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Anti-CRISPR protein applications: natural brakes for CRISPR-Cas technologies

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Clustered, regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) genes, a diverse family of prokaryotic adaptive immune systems, have emerged as a biotechnological tool and therapeutic. The discovery of protein inhibitors of CRISPR-Cas systems, called anti-CRISPR (Acr) proteins, enables the development of more controllable and precise CRISPR-Cas tools. Here we discuss applications of Acr proteins for post-translational control of CRISPR-Cas systems in prokaryotic and mammalian cells, organisms and ecosystems.

Coevolutionary arms races are fertile ground for innovation. The strong selective pressures exerted by mobile genetic elements (MGEs) on their prokaryotic hosts exemplify this, yielding robust technologies such as restriction enzymes and CRISPR-Cas nucleases. CRISPR-Cas systems provide prokaryotes with adaptive immunity from their pathogens. These multiprotein (class 1) or single-protein (class 2) nuclease complexes use a guide or CRISPR RNA (gRNA or crRNA) to target invasive nucleic acids. The specificity and easily programmable nature of CRISPR-Cas nucleases have been repurposed for various molecular, biotechnological and medical applications, including gene editing, gene knockouts and gene regulation[1,2]. However, despite the revolutionary advantages offered by the growing CRISPR-Cas toolbox, several challenges remain for the efficacy and safety of these technologies. For example, Cas nuclease activity in cells during in vivo or ex vivo editing can lead to off-target effects[3], unexpected on-target effects[4], cellular toxicity[5,6] and immunogenicity[7,8]—all of which need to be addressed for the development of safe genome editing applications.

Acr proteins are a collective arsenal of natural bona fide CRISPR-Cas antagonists encoded by diverse MGEs, such as plasmids and phages, that inhibit CRISPR-Cas immune function at various stages[9,10]. To date, 45 non-homologous Acr proteins (24 for class 1 CRISPR-Cas, 21 for class 2) have been discovered, with distinct mechanisms and structures and no significant sequence similarities to each other[11–13] (Box 1). Distinct *acr* genes can often be found next to each other, which has enabled their discovery[14]. The ability of many Acr proteins to directly interfere with CRISPR-Cas functions in heterologous hosts provides genetically encodable, post-translational regulation for CRISPR-Cas-derived technologies.

Characterized Acr proteins inhibit CRISPR-Cas function by interacting directly with a Cas protein to prevent target DNA binding, cleavage, crRNA loading or effector-complex formation (Table 1 and Fig. 1). Some Acr proteins that inhibit type I CRISPR-Cas systems, for example, interact with the crRNA-guided Cascade complex and prevent DNA binding, whereas others prevent recruitment of the Cas3 nuclease[15]. The mechanisms elucidated for type II Acr proteins have presented similar conclusions, where a direct interaction with Cas9 limits DNA binding through steric occlusion[16,17] or prevents the activation of the HNH nuclease domain—allowing DNA binding but blocking target cleavage[18]. The Cas12a inhibitor AcrVA1 operates through an enzymatic mechanism, cleaving the gRNA when bound to Cas12a[12].

Acr proteins are named for the system that they inhibit in the order in which they were discovered[19]. For example, the widely used AcrIIA4 protein was the fourth type II-A Acr protein discovered. Several Acr proteins have already proven successful at regulating gene-editing activities in different cell types, most notably two SpyCas9 inhibitors (AcrIIA2 and AcrIIA4)[20] and two NmeCas9 inhibitors (AcrIIC1 and AcrIIC3)[21]. Here we discuss these proteins and others as useful technologies for regulating CRISPR-Cas-derived tools in both bacterial and eukaryotic cells, including their advantages and disadvantages (Boxes 2 and 3). Additionally, we highlight the use of Acr proteins for inhibiting gene drives, controlling catalytically dead CRISPR-Cas-based applications, and we propose potential future applications. For more details on the current CRISPR-Cas toolkit, we refer readers to recent reviews[1,22].

Acr proteins in prokaryotes

CRISPR-Cas is a powerful tool for many prokaryotic applications[23]. Cas9-based editing has been used in numerous bacteria, from model organisms such as *Escherichia coli*[24] to industrially relevant species belonging to *Clostridium*, *Lactobacillus* or *Streptomyces* genera[25]. Endogenous CRISPR-Cas systems can also be used for editing applications, as approximately 40% of all sequenced, culturable bacteria and 87% of archaea carry a CRISPR-Cas system of some type[26]. In these applications, Acr proteins have dual utility: (i) identifying strains where rampant endogenous CRISPR-Cas inhibition suggests that editing will not be effective and (ii) enhancing temporal control, which can enable editing of bacterial and phage genomes that was not previously possible (Fig. 2a).

In general, there are two routes to achieve genomic editing or targeted gene repression with CRISPR-Cas in bacteria: either introduce an entire exogenous system into the given organism[27] or reprogram an endogenous system by expressing a self-targeting crRNA[28]. Chromosomal targeting with both strategies kills bacteria at high efficiencies, with genome-edited cells constituting a fraction of survivor cells. The presence of Acr proteins could greatly impede the high efficiency of these processes. In fact, genome-encoded Acr

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Box 1 | Acr protein discovery

