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So Yeon Lee & Hyun Ho Park

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AcrIIA7 hijacks tracrRNA to block CRISPR-Cas system

So Yeon Lee^{1,2} and Hyun Ho Park^{1,2,*}

¹College of Pharmacy, Chung-Ang University, Seoul 06974, Republic of Korea

²Department of Global Innovative Drugs, Graduate School of Chung-Ang University, Seoul 06974, Republic of Korea

*Correspondence to:
Hyun Ho Park (email: xrayleox@cau.ac.kr)

ABSTRACT

The CRISPR–Cas9 system provides adaptive immunity against invading genetic elements through a dual-RNA-guided DNA cleavage mechanism. This system relies on the precise assembly of a ribonucleoprotein (RNP) complex composed of the Cas9 endonuclease, a CRISPR-derived RNA (crRNA), and a trans-activating CRISPR RNA (tracrRNA). Around 100 anti-CRISPR proteins that inhibit CRISPR–Cas systems have been identified, and the mechanisms by which they act are

increasingly being elucidated. However, the inhibitory mechanisms of many Acrs, including AcrIIA7, remain poorly understood. Here, we present the structure of AcrIIA7 and uncover a previously unrecognized mechanism by which it inhibits Cas9 function. Structural and biochemical analyses reveal that AcrIIA7 specifically binds to tracrRNA, preventing its association with crRNA and thereby blocking formation of the active Cas9 RNP complex. This tracrRNA hijacking mechanism represents a unique strategy for CRISPR inhibition, in which an anti-CRISPR protein targets an RNA scaffold essential for Cas9 activation rather than interacting directly with the Cas9 protein. Our findings provide the first structural insight into tracrRNA-targeted anti-CRISPR activity and highlight RNA–RNA interaction interfaces as vulnerable nodes in CRISPR–Cas immunity.

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INTRODUCTION

CRISPR–Cas systems provide adaptive immunity in bacteria and archaea by recognizing and destroying invading genetic elements such as bacteriophages and plasmids¹⁻⁴. CRISPR–Cas systems are categorized into two main classes, Class1 and Class2, based on their locus composition and effector protein organization, and further divided into six types (I–VI)^{5,6}. Class 1 CRISPR–Cas systems, which include types I, III, and IV, are composed of multi-subunit RNA-guided surveillance complexes, while Class2 CRISPR–Cas systems, exemplified by type II systems, utilize a single effector protein, such as Cas9⁷⁻⁹. Among these, the Type II-A CRISPR–Cas9 system from *Streptococcus pyogenes* (SpyCas9) has been most extensively characterized and widely repurposed for genome engineering^{10,11}. The Cas9 endonuclease is guided to its DNA target by a dual-RNA complex composed of a CRISPR-derived RNA (crRNA) and a trans-activating CRISPR RNA (tracrRNA), which together form a stable RNA duplex that enables specific recognition and cleavage of target DNA^{10,12}. The assembly of this ribonucleoprotein (RNP) complex is a critical step in the activation of Cas9 function^{8,13-15}.

To counteract CRISPR-mediated immunity, bacteriophages and mobile genetic elements have evolved anti-CRISPR (Acr) proteins that inhibit various stages of the CRISPR–Cas immune pathway¹⁶⁻¹⁹. Since the initial discovery of Acr proteins in 2013¹⁸, approximately 100 distinct variants have been identified through a combination of functional screening and bioinformatic analyses²⁰⁻²². Due to the frequent lack of sequence homology and shared structural features among Acrs, their classification is primarily based on the specific CRISPR–Cas systems they inhibit^{18,23,24}.

These proteins employ diverse mechanisms to disrupt CRISPR–Cas activity, including inhibition of DNA binding, interference with nuclease activity, or sequestration of essential cofactors^{17,22,25-28}. Most recently, AcrIF25 was reported to inhibit CRISPR function by binding to Cas7 and preventing the formation of a DNA-cleaving complex²⁸. Among these, AcrIIA proteins are known to inhibit Cas9, yet for many members of this family, the molecular mechanisms of inhibition remain poorly defined.

AcrIIA7 is one such anti-CRISPR protein that has been previously shown to inhibit SpyCas9, but its mechanism of action remained elusive²⁹. Recent study also showed that AcrIIA7 might be a phage-encoded anti-CBASS protein that inhibits host cyclic-oligonucleotide-based immunity by sequestering cyclic dinucleotide (CDN) molecules³⁰. Our current study was designed to elucidate the molecular mechanism underlying the inhibition of Cas9 by AcrIIA7. In this study, we determined the high-resolution crystal structure of AcrIIA7 from *Phocaeicola dorei* (hereafter called pdAcrIIA7) and conducted structure-based functional analyses to elucidate its inhibitory mechanism. The *pdAcrIIA7* gene originates from a bacteriophage, but as is the case for many Acr genes, it is incorporated into the bacterial chromosome through phage infection. Strikingly, we found that AcrIIA7 does not interact with Cas9 directly, but instead binds to tracrRNA with high specificity and affinity. This interaction prevents tracrRNA from forming a duplex with crRNA, thereby blocking the formation of the functional Cas9 RNP complex and ultimately inhibiting Cas9 activity. This mechanism represents a previously uncharacterized mode of anti-CRISPR action that we refer to as tracrRNA hijacking.

Our findings provide the first structural and mechanistic insight into how an anti-CRISPR protein targets the RNA component of the CRISPR–Cas9 system rather than the Cas protein itself. This discovery not only expands our understanding of anti-CRISPR diversity but also highlights RNA–RNA interactions as a underexplored axis of vulnerability in CRISPR-mediated immunity. The tracrRNA hijacking strategy employed by AcrIIA7 may represent a broader class of anti-CRISPR mechanisms and opens avenues for the design of synthetic CRISPR inhibitors that operate via RNA targeting.

RESULTS

The structural studies revealed that AcrIIA7 from *Phocaeicola dorei* is composed of three domains, and among them, the head domain is flexible

The investigation into the inhibition mechanism of AcrIIA7 commenced with the overexpression and purification of the full-length pdAcrIIA7, paving the way for subsequent structural analysis. The purification process involved Ni-NTA affinity chromatography followed by size exclusion chromatography (SEC), where distinct peak was observed. SDS-PAGE analysis confirmed the purity of the pdAcrIIA7 protein in the main peak eluted around 14mL (Fig. 1A). Crystallizations trials using protein samples eluted from the main peak were successful, and diffraction data from a single crystal were collected at the PAL synchrotron. Initial molecular replacement (MR) phasing, using a predicted structural model from AlphaFold2, was unsuccessful. To resolve the phasing issue, we performed SelenoMet labelling for the purpose of Se-SAD phasing, which ultimately determined the 2.16 Å structure of pdAcrIIA7. The final structural model was refined to $R_{\text{work}} = 20.12\%$ and $R_{\text{free}} = 25.14\%$. The diffraction data and refinement statistics are summarized in Supplementary Table 1.

