 to -2, was subject to CRISPR interference. This indicates that this region is critical for protection of the CRISPR locus. Mutagenesis of this and other regions of *spc1* upstream flanking sequences (Fig. 3b, c and Supplementary Fig. 5b) indicated that at least two consecutive mismatches from positions -4 to -2 are required to eliminate protection of the CRISPR locus (Fig. 4b).

CRISPR systems show a high degree of diversity in *cas* gene content^{4, 9, 15, 16}, and therefore mechanistic differences between different CRISPR/*cas* subtypes are likely to exist. However, differential crRNA pairing potential with CRISPR loci and invasive targets outside of the spacer region (Fig. 4c) is intrinsic to all CRISPR systems, and therefore the mechanism of self/non-self discrimination that we have defined in *S. epidermidis* could apply broadly. The specific bps that are monitored could vary between CRISPR/*cas* systems, and we speculate that protein components of the system may “proofread” paired vs. unpaired structures at these sites in a manner that either aborts or enables later steps in interference, respectively. Our findings also highlight the importance of the previously noted 5'-terminal homogeneity of crRNAs^{2, 3, 17}, which consistently contain ~8 nt of upstream repeat sequences. Finally, our results are inconsistent with a critical role for the CRISPR motif^{12, 25, 27} during the interference phase of CRISPR immunity. No significant similarity exists between the two known targets of the *S. epidermidis* CRISPR locus (Supplementary Fig. 5c) and therefore we cannot determine if the nucleotides that are important for discrimination are also part of a CRISPR motif. However, our mutagenesis experiments indicate that no specific flanking nucleotides are required for interference: the decisive characteristic is noncomplementarity with the crRNA rather than nucleotide identity *per se*. By extension, it is conceivable that CRISPR motif mutants previously shown to evade interference^{12, 25} could reflect a failure of self/non-self discrimination via crRNA/target base pairing, rather than a requirement for specific nucleotide identity within the CRISPR motif. We therefore suspect that CRISPR motifs are more important during the acquisition of new spacers²⁷. In summary, our results reveal the mechanism of self/non-self discrimination in this recently discovered immune system and will facilitate efforts to exploit CRISPR interference for biotechnological applications.

Methods summary

Bacterial strains and growth conditions

S. epidermidis wildtype (RP62a, ref. ²⁰) and *crispr* (LAM104, ref. ³) and *S. aureus* (RN4220, ref. ²⁹) strains were grown in brain-heart infusion (BHI) and tryptic soy broth media, respectively. When required, the medium was supplemented with antibiotics as follows: neomycin (15 µg/ml) for selection of *S. epidermidis*; chloramphenicol (10 µg/ml) for selection of pC194-based plasmids; and mupirocin (5 µg/ml) for selection of pG0400-based plasmids. *E. coli* DH5α cells were grown in LB medium, supplemented with ampicillin (100 µg/ml) or kanamycin (50 µg/ml) when necessary.

Conjugation and transformation

Conjugation and transformation were performed as described previously³ with the following modification: transformations of *S. epidermidis* were recovered at 30 °C in 150 µl of BHI for 6 hs. Corroboration of the presence of the desired plasmid in transconjugants or transformants was achieved by extracting DNA of at least two colonies or agar of empty plates, performing PCR with suitable primers and sequencing the resulting PCR product.

Online Methods

DNA cloning

All plasmids used in this study were constructed by cloning CRISPR or *nes* sequences into the *Hind*III site of pC194 (ref. ³⁰). Inserts were generated by PCR using primers and templates described in Supplementary Table 2. Supplementary Table 3 contains primer sequences. To introduce mutations, internal primers carrying the desired substitution were used with external primers, thus generating two PCR products that were then joined by nested PCR using the external primers. Amplified DNA was cloned into pCR2.1 vector (Invitrogen), the resulting plasmid purified and the insert sequenced. Inserts were cut from pCR2.1 with *Hind*III, purified and ligated into pC194. Ligation products were transformed into *S. aureus* OS2 cells³¹ and colonies containing the desired insert were identified by PCR using primers P86/P87.

pG0400 mutagenesis

Mutations were introduced by allelic exchange as previously described³. P15 and P18 primers were used with reverse and forward primers containing the desired mutation, respectively, to amplify 1 kb of *nes* sequence. PCR products were then joined by nested PCR using P15/P18 primers and recombined into pKOR1 (ref. ³²). Recombination products were transformed into *E. coli* DH5α, and the resulting plasmids were purified, sequenced and transformed into *S. aureus* RN4220 (pG0400) (ref. ³³) for allelic exchange. pG0400(G-2A), pG0400(G-2C) and pG0400(G-2T) mutants were generated using the knock-out constructs pLM397, pLM406 and pLM405, respectively. Inserts contained in these plasmids were generated by joining the PCR products obtained with the following primer pairs: pLM397, P15/P190 and P191/P18; pLM406, P15/P208 and P209/P18; pLM405, P15/P206 and P207/P18. See Supplementary Table 3 for primer sequences.