Likely as a consequence of the fast-paced evolutionary arms race, Acr proteins display little sequence or structural similarities with each other or proteins of known function. Apart from sharing a typically low molecular weight, Acr proteins lack a common denominator, rendering de novo prediction challenging. Current discovery approaches have used the clustering of *acr* genes into '*acr* loci', typically in the vicinity of conserved *aca* transcriptional regulators[14,81]. An alternative discovery strategy is based on the detection of 'self-targeting' genomes—that is, genomes in which there are CRISPR spacers that match MGEs integrated in the same genome (for example, prophages)[20,67,98]. In principle, the resultant autoimmune state should trigger cell death, and consequently, tolerance to self-targeting suggests that the integrated MGEs encode *acr* genes. Additionally, *acr* genes have been found by screening lytic phages to find those that evade CRISPR-Cas immune targeting[87] and through metagenomics screens[92,95]. Putative *acr* genes are functionally verified using in vivo or in vitro functional assays of CRISPR interference inhibition[20,67,82,98,99]. Future work in this area will likely feature novel screening approaches focused on a specific mode of action, algorithms for Acr prediction (for example, machine learning), and the thorough characterization of the spectrum of activity for Acr proteins. Additionally, the identification of novel inhibitory mechanisms, such as catalytic Acr proteins (for example, AcrVA1 and AcrVA5)[12,13], will likely prove highly useful.

proteins are highly prevalent in bacteria; >30% of *Pseudomonas aeruginosa* strains carrying a CRISPR-Cas system also encode one or more cognate *acr* genes (with the number likely to be higher as more of these gene families are identified)[29]. Similarly, Acr proteins that inhibit Cas9 are found in >50% of *Listeria monocytogenes* strains encoding Cas9 (ref. [20]), indicating that this may present a frequent obstacle when attempting to use CRISPR-Cas systems to edit or kill bacteria. If a bacterial strain encodes an *acr* gene against an endogenous CRISPR system, then using an exogenous system for editing may be a more productive approach. In bacteria that naturally have Cas9 orthologs, such as *L. monocytogenes*, the commonly used SpyCas9 protein may not be a viable approach because of the presence of *acr* genes that inhibit it. The continued identification of Acr proteins and mechanisms to inhibit or block their activity is needed to broadly enable CRISPR-based editing in bacteria.

Acr proteins can also be directly used to enhance microbial gene-editing strategies (Fig. 2a–c). For example, the low transformation efficiencies of many microbes limit one's ability to recover transformants after expressing a genome-targeting crRNA. The controlled expression of Acr proteins (potentially on the same construct expressing the synthetic crRNA) may mitigate the toxic effects of genomic targeting, enabling stable transformation (Fig. 2b). Upon repression or de-induction of Acr protein production, editing can commence from a larger starting population. Additionally, using *acr* genes as selection markers to confer resistance against native CRISPR-Cas systems can provide a new route for the engineering of viruses, for which there are a paucity of selectable markers (Fig. 2c). This strategy was recently used to knock out genes in a difficult-to-engineer archaeal virus *Sulfolobus islandicus* rod-shaped virus 2 (SIRV2)[30]. *acrID1* was used to replace a gene of interest, thereby providing positive selection for edited viruses when challenged with the native *S. islandicus* type I-D CRISPR-Cas system.

CRISPR-Cas systems have previously been engineered to repress gene expression in bacteria[31]. Although these CRISPR interference (CRISPRi) systems have mostly used exogenous catalytically dead class 2 enzymes, endogenous type I CRISPR-Cas systems can be repurposed to achieve specific gene silencing[28]. This would require, however, the inactivation or deletion of the effector nuclease (Cas3 in this case). AcrIF3 and AcrIE1 prevent Cas3 recruitment to the surveillance complex at the genomic target[15,32], thereby 'activating' CRISPRi in the absence of any genome manipulation (Fig. 2d). Similarly, broad-spectrum AcrIIC1 allows Cas9 to bind DNA but prevents cleavage, likely enabling CRISPRi in bacteria with II-C CRISPR systems[18].

Acr proteins may also be useful in antibacterial applications. As CRISPR-Cas systems have been proposed to regulate bacterial virulence[33], Acr proteins could disrupt the CRISPR-dependent virulence mechanisms of these bacterial pathogens (Fig. 2e). Non-canonical Cas functions, however, may not be inhibited by phage proteins; therefore, investigation of this prediction is needed.

Lastly, *acr* genes may also be used to augment phage therapy approaches, as they can expand phage host range (Fig. 2f). With the global propagation of antibiotic-resistant bacteria, phage therapy has reemerged as an alternative method for combating bacterial infections[34]; however, many bacterial pathogens are naturally equipped with active CRISPR-Cas systems that may limit the efficacy of phage-based therapeutic approaches. Because of their small size, an arsenal of Acr proteins could be engineered into therapeutic phages to combat CRISPR-based phage resistance, leading to improved efficacy for these antibacterial strategies.

Acr proteins in eukaryotes

CRISPR-Cas systems have been heterologously expressed in many eukaryotic systems, including fungal[35], plant[36] and mammalian cells[37,38]. Cas9 and Cas12a have primarily been used due to their ease of programmability and expression in many hosts[39,40]. However, strategies to limit and/or control Cas nuclease activity are limited. Moreover, Cas nucleases have been shown to cause variable degrees of off-target editing[3,41,42], which could be remedied with 'off switches'. Strategies to prevent off-target editing have generally focused on limiting nuclease activity and expression, such as injecting preformed ribonucleoprotein (RNP) complexes[42–44], introducing additional regulatory domains[45,46] or mutating Cas9 (refs. [47,48]). Although these strategies have reduced off-target effects, they have important limitations. Regulatory domains can substantially increase the size of Cas9 and often require additional ligands. High-fidelity Cas9 variants can work well for certain guides and delivery modalities but are not universally efficacious and require extensive engineering for each Cas ortholog[47–49]. Although limiting the duration of CRISPR-Cas activity with RNP delivery can be effective for reducing off-target effects, this strategy is not adaptable for genetically encoded systems, such as gene drives and in vivo delivery via viral vectors. Moreover, the duration of RNP activity cannot be tightly controlled without an additional level of regulation.