The crystal belonged to space group $P2_12_12_1$ and contained four molecules (Molecule A ~D) in the asymmetric unit (ASU) (Fig. 1B). The final structural model showed that molecules B and C of pdAcrIIA7 covered most of pdAcrIIA7 (residues M1 to E150), while molecules A and D excluded 22 and 28 N-terminal amino acids, respectively, due to unclear electron density. The structure of pdAcrIIA7 consisted of seven α -helices ($\alpha 1$ – $\alpha 7$) and seven β -sheets ($\beta 1$ – $\beta 7$) (Fig. 1C and D). Following the two long helices of head domain ($\alpha 1$ and $\alpha 2$), the core domain is formed by five β -sheets ($\beta 1, \beta 2$ and $\beta 5$ – $\beta 7$) and three α -helices ($\alpha 3, \alpha 4$ and $\alpha 7$), extending further two α -helices ($\alpha 5$ – $\alpha 6$) and two β -sheets ($\beta 3$ – $\beta 4$) as extended domain (Fig. 1C and D). Four molecules in the same ASU exhibited slight structural differences in their head domains ($\alpha 1$ – $\alpha 2$) and in the $\alpha 7$ regions of their core domains (Fig. 1E~G). RMSD analysis indicated that molecules B and A differed by 1.8Å, whereas molecules B and C, as well as molecules B and D, showed relatively larger disparities with RMSD values of 2.6Å and 2.2Å, respectively (Fig. 1E). Furthermore, the $\alpha 1$ and $\alpha 2$ helices in the head domain of molecules B and C were found to be inclined at different angles (Fig. 1F). In the core domain, the alpha helix was well conserved in molecules A and B, while in molecules C and D, these regions formed a loop configuration rather than alpha helix (Fig. 1G).

An analysis of the electrostatic potential on the molecular surface revealed a distinct distribution of charges; negative charges were predominantly localized in the head and the extended domains, whereas positive charges were primarily concentrated in the core domain (Fig. 1H). B-factor analysis highlighted notable rigidity in the core and head domains, evidenced by their low B-factor values (average 48.12Å). In contrast, the extended domain exhibited high B-factor values (average 68.98Å), indicating greater structural flexibility (Fig. 1I).

The utilization of the DALI server to explore proteins structurally akin to pdAcrIIA7 revealed the sulfate transporter protein as the most structurally similar (Fig. 1J). Despite the sulfate transporter emerging as the closest match, the modest Z-score (6.0) and sequence identity (5%) underscored a discernible lack of structural congruence. In addition, the other selected proteins exhibited similarly low Z-scores (ranging from around 5.7-4.6) and sequence identities (2~10%), collectively signifying the novelty of the AcrIIA7 structure. Indeed, the fold of pdAcrIIA7 appears to be unprecedented within the existing structural repertoire. While we were preparing our manuscript, the structure of Tad2 (AcrIIA7 from human gut metagenomic libraries) was published, which represented a form of AcrIIA7 lacking the head domain and anti-CRISPR activity³⁰.

Since the MR phasing method using the predicted structural model by AlphaFold2, failed, we were intrigued to compare the predicted model with the experimental structure. To achieve this, we aligned two structures. The structural alignment of pdAcrIIA7 with the AlphaFold2 predicted model revealed notable differences. Although the overall fold was the consistent between the two models, significant structural discrepancies were observed in the head domain. The $\alpha 1$ and $\alpha 2$ helices in the predicted

model were notably distorted, and several regions did not match when compared to the crystal structure of pdAcrIIA7 (Fig. 1K). These structural discrepancies could explain the unsuccessful MR phasing. Overall, based on the structural studies of pdAcrIIA7, we concluded that pdAcrIIA7 is composed of three domains, and among them, the head domain is flexible.

AcrIIA7 forms a distinct and robust homo-tetrameric structure in solution

Indeed, although numerous Acrs inhibit Cas9 activity as monomers, previous research has demonstrated that some Acrs operate in a dimeric configuration^{31,32}. In the case of pdAcrIIA7, the SEC results indicated that pdAcrIIA7 eluted between the molecular sizes of 158kDa and 44kDa, producing a prominent peak in the chromatogram (Fig. 1A). However, SEC alone was insufficient to determine the stoichiometry of pdAcrIIA7. To confirm its stoichiometry, we employed multi-angle light scattering (MALS) analysis. MALS yielded an experimental molecular weight of 74.86 kDa, with a fitting error of 1.12 % (Fig. 2A). Given the theoretically calculated molecular weight of monomeric pdAcrIIA7 with a C-terminal 6X histidine tag is 17.82 kDa, the MALS data conclusively indicated that pdAcrIIA7 exists as a tetramer in solution. This tetrameric structure of pdAcrIIA7 provides further insights into the diverse mechanisms of Acr function, revealing that higher-order oligomerization may play a crucial role in their ability to inhibit Cas9 activity.

Further crystallographic packing analysis revealed three types of putative dimers: Mol B(D)/Mol A(C), Mol B(A)/Mol C(D), Mol B(A)/Mol D(C) (Fig. 2B and C). To ascertain the biologically relevant form, we conducted protein-protein interface (PPI) analysis using the PDBePISA server³³. The PPI analysis showed complexation significance score (CSS) for each dimer: Mol B(D)/Mol A(C) (0.000), Mol B(A)/Mol C(D) (0.100), and Mol B(A)/Mol D(C) (1.000). Notably, the Mol B(A)/Mol D(C) dimer exhibited the greatest biological relevance, as indicated by its superior CSS compared to other dimers with lower scores (Fig. 2B).