Northern blot analysis

RNA extraction of *S. epidermidis* and oligonucleotide labelling was performed as indicated in ref. ³. Northern blot analysis was carried out according to Pall *et al.* ³⁴ using UV crosslinking and a hybridization temperature of 37°C.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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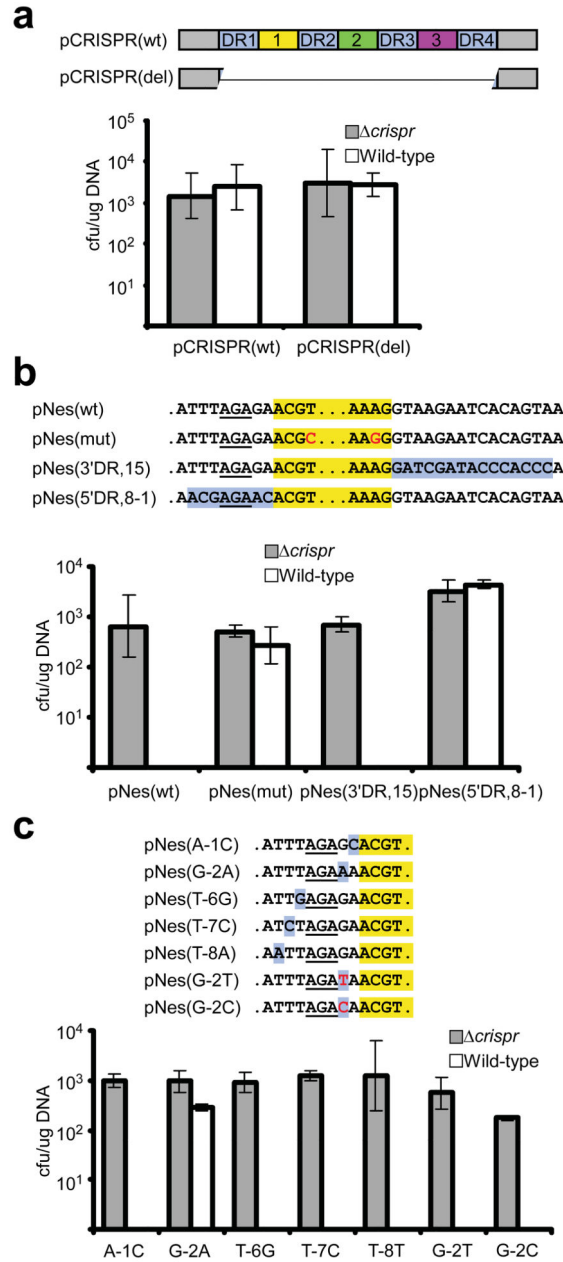


Figure 1. Protection of *nes* target by spacer flanking sequences

a, Direct repeats (DR1–4, purple boxes) and spacers (1–3, colored boxes) of the *S. epidermidis* RP62a CRISPR locus were cloned into pC194 generating pCRISPR(wt) and its deletion variant, pCRISPR(del). **b**, pNes(wt) and pNes(mut) contain wild-type and mutated *nes* target sequence of pG0400 (highlighted in yellow, with mutations in red). In pNes(5'DR, 8-1) and pNes(3'DR,15) *nes* target flanks were replaced by repeat sequences present upstream and downstream of *spc1* (highlighted in purple), respectively. **c**, Individual nucleotides upstream of *nes* target were replaced by those present upstream of *spc1* (highlighted in purple). The G at position –2 was also changed to C and T (in red). The AGA sequence (underlined) is shared by both the *nes* target and spacer 5' flanking

sequences. All plasmids were transformed into *S. epidermidis* RP62a and its isogenic *crispr* mutant. The average of at least three independent measures of the transformation efficiency (determined as cfu/ μ g DNA) is reported and error bars indicate 1 s.d.