The delayed introduction of an Acr protein presents a flexible and tunable mechanism to limit off-target editing while using the wild-type version of the Cas enzyme. For example, delivery of AcrIIA4, either encoded on a plasmid or as purified protein, 6 h after the introduction of Cas9 RNPs was found to reduce off-target editing in human cells[50]. This was effective for cells using different single guide RNAs (sgRNAs) that target β-globin (*HBB*) and vascular endothelial growth factor A (*VEGFA*) with reported off-target sites. The underlying assumption is that off-target edits begin to accrue after a majority of on-target editing has occurred. If so, there should be an ideal and empirically measurable moment where anti-CRISPR activity is optimal, which may vary by cell type and target.

The relatively small size of most Acr proteins (~50–200 amino acids) also makes them promising CRISPR-Cas modulators during delivery in situ via adeno-associated viral (AAV) vectors. These vectors are commonly used to deliver genes to cells within specific tissues but are tightly limited on cargo size. Regulation of Cas nuclease activity in tissues is particularly important, given reports of

Table 1 | Summary of identified Acr mechanisms

Type	Stage inhibited	Subtype inhibited	Acr name	Cas ortholog	Refs.
I	DNA binding	I-F	AcrIF1	PaeCascade (I-F), PecCascade (I-F)	15,81,82
			AcrIF2	PaeCascade (I-F), PecCascade (I-F)	15,81,82
			AcrIF4	PaeCascade (I-F)	82
			AcrIF10	PaeCascade (I-F), PecCascade (I-F)	81,83
		I-D	AcrID1	SisCas10d (I-D)	84
	DNA cleavage	I-E	AcrIE1	PaeCas3 (I-E)	32
		I-F	AcrIF3	PaeCas3 (I-F)	15,82
	Unknown	I-C	AcrIC1	PaeCascade/Cas3 (I-C)	67
		I-E	AcrIE2–7	PaeCascade/Cas3 (I-E)	67,85
		I-F	AcrIF5–9 AcrIF11–14	PaeCascade/Cas3 (I-F)	67,81,82
II	DNA binding	II-A	AcrIIA2[a]	SpyCas9, LmoCas9	16,20
			AcrIIA4[a]	SpyCas9, LmoCas9	16,20
			AcrIIA6[a]	St1Cas9	86,87
		II-C	AcrIIC3[a]	NmeCas9, Nme2Cas9, HpaCas9, SmuCas9	18,21, 88,89
			AcrIIC4[a]	NmeCas9, Nme2Cas9, HpaCas9, SmuCas9	88
			AcrIIC5[a]	NmeCas9, HpaCas9, SmuCas9	88
	Guide loading		AcrIIC2[a]	NmeCas9, SmuCas9, HpaCas9	21,88, 90,91
	DNA cleavage		AcrIIC1[a]	NmeCas9, Nme2Cas9, CjeCas9, GeoCas9, SmuCas9, HpaCas9	18,21, 88,91
		II-A	AcrIIA11[a]	SpyCas9, TdeCas9	92
	Unknown	II-A	AcrIIA1	LmoCas9, SpyCas9	20,93
			AcrIIA3	LmoCas9, SpyCas9	20
			AcrIIA5[a]	St1Cas9, St3Cas9, SpyCas9	87,94
			AcrIIA7–10	SpyCas9	95
III	Interferes with Csx1 RNase	III-B	AcrIIIB1	SisCmr-α; SisCmr-γ	96
V	DNA binding	V-A	AcrVA1[a]	MbCas12a, AsCas12a, LbCas12a, FnCas12a	12,67, 97,98
			AcrVA4[a]	MbCas12a, LbCas12a	12,97,98
			AcrVA5[a]	MbCas12a, LbCas12a	13,98
	Unknown		AcrVA2	MbCas12a	67
			AcrVA3.1[b]	MbCas12a, PaeCascade/Cas3 (I-C)	67
VI	Unknown	VI-B	Csx27[c]	BzoCas13b, PbuCas13b	80

[a]Acr protein functions in human cells. [b]Some Acr proteins inhibit multiple subtypes. AcrVA3.1 has been shown to inhibit V-A and I-C CRISPR-Cas systems. AcrIE4-IF7, a fusion of AcrIE4 and AcrIF7, has been shown to inhibit both I-F and I-E subtypes[67]. [c]Csx27 has Acr function but appears to be a Cas protein, likely serving a regulatory role.

off-target effects and cytotoxicity associated with excessive nuclease activity[3,5,6,51]. Indeed, cytotoxicity and poor engraftment outcomes for CD34+ ex vivo hematopoietic stem cells expressing Cas9 have been reported[6]. The delivery of AcrIIA2 and AcrIIA4 on a single adenovirus vector two days after Cas9 significantly improved engraftment outcomes in mice with on-target editing rates unaffected. Another study recently reported editing of the *CEP290* gene in photoreceptor cells in mice and non-human primates using AAV-mediated delivery of SaCas9 and sgRNA[52]. This study reported robust SaCas9 expression in the eye up to 40 weeks after a single dose, even though editing was complete after ~1 week. The persistence of Cas9 expression indicates that a mechanism to inactivate Cas9 would be advantageous, given that the enzyme is presumably active. Inactivation could be especially important for more promiscuous sgRNAs or for more immunocompetent tissues than the eye. Progress toward Acr deployment in vivo has recently been made with AcrIIC3 successfully inhibiting Nme2Cas9 editing in mice, without any apparent toxicity[53]. Delivery of AcrIIC3 and the sgRNA on one AAV with Nme2Cas9 on another led to near-complete inhibition of editing in the heart and liver. Moreover, a miRNA-based strategy to prevent AcrIIC3 production in specific tissues was successful[53].