Upon closer examination of each dimer's structural details, it was observed that the Mol B/Mol A dimer formed through a loop connecting $\beta 5$ to $\alpha 7$ in the core domain, forming 2 hydrogen bonds between G133 and Q138 of each molecule. (Fig. 2D) The Mol B(A)/Mol C(D) dimer predominantly interacted through 5 hydrogen bonds in the core domain, with an additional hydrogen bond in the head domain (Fig. 2E). In the case of Mol B/ Mol D dimer, which scored the highest in CSS, displayed a more intricate interaction pattern with 14 hydrogen bonds and 6 salt bridges between two molecules, primarily in both the core domain and the extended domain (Fig. 2F).

Anti-CRISPR activity of AcrIIA7 relies on the absence of its head domain

To evaluate the inhibitory effect of AcrIIA7 on the enzymatic activity of Cas9, we established an *in vitro* target DNA cleavage assay. We selected *Streptococcus pyogenes* Cas9 (SpyCas9) for this assay due to its known anti-CRISPR activity against SpyCas9 and its widespread use in genome-editing applications. SpyCas9 is a large nuclease composed of 1368 amino acid residues. The assay employed an 1100bp double-stranded DNA (dsDNA) substrate containing the target region with the PAM sequence (5'-NGG-3'), along with a specifically designed single guide RNA (sgRNA) to direct SpyCas9 to the target site. We tested varying concentrations of AcrIIA7 to quantitatively assess its inhibitory potential. As expected, when SpyCas9 was incubated with sgRNA to form the ribonucleoprotein (RNP) complex, the enzyme successfully cleaved the target DNA (Fig. 3A). The inhibitory capacity of AcrIIA7 was further evaluated by varying the incubation order of the components. Despite using a final AcrIIA7 concentration of 10 μ M, which is much higher than the effective concentrations of other AcrIIA family members, AcrIIA7 did not inhibit DNA cleavage of SpyCas9 under any tested condition (Fig. 3A). Given that previous studies have identified AcrIIA7 from human gut metagenome (meAcrIIA7) as an inhibitor of SpyCas9, we decided to align the sequences of

pdAcrIIA7 and meAcrIIA7 to identify potential differences. Sequence alignment revealed that pdAcrIIA7 possesses an additional 48 residues at the N-terminus (Fig. 3B). Apart from this notable difference, the sequences were largely conserved between the two proteins (Fig. 3B). Structurally, these extra 48 residues found in pdAcrIIA7 correspond to the head domain (Fig. 3C and D). Leveraging the experimental structure of the complete AcrIIA7, we constructed a model depicting the tetrameric arrangement of the meAcrIIA7 (Fig. 3E). To ascertain the functionality of pdAcrIIA7 in the absence of the head domain, we engineered a head domain-deleted pdAcrIIA7 (Δ N48AcrIIA7), subsequently conducting a comparative analysis with the intact pdAcrIIA7, Δ N48AcrIIA7, and the meAcrIIA7. The stoichiometries of Δ N48AcrIIA7 and meAcrIIA7 were analyzed through MALS. The results revealed an experimental molecular weight of 52.46 kDa for the Δ N48 AcrIIA7 and 52.79 kDa for the meAcrIIA7. Considering the theoretically calculated molecular weight of both monomeric Δ N48AcrIIA7 and meAcrIIA7 to be around 12.7 kDa, the MALS data demonstrated that the Δ N48AcrIIA7 and the meAcrIIA7 exist as a tetramer in solution. This observation indicated that the absence of the head domain does not impede tetrameric formation (Fig. 3F and G).

In the *in vitro* cleavage-inhibition assay using SpyCas9, both the meAcrIIA7 and the Δ N48AcrIIA7 were subjected in the same experimental conditions as the full-length pdAcrIIA7. In contrast to the full length pdAcrIIA7, which did not exhibit inhibitory function in this assay, the meAcrIIA7 and Δ N48AcrIIA7 variants displayed significant inhibition of DNA cleavage. Notably, a distinct non-cleaved band was observed, particularly when the AcrIIA7 was pre-incubated with sgRNA (Fig. 3H and I). Further assays examining concentration and order dependency with Δ N48AcrIIA7 demonstrated that inhibition increased with higher concentrations, but only when AcrIIA7 was added prior to Cas9 RNP complex formation (Fig. 3J). These findings suggest that the head domain of pdAcrIIA7 interferes with its inhibitory functions, rendering the full length pdAcrIIA7 unable to impede DNA cleavage. Additionally, DNA recovery was observed only when AcrIIA7 was introduced before the assembly of the SpyCas9-sgRNA complex, indicating that AcrIIA7 likely inhibits Cas9 activity by preventing proper RNP formation and thereby obstructing target DNA recognition.

AcrIIA7 inhibits the DNA cleavage activity of Cas9 without directly binding to the protein

Acr proteins are known to employ diverse strategies to inhibit Cas9, with many acting directly on the Cas9 protein to suppress its DNA cleavage activity^{31,34-36}. Previous studies have demonstrated that numerous Acrs achieve inhibition by physically blocking key functional domains of Cas9, such as the HNH or RuvC nuclease domains³⁵, REC3 domain³⁴, BH domain³⁷, and WED-PI domain³⁸. Building upon these findings, we further investigated the inhibition mechanism of AcrIIA7 through binding assays conducted with full-length Cas9 proteins from multiple species, including *Streptococcus pyogenes* (SpyCas9), *Neisseria meningitidis* (NmeCas9), *Haemophilus parainfluenzae* (HpaCas9), and *Simonsiella muelleri* (SmuCas9). These experiments were designed to identify potential cross-species interactions and to determine whether AcrIIA7 exhibits a conserved inhibitory mechanism across different Cas9 variants. When purified AcrIIA7 was mixed with various Cas9 proteins, no co-migrations were detected with any of the Cas9 variants upon the SEC experiments, suggesting that AcrIIA7 does not directly bind to Cas9 to exert its inhibitory activity (Fig. 4A and Supplementary Fig. 1A~C). Given that the full-length AcrIIA7 was confirmed to be inactive, we conducted the identical binding assay using meAcrIIA7 and the Δ N48AcrIIA7 to determine if the presence of the head domain hindered interactions between AcrIIA7 and Cas9. The assay also showed no binding results, indicating that, regardless of the presence of the head domain, AcrIIA7 inhibits DNA cleavage independently of directly binding to Cas9 (Fig. 4B and C, and Supplementary Fig. 2A~C and 3A~C). We also performed pull-down assays to examine whether AcrIIA7 can interact with SpyCas9 in a cellular context, which would allow detection of weak or transient interactions that may not be captured by SEC co-elution

analysis. Pull-down experiments were performed using both full length pdAcrIIA7 and the Δ N48 variant. In all cases, we did not observe detectable interaction between SpyCas9 and either AcrIIA7 construct under the tested conditions (Supplementary Fig. 4). These results support the conclusion that AcrIIA7 does not directly bind SpyCas9 as part of its inhibitory mechanism, consistent with our SEC analysis.