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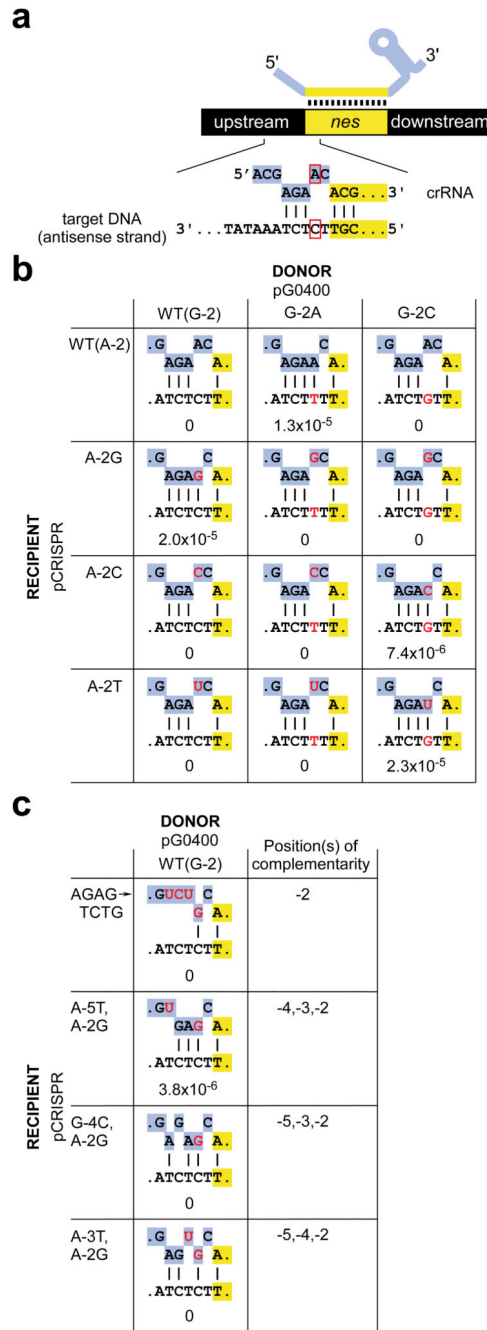


Figure 2. Complementarity between crRNA and target DNA flanking sequences is required for protection

a, Schematic of the complementarity between the flanking sequences of crRNA (top, highlighted in purple) and target DNA (bottom). The red box indicates the nucleotides mutated in the experiments shown in **b**, **c**. **b**, **c**, Conjugation assays of pG0400 and its mutant variants, using as a recipient the *crispr* strain harboring different pCRISPR plasmids. Mutations are shown in red. Conjugation efficiency was determined as transconjugant cfu/recipient cfu; the average of at least 3 independent experiments is reported.

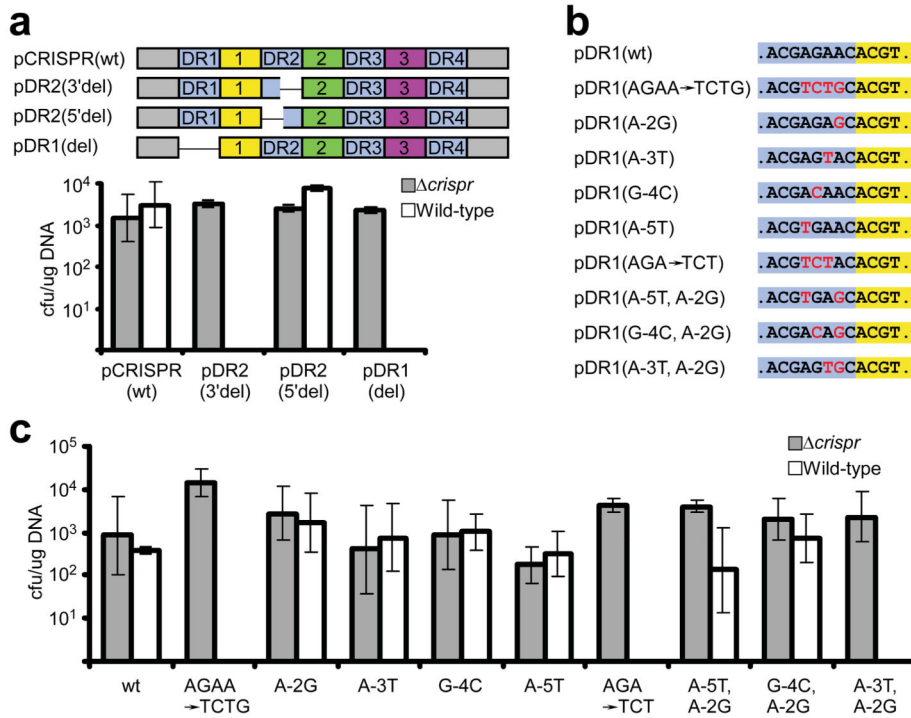


Figure 3. Mutations in upstream flanking sequences of CRISPR spacers elicit autoimmunity
a, Deletions were performed in the flanking repeats of *spc1*: the 3' half of DR2, the 5' half of DR2, and all of DR1 were deleted from pCRISPR(wt) in pDR2(3'del), pDR2(5'del) and pDR1(del), respectively. **b**, **c**, Substitutions (red) were introduced in the 5' flanking sequence (highlighted in purple) of *spc1* (highlighted in yellow), generating different pDR1 variants that were tested by transformation. All plasmids were transformed into *S. epidermidis* RP62a and *crispr* strains. The average of at least three independent measures of the transformation efficiency (determined as cfu/μg DNA) is reported and error bars indicate 1 s.d.