CRISPR-Cas systems are increasingly being harnessed to alter gene expression without cleavage[1]. dCas9 or dCas12a have been fused to various functional domains, including transcriptional activators, repressors and epigenetic modifiers[1] (Fig. 3). These functional domains are then recruited to specific sites by the dCas-guide complex. Because most characterized Acr proteins prevent Cas proteins from binding DNA, they have been used to spatially and temporally regulate these processes and confirm that they result from Cas-dependent activity. For example, AcrIIA4 was recently used to inactivate a dCas9-Tet1 demethylase fusion and demonstrated the remarkable persistence of demethylation in the absence of continued dCas9-Tet1 activity[54] (Fig. 3). Similarly, dCas9 has also been fused to fluorescent reporters to visualize chromatin dynamics in a sequence-specific manner[1,55]. AcrIIC3 was shown to prevent

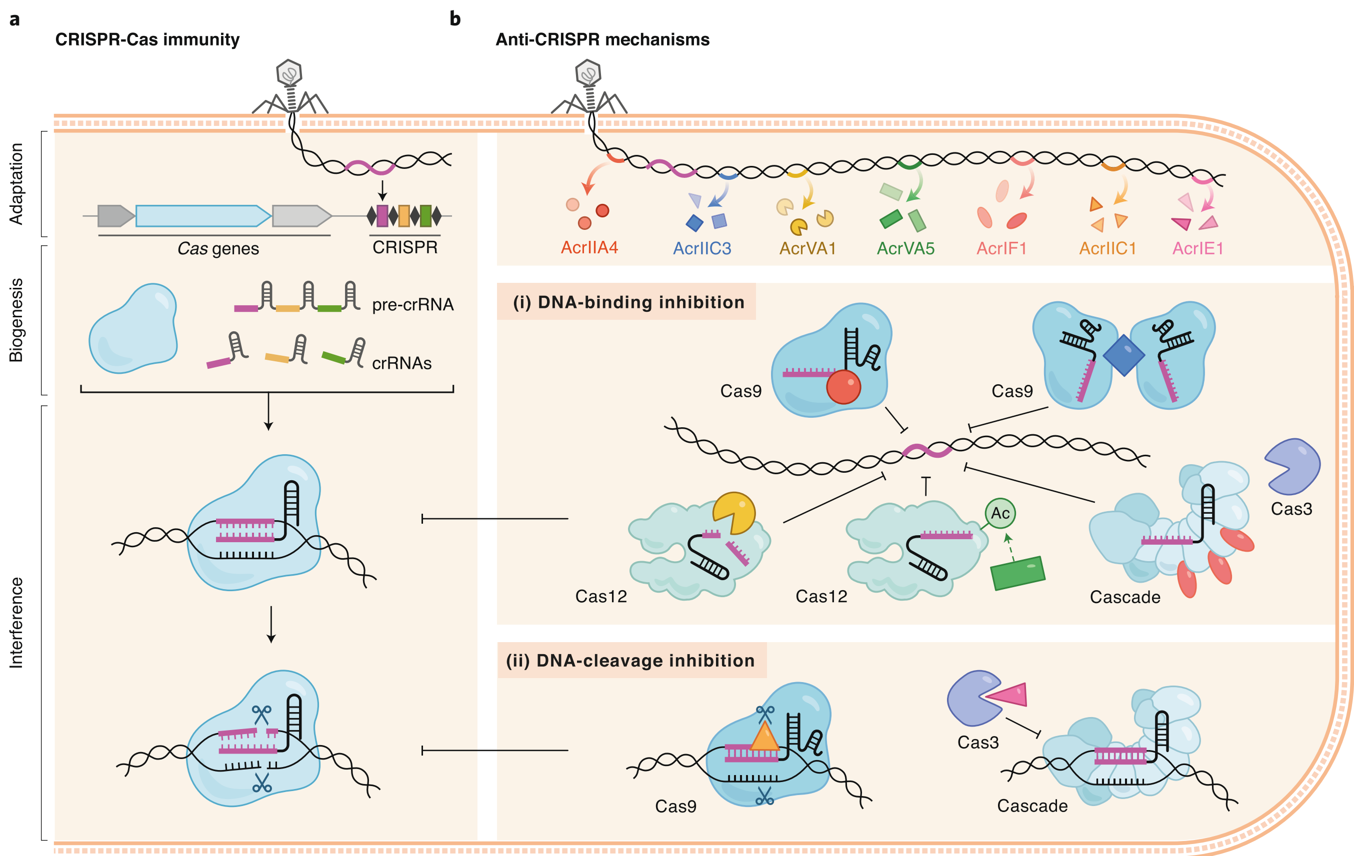

Fig. 1 | Stages of CRISPR-Cas immunity and mechanisms of Acr function. a, Stages of CRISPR-Cas immunity. During adaptation, a fragment of the invading nucleic acid is incorporated as a new spacer (purple) in the CRISPR array. In the biogenesis stage, the CRISPR array is transcribed into a long precursor CRISPR RNA (pre-crRNA) that is processed by Cas proteins into single crRNAs (purple, yellow and green) that act as guides for Cas effector nucleases (blue). During interference, the crRNA-guided effector nucleases survey the cell in search of a cognate sequence, leading to target cleavage upon binding. **b**, Mechanisms of Acr function. Targeted phages and other MGEs can bypass CRISPR-Cas immunity by expressing Acr proteins. Although the inhibitory functions of the Acr proteins characterized to date are highly diverse, they can be broadly classified into two main mechanisms of action. (i) Inhibitors of target DNA binding: for example, AcrIIA4 (red) occludes Cas9's PAM recognition domain, AcrIIC3 (blue) forces Cas9 dimerization, AcrVA1 (yellow) cleaves Cas12a's crRNA, AcrVA5 (green) inhibits Cas12a via post-translational acetylation (Ac), and AcrIF1 (vermilion) prevents target DNA binding by binding to Cascade. (ii) Inhibitors of DNA cleavage: for example, AcrIIC1 (orange) disables Cas9 by binding to the HNH nuclease domain, and AcrIE1 (pink) blocks DNA cleavage by binding to Cas3.

NmeCas9-GFP localization to telomeres in human cells[21]. Given reports of high background fluorescence from free-floating CRISPR complexes[56], Acr proteins were used to determine whether their localization patterns are specifically due to target binding (Fig. 3).

Fusion variants of Cas9 and Cas12a have also been developed for applications other than gene regulation. Base editors, which consist of a catalytically impaired Cas protein fused to a nucleotide deaminase, convert one nucleotide base pair to another at specific sites without inducing double-strand breaks[57–59]. Given recent reports of off-target base editing of RNA in some systems[60,61], Acr proteins may be a useful reagent for this and other recruitment efforts to mechanistically dissect off-target events mediated by the fused enzyme compared to Cas9 itself.