Due to the potential ambiguity of analyzing co-migration tendency of AcrIIA7 with full length Cas9s using SEC based experiments, we additionally performed binding assays with distinct functional domains of SpyCas9, including RuvC, REC, HNH, WED, and PI. Each domain was expressed and purified (REC1-2, REC3, HNH and WED), except for the PI domain, which was insoluble in our experiments (Supplementary Fig. 5A). We conducted the SEC binding tests between each domain of SpyCas9 and both full length pdAcrIIA7 and Δ N48AcrIIA7. These experiments demonstrated that neither full-length pdAcrIIA7 nor Δ N48AcrIIA7 exhibited obvious co-migration with various domains of Cas9, suggesting that the inhibitory mechanism of AcrIIA7 operates independently of direct binding to Cas9 (Supplementary Fig. 5B~I).

AcrIIA7 lacking head domain directly binds RNA, specifically tracrRNA

Despite the inhibitory activity of AcrIIA7 has been validated through *in vitro* assays, its precise mechanism of action remains elusive due to limitations of previous binding assays with Cas9 proteins. To gain further insight into its functional properties, we analyzed the surface electrostatic potential of tetrameric pdAcrIIA7, as the electrostatic characteristics of a protein surface are often closely linked to its biological function. This analysis revealed that a deep groove is formed toward the core domain of the AcrIIA7 tetramer, and that this groove is lined with numerous positively charged residues (Fig. 5A). Based on this observation, we hypothesized that the protein may be capable of binding to nucleotides. To explore whether AcrIIA7 directly binds to nucleotides, we initially performed EMSA using sgRNA that has been used for the formation of active SpyCas9. The result showed a clear RNA band super-shift when RNA was incubated with both Δ N48AcrIIA7 and meAcrIIA7, but no shifts were observed with full-length pdAcrIIA7, indicating that only AcrIIA7 without the head domain directly binds to sgRNA (Fig. 5B). This finding aligns with previous experimental results of *in vitro* assay, which also revealed that only AcrIIA7 without the head domain inhibited DNA cleavage, demonstrating that the head domain-deleted AcrIIA7 directly binds to sgRNA to inhibit the Cas9-mediated DNA cleavage. Interestingly, in the case of sgRNA, an upper shift of the band was observed even in the absence of protein, when only buffer was added (Fig. 5B). This shift was presumed to result from structural changes in the sgRNA under the buffer conditions. However, the protein-induced super-shift appeared at a distinctly higher position, allowing clear differentiation between nonspecific buffer effects and specific binding of the protein (Fig. 5B). Because the C-terminal His-tag of AcrIIA7 could potentially affect its interaction with RNA, we generated an N-terminally tagged version of AcrIIA7 and examined its binding to sgRNA. The N-terminally tagged protein exhibited the same binding behavior (Supplementary Fig. 6), indicating that the presence and position of the tag does not influence RNA interaction.

We next asked whether AcrIIA7 also binds to other types of nucleic acids. EMSAs with ssDNA and dsDNA showed no band shifts in the presence of AcrIIA7, indicating its specific interaction with RNA, but not DNA (Fig. 5C and D).

The guide RNA (gRNA) in bacteria and generated sgRNA used for our assay consists of two components: CRISPR-derived RNA (crRNA), which specifically binds to target DNA with its complementary sequence, and trans-activating CRISPR RNA (tracrRNA), which binds to Cas9 to form Cas9-RNP complex (Fig. 5E). To comprehensively understand the binding mode of AcrIIA7 to gRNA, we performed additional EMSA with crRNA and tracrRNA, respectively. AcrIIA7 did not exhibit band

shifts with crRNA (Fig. 5F), while super-shifts were detected with tracrRNA (Fig. 5G). Interestingly, although active AcrIIA7, without head domain; Δ N48AcrIIA7 and meAcrIIA7 showed super-shifts with tracrRNA, full-length pdAcrIIA7 also showed distinct band shifts with tracrRNA (Fig. 5G). These results suggest that the head domain of pdAcrIIA7 does not significantly affect binding to shorter RNA molecules like tracrRNA but may impose steric hindrance when interacting with longer RNAs such as sgRNA. One additional noteworthy observation is that pdAcrIIA7 constructs containing the head domain show two distinct bands in the EMSA with tracrRNA (Fig. 5G). The presence of two bands might be due to structural heterogeneity resulting from the flexible head domain, leading to different conformational states of AcrIIA7.

AcrIIA7 binds nucleic acids in a structure-dependent fashion

After confirming that AcrIIA7 binds tracrRNA, we wondered whether this interaction is RNA-specific, sequence-specific, or instead driven by recognition of a particular stem-loop structure within the tracrRNA. To address this question, we synthesized the tracrRNA from an *Haemophilus parainfluenzae* (Hpa) strain belonging to the type II-C system, which contains a completely different stem-loop architecture (Fig. 6A), and examined its binding to AcrIIA7 using EMSA. The results showed that neither pdAcrIIA7 nor the head domain-deficient AcrIIA7 bound to the type II-C tracrRNA with the distinct stem-loop structure (Fig. 6A). This finding indicates that AcrIIA7 recognizes and interacts with RNA in a structure-dependent manner, binding only to tracrRNAs that possess a specific structural configuration. These results indicate that AcrIIA7, which has been reported to inhibit type II-A systems, indeed acts in a type II-A-specific manner, and that this specificity arises from recognition of a particular tracrRNA structural feature rather than from RNA sequence alone.

We next asked whether AcrIIA7 binds specifically to RNA, and what would happen if a DNA molecule possessed a stem-loop structure similar to that of tracrRNA. To address this question, we synthesized an ssDNA containing the same stem-loop architecture as the type II-A tracrRNA (Supplementary Fig 7). We refer to this molecule as structured ssDNA (SssDNA) and examined its interaction with AcrIIA7 by EMSA. Interestingly, both meAcrIIA7 and Δ 48AcrIIA7, which lack the head domain, were able to bind SssDNA, whereas pdAcrIIA7 did not show detectable binding (Supplementary Fig 7). Although the presence of the head domain in pdAcrIIA7 may slightly interfere with DNA binding, the observation that AcrIIA7 variants bind to a stem-loop-containing DNA clearly demonstrates that AcrIIA7 does not interact with nucleic acids in an RNA-specific or sequence-specific manner. Rather, AcrIIA7 recognizes and binds nucleotides in a structure-dependent fashion.