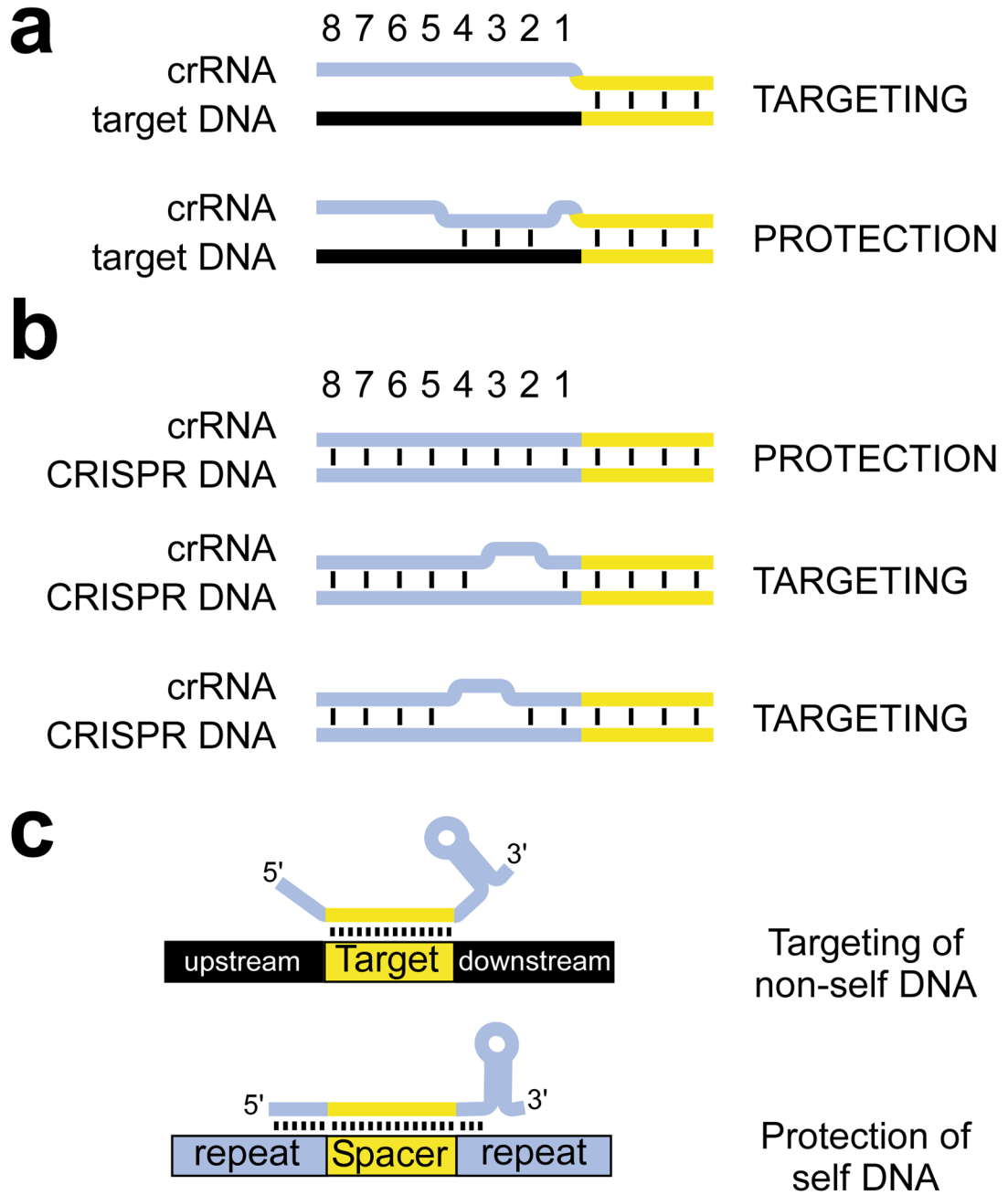


Figure 4. Requirements for targeting and protection during CRISPR immunity

a, In *S. epidermidis*, CRISPR interference is enabled by mismatches between target DNA and crRNA sequences upstream of the spacer. Formation of at least 3 base pairs at positions -4, -3 and -2 eliminates targeting. **b**, Complementarity between the *S. epidermidis* CRISPR locus and the crRNA 5' terminus protects it from interference. Disruption of base pairing at positions -4, -3 or -3, -2 eliminates protection. **c**, General model for the prevention of autoimmunity in CRISPR systems. The ability of crRNA termini (5', 3', or both) to base pair

with potential targets enables discrimination between self and non-self DNA during CRISPR immunity.

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