The ability of some Acr proteins to prevent target binding by Cas proteins has also been harnessed for biosensors and synthetic gene circuits (Fig. 3). For example, a biosensor that couples induction of anti-CRISPR expression or activity with enhancement of fluorescence expression was constructed in *Saccharomyces cerevisiae*[62]. In this case, AcrIIA2 and AcrIIA4 were transcriptionally induced or post-translationally activated by small molecules in a system where dCas9::sgRNA complexes are programmed to constitutively repress green fluorescent protein (*eGFP*) transcription, enabling a simple readout to detect these molecules. Another genetic circuit was developed in mammalian cells using dCas9 fused to VPR, a transcriptional activator domain[63]. Expression of dCas9-VPR simultaneously induced expression of both GFP and AcrIIA4, which subsequently bound dCas9-VPR and prevented further GFP activation. This feedback loop generated a pulse of fluorescence, demonstrating the utility of *acr* genes for creating dynamic gene circuits.

Regulation of Acr proteins

Multiple strategies have been developed to modulate Cas9 expression and activity[1], including inducible promoters[64] and destabilization domains that allow expression only in the presence of certain ligands[46]. Other strategies control the functionality of Cas9 protein using light[45,65] or small molecules[46,66]. These strategies have shown efficacy for SpyCas9 regulation, but they have not been adapted to all of the other natural and engineered Cas variants that differ in size, fidelity and protospacer-adjacent motif (PAM). Engineering all nucleases to include these regulatory domains or finding potent small molecules to inhibit them remains a formidable challenge. Fortunately, Acr proteins have been found for most major Cas proteins, and some can inhibit more than one Cas ortholog (for example, AcrIIA5, AcrIIC1 or AcrVA1). They can therefore be used to inhibit multiple Cas orthologs or different engineered variants of the same ortholog without modifying each nuclease. Furthermore,

Box 2 | Advantages of Acr proteins

Although many strategies for regulating CRISPR-Cas activity have been developed, Acr proteins have several features that make them well suited for certain applications:

Genetically encodable. *acr* genes can be encoded and delivered on vectors to cells in vivo or used to halt gene drives in situ. Because they are separate from the CRISPR-Cas system, they can be deployed to shut off or maintain the desired dynamic range of Cas activity as needed. This can be used to continually protect cells from editing, finely tune the amount and duration of Cas activity, and limit undesired background levels in inducible CRISPR-Cas systems.

Broad spectrum. Many Acr proteins, such as AcrIIA5, AcrIIC1 and AcrVA1, have been found to inhibit multiple orthologs of its target[18,67,87,98]. This broad-spectrum activity can be used to regulate multiple natural and engineered variants of CRISPR-Cas systems without re-engineering each Cas protein.

Diverse in strength and mechanism. Multiple Acr proteins have been discovered to target the same nuclease yet vary substantially in their size, strength of inhibition, and mechanism of action[9,11]. Acr proteins can accordingly be selected and optimized (or weakened) according to the needs of the assay (Table 1).

Easy to use. Acr proteins can easily be integrated into a wide range of in vivo and in vitro systems using a standard molecular biology toolkit, without the need for expensive ligands, equipment or protein engineering. The direct mode of action for many characterized Acr proteins ensures that they function in heterologous hosts. They are also complementary to many existing strategies of regulation.

Box 3 | Limitations of Acr proteins

Additional component. External regulation with Acr proteins introduces an extra component to the system. The use of genetically encoded *acr* sequences may necessitate an additional vector or increase vector size. For transient inhibition, purified Acr protein can be used instead, but unlike small-molecule inhibitors of Cas9 (ref. [66]), Acr proteins are not cell-permeant and must be delivered into cells.