If AcrIIA7 binds nucleotides containing three stem-loop structures, an important next question is which stem loops (SL) are specifically recognized and what constitutes the minimal binding motif within tracrRNA. To address this question, we synthesized a series of gRNA variants (SL12, SL1, SL0) in which individual stem-loop elements were selectively removed (Fig. 6B) and examined their interactions with AcrIIA7 using EMSA (Fig. 6C and D). Removal of SL3 did not abolish binding, although the affinity for AcrIIA7 was noticeably reduced (Fig. 6C and D). In contrast, gRNA variants lacking both SL2 and SL3, or lacking all three stem-loops (SL1–SL3), showed little to no detectable binding to AcrIIA7 (Fig. 6C and D). These results indicate that SL3 is not strictly required for binding, whereas SL1 and SL2 are essential. We therefore conclude that SL1 and SL2 constitute the minimal structural motif necessary for AcrIIA7 recognition.

tracrRNA binds within the deep groove formed by the AcrIIA7 tetramer core, which is lined with positively charged residues

To investigate how AcrIIA7 recognizes and binds to tracrRNA, we first identified functionally important and evolutionarily conserved residues within AcrIIA7 using the Consurf server³⁹. This

analysis revealed that conserved residues are primarily located in the core domain, with a few also present in the head domain, including E6 and E10 (Fig. 7A~C). Among these, we focused on conserved positively charged residues positioned within the deep groove formed by the core domains upon tetramerization, as they may play a critical role in RNA binding (Fig. 7D). Based on this reasoning, we selected five conserved, positively charged residues—K58, K66, K71, K93, and R101—as candidates likely involved in RNA interaction. Supporting this hypothesis, an AlphaFold3 model of the AcrIIA7–tracrRNA complex showed that tracrRNA fits into the deep groove of the core domain, forming interactions with these positively charged residues, further reinforcing our prediction (Fig. 7E). To experimentally validate these predictions, we introduced charge- or size-disrupting mutations by substituting each residue with aspartic acid or tryptophan, generating five mutants: K58D, K66W, K71W, K93W, and R101W. Since the N-terminally truncated form (Δ N48) of AcrIIA7 shows the most distinct RNA binding behavior (Fig. 5B and G), all mutations were introduced into the Δ N48 background. We then examined the RNA-binding ability of these mutants using EMSA with sgRNA. As expected, the K58D, K66W, and K71W mutants failed to form any detectable complex with sgRNA, showing complete loss of RNA binding (Fig. 7F). In contrast, K93W and R101W showed an upper-shifted band indicative of complex formation, but the shifts differed from that of the wild-type, suggesting altered or weakened binding (Fig. 7F).

To further corroborate these findings, an *in vitro* cleavage-inhibition assay was performed using the mutant variants. The results were consistent with those of the sgRNA binding assays: the K58D and K71W mutants displayed significantly reduced Cas9 inhibitory activity due to their diminished affinity for sgRNA (Fig. 7G). For the K93W and R101W mutants, which showed slightly weaker band shifts in the sgRNA EMSA, a corresponding reduction in cleavage-inhibition activity was also observed compared to the wild type (Fig. 7G).

Although the K66W mutant, previously shown to have completely lost sgRNA-binding affinity, exhibited instability at 37°C, preventing target DNA detection in the *in vitro* assay, results from other mutants collectively confirmed that sgRNA binding by pdAcrIIA7 is essential for its Cas9 cleavage-inhibition mechanism. These findings underscore the critical role of conserved, surface-exposed positively charged residues within the deep groove of AcrIIA7 tetramer in mediating tracrRNA interactions and inhibiting Cas9 activity (Fig. 7F and G).

The proposed scenario of AcrIIA7-mediated Cas9 inhibition

Based on our series of experimental results, we propose a previously uncharacterized inhibitory mechanism for AcrIIA7, in which it hijacks tracrRNA to prevent gRNA formation, thereby inhibiting Cas9 activity. This mode of inhibition represents a previously uncharacterized strategy among known anti-CRISPR proteins. To validate that tracrRNA hijacking by AcrIIA7 directly impairs gRNA formation, we synthesized crRNA_repeat and anti-repeat_tracrRNA, and incubated them in annealing buffer at 37°C for 2 hours to mimic gRNA formation. The gRNA, formed via annealing of the complementary repeat and anti-repeat regions, was visualized as an upper band on native gel. Despite testing multiple annealing conditions, optimal gRNA formation remained challenging. Nevertheless, a distinct band corresponding to gRNA was detected, allowing us to assess AcrIIA7-mediated inhibition under these conditions (Supplementary Fig. 8A~C).

Both full length pdAcrIIA7 and meAcrIIA7 were tested for their ability to inhibit gRNA formation. As protein concentrations increased, the intensity of the gRNA decreased, indicating that AcrIIA7 inhibits gRNA assembly in a concentration-dependent manner through tracrRNA sequestration (Fig. 8A and B, Supplementary Fig. 9A and B). Notably, pdAcrIIA7 inhibited gRNA formation completely at 20 μ M, whereas meAcrIIA7 achieved the same effect at just 2 μ M, demonstrating a tenfold greater potency. This

supports the idea that while the head domain of pdAcrIIA7 does not eliminate its ability to bind tracrRNA, it reduces binding efficiency, particularly in the *Phocaeicola dorei*-derived AcrIIA7 variant. Order of addition experiments further confirmed the role of AcrIIA7 in gRNA formation. For full length pdAcrIIA7, when crRNA and tracrRNA were pre-incubated before AcrIIA7 addition, gRNA formation proceeded effectively (Fig. 8C and D, Supplementary Fig. 9C and D). However, when AcrIIA7 was added prior to or simultaneously with both RNAs, gRNA formation was significantly impaired (Fig. 8C and Supplementary Fig. 9C). In contrast, meAcrIIA7 exhibited potent inhibition regardless of the incubation order, consistent with its higher affinity for tracrRNA (Fig. 8D and Supplementary Fig. 9D). These findings collectively demonstrate that AcrIIA7 directly disrupts gRNA formation through tracrRNA hijacking, thereby blocking CRISPR-Cas activity.

Although our model predicted that hijacking of tracrRNA would prevent proper gRNA assembly and subsequently block its loading onto Cas9, we sought to obtain more explicit experimental evidence supporting this mechanism. To this end, we separately synthesized crRNA and tracrRNA and performed experiments in which their order of addition with Cas9 and AcrIIA7 was systematically varied. Although activation of Cas9 using separately prepared crRNA and tracrRNA produced lower activity than using a pre-assembled sgRNA, Cas9 activity was still detectable (Supplementary Fig. 10). When tracrRNA or gRNA encountered AcrIIA7 before interacting with Cas9, Cas9 activity was completely inhibited in all cases. However, when the RNAs first interacted with Cas9 to form a fully assembled RNP complex, AcrIIA7 was no longer able to inhibit Cas9 activity (Supplementary Fig. 10). These results clearly demonstrate that AcrIIA7 cannot inhibit Cas9 once the RNP complex has been formed and that AcrIIA7 exerts its inhibitory effect by binding to tracrRNA or gRNA prior to RNP assembly. In conclusion, inhibitory strategy of AcrIIA7 centers on tracrRNA hijacking, which serves as the foundation for its two inhibitory effects: (1) halting gRNA maturation through tracrRNA sequestration and (2) preventing the assembly of the Cas9-RNP complex by blocking gRNA loading onto Cas9 (Fig. 9).

DISCUSSION

The discovery of AcrIIA7 as a Cas9 inhibitor has underscored its importance in understanding the broader ecological and evolutionary dynamics of CRISPR-Cas systems^{20,26}. Unlike many other Acr proteins, AcrIIA7 is broadly distributed across diverse bacterial phyla and exhibits unique features that distinguish it from classical anti-CRISPR proteins^{22,26}. Despite its ecological prevalence and evolutionary relevance, the precise mechanism underlying inhibitory activity has remained unclear, limiting our understanding of its biological function.

Over the past decade, more than 100 anti-CRISPR proteins have been identified, spanning CRISPR-Cas types I through VI. Among these, 32 families of AcrIIA proteins have been discovered through approaches such as self-targeting analyses, machine learning predictions, and experimental validation^{23,26,40,41}. These AcrIIA proteins neutralize Cas9 activity via three primary mechanisms: inhibition of crRNA loading, prevention of DNA binding, and blockage of DNA cleavage. This mechanistic diversity reflects the evolutionary sophistication of Acrs in countering CRISPR-mediated defense systems.

In this study, we reveal that AcrIIA7 employs a distinct inhibitory mechanism based on tracrRNA hijacking, a strategy not previously described among known Acrs. Through a combination of structural prediction, biochemical binding assays, and *in vitro* inhibition experiments, we show that AcrIIA7 inhibits Cas9 by disrupting gRNA formation at two critical stages. First, AcrIIA7 binds directly to tracrRNA, sequestering it and blocking its function in crRNA maturation. This impedes the formation of mature gRNA, thus disrupting the upstream steps required for Cas9 activation. Second, AcrIIA7 interferes with the assembly of the Cas9-RNP complex by preventing gRNA loading, thereby halting

DNA cleavage activity. These dual actions suggest that AcrIIA7 functions by targeting the pre-RNP phase of the CRISPR-Cas9 pathway—a characteristic mechanism of early-stage Acr inhibitors. Interestingly, functional distinctions were observed between full-length AcrIIA7 (pdAcrIIA7) and its head domain–deleted variant (Δ N48AcrIIA7). Notably, a head domain–missing variant of AcrIIA7 was also discovered in the human gut metagenome, suggesting that this form represents a naturally occurring variant. While full-length pdAcrIIA7 could bind tracrRNA, it showed limited inhibition of Cas9 activity when tested with pre-assembled sgRNA, suggesting that the head domain may obstruct binding to mature gRNA. In contrast, Δ N48AcrIIA7 retained the ability to bind both tracrRNA and sgRNA and robustly inhibited DNA cleavage, even in the presence of pre-synthesized sgRNA. Structural modeling supports this view, showing that the crRNA–tracrRNA duplex (or sgRNA) lies within a positively charged groove near the core domain of AcrIIA7, a region that would sterically clash with the head domain in the full-length form (Supplementary Fig. 11). Thus, we propose that full-length AcrIIA7 primarily inhibits gRNA formation via tracrRNA sequestration, whereas the headless variant can inhibit both gRNA formation and Cas9-RNP formation. If so, could AcrIIA7 also dissociate the crRNA–tracrRNA duplex after the gRNA has been fully assembled. Based on our current data, however, AcrIIA7 does not appear to disrupt the crRNA-tracrRNA complex once it is formed. In our gRNA formation assays, pdAcrIIA7 did not reduce the gRNA band when added after crRNA-tracrRNA duplex formation (Fig. 8C), indicating that it does not dissociate the pre-assembled gRNA. In contrast, meAcrIIA7, which has an affinity for gRNA, produced band shifts even in the presence of gRNA, but this likely reflects its binding to the stem-loop region of tracrRNA within the gRNA structure, not dissociation of the duplex itself (Fig. 8D). Because the AcrIIA7 binding site does not overlap with the crRNA-tracrRNA region, it is unlikely that AcrIIA7 actively separates the two RNAs. Instead, AcrIIA7 appears to bind the structured region of tracrRNA or gRNA without disrupting the crRNA-tracrRNA pairing. Together, these results suggest that AcrIIA7 inhibits Cas9 not by dissociating crRNA and tracrRNA, but rather by binding to the stem-loop region of tracrRNA and preventing proper gRNA formation and loading onto Cas9.

Based on our experimental results, the gRNA formation assay—where crRNA and tracrRNA are annealed *in vitro* to generate gRNA—shows very low efficiency (Fig. 8). At first glance, one might suspect that the faintly formed gRNA species are artifacts of RNA annealing and may not be biologically meaningful. However, our data strongly argue against this interpretation. Specifically, the inefficient gRNA formation observed *in vitro* correlates well with the substantially reduced Cas9 activity when separately synthesized crRNA and tracrRNA are used instead of sgRNA (Supplementary Fig. 10), and the inhibition pattern of AcrIIA7 precisely mirrors the efficiency of gRNA assembly (Supplementary Fig. 10). In other words, whenever gRNA formation is compromised, Cas9 activity decreases accordingly, and AcrIIA7 inhibition directly corresponds to the extent to which gRNA assembly is blocked. Taken together, these results demonstrate that although gRNA formation is inefficient under RNA-only *in vitro* conditions, the faintly assembled gRNA species are biologically meaningful, as they directly correlate with Cas9 activity and AcrIIA7-mediated inhibition.