Slow reversibility. Although the degree of CRISPR-Cas inhibition can be titrated with Acr proteins of varying potency, a single inhibition event is not readily reversible without additional engineering. Stoichiometric CRISPR-Cas inhibition by Acr proteins can be overcome by increasing the amount of Cas protein or decreasing Acr expression, but this may be slower than other regulatory methods, such as small molecules or optogenetics directly acting on Cas enzymes. Fortunately, light- and ligand-inducible variants of AcrIIA4 have been engineered to improve reversibility and temporal control[63,69], but this has yet to be developed for other Acr proteins.

Potential toxicity or immunogenicity. Two Acr proteins have been expressed in mice without causing apparent tissue damage[53], but it is not yet known if they interact with other host proteins or provoke a host response. Expression of some Acr proteins has displayed toxicity[67]. Additional parameters must be considered in vivo, including Acr protein stability, optimal expression levels, and potential for off-target interactions.

'regulating the regulator' of CRISPR-Cas activity will likely provide tighter on–off control than directly regulating the Cas enzyme because one can induce the Acr protein, which represses Cas activity, instead of relying on a leaky basal off-state.

To achieve more rapid and dynamic control of CRISPR-Cas activity, several methods for regulating Acr expression and activity have been discovered or developed, including (i) transcriptional, (ii) post-transcriptional, (iii) optogenetic and (iv) ligand-based strategies[63,67–69] (Fig. 3). (i) Anti-CRISPR-associated (*aca*) genes have recently been shown to repress *acr* transcription in bacteria by binding to the native *acr* promoter[70]. These *aca* genes are usually encoded within the same operon as *acr* genes and provide a means to downregulate initially high levels of *acr* transcription. In heterologous systems, *acr* genes have been transcriptionally regulated using inducible promoters[17,62,67]; however, temporal regulation could also be achieved with promoters expressed at a specific stage of the cell cycle or in response to cellular events. (ii) Post-transcriptional regulation of Acr proteins using tissue-specific microRNAs has recently been developed to control Cas9. This was achieved by modifying the 3′ untranslated region of *acrIIA4* and *acrIIC3* transcripts to include binding sites for microRNAs (miRNAs) that are highly expressed in liver cells[53,68,71]. These miRNAs effectively downregulate AcrIIA4 and AcrIIC3 in hepatocytes, where Cas9 activity is desired, but allow it to inhibit Cas9 in other cell types. This strategy effectively inhibited in vivo Nme2Cas9 editing in heart tissue but allowed editing in liver cells[53]. (iii) A photo-controllable AcrIIA4 variant was developed by inserting the LOV2 domain from *Avena sativa* phototrophin-1 into an AcrIIA4 surface-exposed loop and optimizing for functionality[69]. In the absence of light, AcrIIA4-LOV2 can bind and inhibit SpyCas9, but upon photoexcitation, the LOV2 domain loses its structural conformation and causes AcrIIA4 to misfold and lose affinity for Cas9. (iv) A ligand-inducible Acr protein was also recently developed by fusing AcrIIA4 to a destabilization domain (DD)[63]. In the presence of Shield-1, the DD is stabilized, allowing AcrIIA4 to maintain structural integrity and inhibit Cas9. In the absence of Shield-1, AcrIIA4 misfolds and becomes degraded, thereby liberating Cas9. In that study[63], DD-AcrIIA4 inhibited a dCas9-VPR transcriptional activator in an inducible manner. Fusing dCas9-VPR directly to a destabilization domain did not render dCas9 inducible, perhaps owing to limitations imposed by the VPR domain. This finding demonstrates the value of using inducible Acr proteins to control Cas proteins that may not be amenable to direct regulation or further engineering.

Application of Acr proteins for gene drives

The advent of CRISPR-Cas9-based technologies has accelerated the potential for ecological engineering through the use of 'gene drives', which spread engineered traits within a population through a super-Mendelian mechanism[72] (Fig. 4a,b). Gene drives often feature a transgenic organism with chromosomally encoded Cas9 that is programmed to target the homologous region on the sister chromosome. When the targeted region repairs the cut using the drive sequence as a template, Cas9 and its associated cargo become encoded on both chromosomes. Gene drives have the potential to greatly benefit human health in various ways, including curtailing insect-borne diseases such as malaria or dengue[73,74], eliminating invasive species, and increasing agricultural sustainability[75]. However, gene drives have been met with calls for caution[76], as they could have unforeseen consequences or be co-opted for nefarious purposes, leading to large-scale devastation. For these reasons, multiple robust safety measures are needed before gene drive technologies can be used in the wild.

Acr proteins currently present the most direct and broadly acting (that is, independent of sgRNA sequence) method for inhibiting or modulating drive strength and could be deployed concomitantly with or after a gene drive (Fig. 4c). It was recently demonstrated that both AcrIIA2 and AcrIIA4 can inhibit gene drives, at varying levels, with AcrIIA4 showing > 99.9% suppression in a yeast model system[77]. AcrIIA2 was slightly weaker at inhibiting gene