Cells contain numerous RNA species, raising the question of how AcrIIA7 selectively distinguishes and binds tracrRNA from other cellular RNAs. Our results indicate that this specificity arises from AcrIIA7's recognition of a unique structural feature of tracrRNA. Specifically, AcrIIA7 did not bind the type II-C tracrRNA (Fig 6A), which possesses a markedly different stem-loop architecture, whereas it did bind ssDNA engineered to adopt the same stem-loop structure as type II-A tracrRNA (Supplementary Fig. 7). These findings demonstrate that AcrIIA7 does not recognize nucleic acid type or specific sequences, but instead binds nucleotides in a structure-dependent manner. Consequently, AcrIIA7 is likely to target type II-A CRISPR–Cas systems specifically and to selectively act on tracrRNA while discriminating against other cellular RNAs.

Importantly, we observed that AcrIIA7 also binds the tracrRNA of another type II-A *SauCas9* and effectively inhibits its activity *in vitro*, demonstrating cross-reactivity beyond *SpyCas9* (Supplementary Fig. 12A and B). This also suggests that AcrIIA7 recognizes conserved structural features in type II-A tracrRNA, such as stem-loop elements, allowing it to target multiple type II-A *Cas9* orthologs. The broad-spectrum inhibition potential of AcrIIA7 aligns with its widespread distribution and evolutionary conservation, supporting its classification as a generalist Acr protein capable of modulating various type II-A CRISPR systems.

Recent studies have proposed an alternative interpretation of AcrIIA7 function, based on a homolog identified from the human gut microbiome (*HgmTad2*), suggesting it primarily inhibits the CBASS and Thoeis immune systems by sequestering cyclic signaling molecules rather than acting as an anti-CRISPR protein³⁰. Notably, these studies could not reproduce inhibitory activity of AcrIIA7 against *SpyCas9*. In contrast, our findings provide strong biochemical and functional evidence that AcrIIA7 is indeed a bona fide anti-CRISPR protein, inhibiting *SpyCas9* through a previously uncharacterized RNA-targeting mechanism.

To understand the functional differences observed between *HgmTad2* and our AcrIIA7, we compared their structures and stoichiometry. The two proteins showed nearly identical structures, with an RMSD of 0.7 Å, and both formed tetramers (Supplementary Fig. 13). Furthermore, native-PAGE analysis revealed that, similar to *HgmTad2*, both *meAcrIIA7* and *pdAcrIIA7* containing the head domain were capable of binding cyclic signaling molecules (Supplementary Fig. 14A~C). These findings indicate that *pdAcrIIA7* is also likely to inhibit CBASS and Thoeis immune systems, as reported for *HgmTad2*. Given the comparable structures and ligand-binding properties, we examined why *HgmTad2* failed to exhibit Acr activity against *Cas9*, whereas our AcrIIA7 did. In the previous study, Acr function was tested only after *Cas9* and gRNA had already formed a mature RNP complex. Under such conditions, AcrIIA7 would no longer be able to hijack tracrRNA before *Cas9* becomes activated. Consistently, in our analyses, AcrIIA7 also failed to inhibit *Cas9* once the RNP complex had already formed. Taken together, the earlier results and our current findings strongly support a model in which AcrIIA7 acts by binding to and hijacking tracrRNA prior to RNP assembly, suggesting that it may function as a bifunctional protein.

The adaptability not only underscores its evolutionary importance in phage-host dynamics but also highlights its potential as a versatile tool for gene-editing regulation. Given its unique mechanism of action and cross-species reactivity, AcrIIA7 could serve as a blueprint for engineering RNA-targeting Acr variants tailored to specific *Cas9* systems. Future studies focused on the structural and functional plasticity of AcrIIA7 across diverse CRISPR-Cas platforms may offer valuable insights into bacterial immunity and phage evolution, as well as distinct strategies for controlling genome editing technologies.

In conclusion, this study clarifies the molecular basis of AcrIIA7 function, revealing it as a legitimate and mechanistically distinct anti-CRISPR protein that inhibits *Cas9* by hijacking tracrRNA. By reconciling conflicting interpretations and providing structural and biochemical validation, we establish AcrIIA7 as a multifaceted modulator of CRISPR-Cas activity and a promising model for studying anti-CRISPR strategies in both natural and applied settings.

METHODS

Cloning, overexpression, and purification of AcrIIA7s, various Cas9 from different species, and various Cas9 domains for structural and biochemical studies

The gene encoding AcrIIA7 (residues 1–150) from *Phocaeicola dorei* (accession number: WP_038611802) was synthesized by Bionics (Daejeon, Republic of Korea) and subsequently inserted into a pET21a plasmid vector (Novagen, Madison, WI, USA), equipped with a C-terminal hexa-

histidine tag for affinity chromatography. Cloning was executed utilizing *NdeI* and *XhoI* restriction sites. Following transformation into *Escherichia coli* BL21(DE3) competent cells, a single colony was selected and cultured in lysogeny broth (LB) medium for protein expression. For preparation of selenomethionine-labeled protein used in crystallographic studies, cells were alternatively grown in 1L SelenoMet Medium base supplemented with 50mL SelenoMet Nutrient mix and 4 mL SelenoMet solution (Molecular Dimension). For generation of the N-terminally histidine-tagged AcrIIA7, the AcrIIA7 coding sequence was additionally cloned into the pET28a vector using the same *NdeI* and *XhoI* restriction sites, with a stop codon introduced at the C-terminus to prevent C-terminal tagging.

Following transformation into *E. coli* BL21(DE3) competent cells, protein expression was carried out in LB medium. For all expression cultures, cells were grown at 37°C with 50 µg/mL ampicillin or 25 µg/mL kanamycin, until the optical density at 600 nm reached 0.7–0.8, upon which 0.25 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added. The subsequent incubation, at 20°C for 18 hours in a shaking incubator, facilitated protein expression.

Following culture harvest via centrifugation at 2350 × g and 20°C for 15 minutes, the cells were resuspended in 20 mL lysis buffer (20 mM Tris–HCl pH 8.0 and 500 mM NaCl) and lysed via ultrasonication at 4°C. The resulting lysate and supernatant were separated by centrifugation at 27,000 × g and 4°C for 30 minutes. The supernatants were collected, combined with Ni-nitrilotriacetic acid (NTA) affinity resins, and incubated at 4°C for 2 hours. Subsequently, the mixture was loaded onto a gravity-flow column, and after elution, the resin-bound AcrIIA7 was eluted using elution buffer (20 mM Tris–HCl pH 8.0, 500 mM NaCl, and 250 mM imidazole).