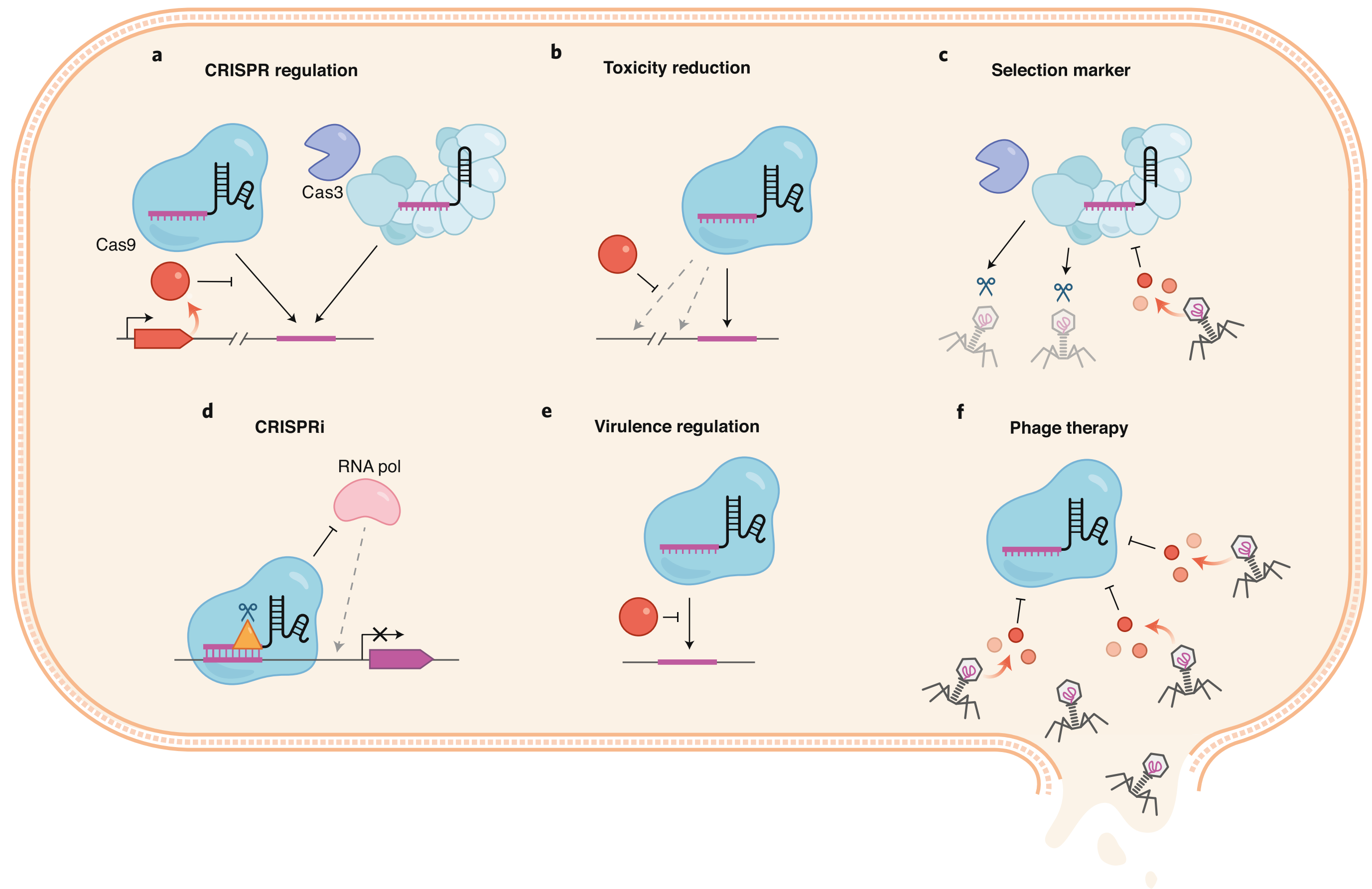

Fig. 2 | Applications of *acr* genes in prokaryotes. a, Acr proteins (red circle) may block editing by endogenous or exogenous CRISPR-Cas systems. **b**, Acr proteins (red circle) may help reduce the occurrence of any unwanted genomic editing events that contribute to toxicity. **c**, *acr* genes can serve as selectable markers in the genetic editing of bacteriophages. **d**, Acr proteins (for example, AcrIIC1, AcrIF3, AcrIE1, yellow triangle) can disable the endonuclease activity of the effector nuclease, thereby repurposing it as a CRISPRi system for gene knockdown applications. RNA pol, RNA polymerase. **e**, CRISPR-Cas machinery can contribute to bacterial virulence, for example, through binding to genomic DNA. Administration of Acr proteins may prevent these non-canonical functions of CRISPR-Cas systems. **f**, The viral expression of Acr proteins can potentially broaden the host range of phage therapeutics by inhibiting CRISPR-Cas immunity.

drive, consistent with early work demonstrating that AcrIIA2 is weaker than AcrIIA4 in some contexts[17,20]. This study also revealed that the exact level of gene drive inhibition is titratable depending on specific mutations within the *acr* genes as well as their levels of expression. Using a mutant Acr or a natural variant that does not completely inhibit Cas9 to weaken a drive may provide the ideal scenario for achieving drive persistence by avoiding strong selection toward complete inactivation. To rapidly halt the spread of an ongoing drive, an alternate Cas enzyme could be used to drive the *acr* gene through the population and thereby reverse the effect of the original. Acr proteins thus present a means for finely tuned control of gene drives, which may enable safe deployment of this promising technology. Future work in animal-based gene drives will be needed to test Acr efficacy — specifically, whether mating a gene drive animal with an Acr animal returns inheritance to Mendelian frequencies or has unforeseen outcomes.

Applications of Acr proteins in vitro

Acr proteins have also been used as lab reagents. In one case, CRISPR-Cas9 RNPs were detected and quantified using AcrIIA4 as an immobilized capture ligand[78]. Fixing AcrIIA4 on glassy carbon electrodes enabled the specific detection of sgRNA-loaded Cas9 using electrochemical, colorimetric and fluorescent readouts, which can be used to measure Cas9 delivery efficacy and persistence in biological samples. In another case, Acr proteins were used to facilitate adenoviral vector production[79]. A helper-dependent adenovirus was engineered to express Cas9 for genome editing and self-cleavage after transduction into cells, thereby allowing editing but preventing Cas9 persistence. To prevent self-cleavage during vector production before transduction, expression of AcrIIA2 and AcrIIA4 was combined with *Cas9* mRNA downregulation. Acr proteins can similarly be used to inactivate Cas protein in other systems where leaky Cas activity is detrimental or confounding.

Conclusions

Moving forward, we anticipate that the discovery of Acr proteins can match the discovery and development of new CRISPR-Cas systems. MGEs encoding *acr* genes have yet to be identified for several CRISPR-Cas types and subtypes, most notably the type VI system. The CRISPR-associated protein Csx27 has been shown to repress type VI-B function[80], but phages and plasmids may also encode inhibitors of Cas13. The discovery of *acr* genes for these systems will provide regulatory tools for Cas proteins and may reveal mechanistic novelty that has yet to be uncovered for these systems. The recent discovery of a catalytic Acr protein that functions at sub-stoichiometric levels[12] suggests that similarly novel and potent CRISPR inhibitory mechanisms are waiting to be identified. Lastly, Acr proteins that directly interfere with other stages of CRISPR immunity

Fig. 3 | Applications and regulation of Acr proteins. Acr proteins can be regulated using inducible promoters, tissue-specific miRNAs, light, and small molecules to achieve rapid and dynamic control of CRISPR-Cas activity. RNA pol, RNA polymerase.

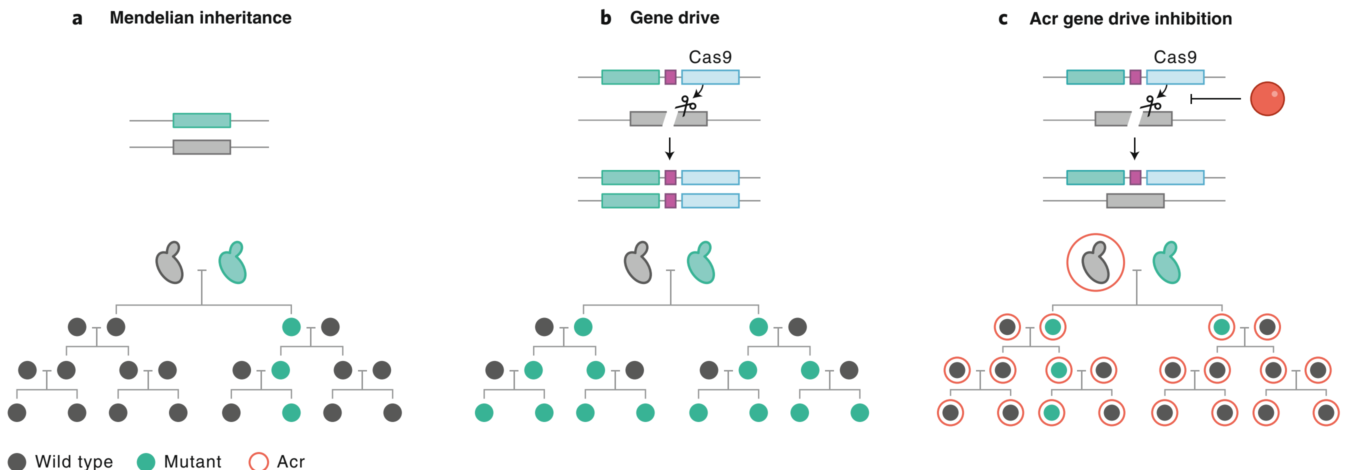

Fig. 4 | Use of Acr proteins for controlling gene drives. a, In Mendelian inheritance, a heterozygous mutant allele (marked with blue), is inherited in 25% of the offspring when mated with a homozygous non-mutant. **b**, The presence of a gene-drive element containing the Cas9 endonuclease rapidly spreads the Cas9 allele throughout the population. **c**, A population of Acr-expressing homozygotes inhibiting Cas9 can impede the spread of the gene drive construct (the presence of the Acr is denoted with a red circle). A scenario is depicted where a population expressing an Acr is protected from a gene drive. It is assumed that the gene-drive expressing individuals will mate with engineered Acr-expressing individuals.

such as crRNA processing and spacer acquisition, rather than targeting, have yet to be reported.

Additional work must also be done to determine whether Acr proteins are safe and effective off switches in vivo. Although Acr proteins have been shown to inhibit editing by Cas9 in mice[53], it is not known if they ever induce toxicity or provoke a host response, which must be determined for their safe implementation in animals. The efficacy of Acr proteins for preventing off-target editing or halting gene drives in animals also remains to be demonstrated. The applications described here for CRISPR-Cas systems and their

antagonists represent an early stage for these technologies, and much innovation is likely to come.

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Author contributions

N.D.M. wrote the sections on the applications of Acr proteins for eukaryotic and in vitro systems and regulation of Acr proteins, and the informational boxes on the advantages and limitations of Acr proteins. R.P.R. wrote the introduction, informational box for Acr protein discovery, and Table 1. B.C. wrote the sections on controlling gene drives with Acr proteins and applications of Acr proteins for prokaryotic systems. J.B.-D. supervised and wrote the manuscript with N.D.M, R.P.R. and B.C. All authors contributed to figure content and edited the manuscript.

Competing interests

J.B.-D. is a scientific advisory board member of SNIPR Biome and Excision Biotherapeutics and a scientific advisory board member and co-founder of Acrigen Biosciences. J.B.-D. and N.D.M. have filed patents on technology related to anti-CRISPR proteins. R.P.R. is a consultant for Ancilia Inc.

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