Further purification of target protein samples was performed by size exclusion chromatography (SEC) on a Superdex 200 Increase 10/300 GL column connected to an ÄKTA Explorer system. Eluted fractions were pooled, concentrated to 10 mg/mL, and subjected to structural and biochemical analyses. Protein sample purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Additionally, expression constructs for *Streptococcus pyogenes* Cas9 (#62731), *Neisseria meningitidis* Cas9 (#71474), *Haemophilus parainfluenzae* Cas9 (#121540), and *Simonsiella muelleri* Cas9 (#121541) were procured from Addgene. The SpyREC12, SpyREC3, SpyHNH, and SpyWED domain expression constructs were generated via cloning assays utilizing *S. pyogenes* Cas9 plasmids as templates. All PCR products were subsequently cloned into the pET28a vector using *NdeI* and *XhoI* restriction sites. The Cas9 proteins and domains were cultured in lysogeny broth (sigma-lennox), expressed, and purified using the same methodology as for AcrIIA7.

Oligonucleotide sequences and plasmid information are provided in Supplementary Data 1. Plasmids generated in this study are available from the corresponding author upon reasonable request.

Crystallization and X-ray diffraction data collection

Crystallization of pdAcrIIA7 was executed in a controlled environment at 20°C employing the sitting drop vapor diffusion technique. Initial crystals were achieved by combining 1 µL of protein solution (10.23 mg/mL protein in SEC buffer) with 1 µL of reservoir solution (comprising 20%(w/v) PEG 3000 and 0.1M acetate pH 4.5) and equilibrating the mixture using 0.5 mL of reservoir solution. The definitive crystals utilized for X-ray diffraction analysis were cultivated on plates, with a solution comprising 1 µL of protein solution and 1 µL of reservoir solution containing 15% (w/v) PEG 3000 and 0.1M acetate pH 4.7 over three days.

Following crystal growth, a single crystal was carefully chosen and immersed in a reservoir solution augmented with 23% (v/v) glycerol for cryoprotection. X-ray diffraction data acquisition was conducted at -178°C utilizing the BL-5C beamline at the Pohang Accelerator Laboratory (Pohang, Korea).

Subsequent data processing, encompassing indexing, integration, and scaling, was performed using HKL2000 software ⁴².

Structure determination and refinement.

The initial structure of pdAcrIIA7 was determined using *AutoSol*, SAD phasing, an automated experimental phasing with model building software, in the package of PHENIX ⁴³. Subsequent model building with refinement was performed using *phenix.autobuild*, *phenix.refine*, and *Coot* ^{44,45}. The structure quality and stereochemistry were validated using the MolProbity⁴⁶. All structural figures were generated using the PyMOL program ⁴⁷. Representative electron density map was provided at Supplementary Fig. 15

Multi-angle light scattering analysis (MALS)

SEC-coupled multi-angle light scattering (SEC-MALS) experiments were carried out to determine the absolute molecular mass of AcrIIA7 in solution. Purified AcrIIA7 protein in SEC buffer was injected into a Superdex 200 Increase 10/300 GL column pre-equilibrated with SEC buffer. SEC-MALS was performed at 20 °C with a 0.5 mL/min buffer flow rate. The ÄKTA explorer system (GE Healthcare) was coupled with a DAWN-Treos MALS detector (Wyatt Technology, Santa Barbara, USA). A reference molecular mass value was established using bovine serum albumin for calibration. Data were processed and assessed using ASTRA software (Wyatt Technology).

In vitro anti-CRISPR activity assay

To assess the inhibitory potential of AcrIIA7 and its mutants on Cas9-mediated target cleavage, a series of *in vitro* target DNA cleavage assays were conducted. SpyCas9 enzyme, acquired from NEB, a single guide RNA (sgRNA), generated using the EnGen® sgRNA Synthesis Kit, *S. pyogenes* (NEB), and each AcrIIA7 wild-type and its mutant variants were added and combined in different order. For the first binding, the mixture was incubated at 25°C for an hour. In the subsequent components were added to the mixture, then incubated at 25°C for 30 minutes. Finally, the target DNA was added to the reactions and incubated in NEBuffer r3.1(NEB) at 37°C for 25minutes. Proteinase K was added and incubated for 10 minutes to terminate the reaction.

All reaction products were subjected to loading and separation through electrophoresis on 4% polyacrylamide gels. The visualization of DNA cleavage products was accomplished by staining the gels with SYBR GOLD. The resulting gel images were analyzed to evaluate the inhibitory impact of the wild-type AcrIIA7 and the various mutant forms on Cas9-mediated target cleavage.

Size exclusion chromatography assay for complex formation

Size exclusion chromatography (SEC) was employed to investigate whether AcrIIA7 forming complexes between various Cas9s from different species, or SpyCas9 domains. Each wild-type and N-term deleted AcrIIA7 from *Phocaeicola dorei* and AcrIIA7 from metagenome were mixed with various Cas9s and each Cas9 domain, followed by incubation at 4 °C for 1 hour. Then, the resulting mixture was loaded onto a Superdex 200 Increase 10/300 GL column (GE Healthcare) pre-equilibrated with SEC buffer (20 mM Tris–HCl pH 8.0, 150 mM NaCl). The eluted fractions corresponding to the peaks of interest were collected, and the samples were subsequently analyzed by SDS-PAGE stained with Coomassie Brilliant Blue. To assess the complex formation between AcrIIA7 and the Cas9 proteins or each domain, the migration patterns, and the co-migration of protein bands within the gel were examined and analyzed.

Pull down assay

6x His-tagged SpyCas9 cloned into the pET21a vector and untagged AcrIIA7 cloned into the pET24a vector were co-transformed into *E. coli* BL21(DE3) competent cells. A single co-transformed colony was selected and cultured under the same conditions used for AcrIIA7 expression. Cells were harvested by centrifugation and resuspended in lysis buffer containing 20 mM Tris-HCl pH 8.0, 150 mM NaCl. The cell suspension was lysed by sonication, and the clarified lysate was incubated with Ni-NTA agarose beads for 2 hours to capture 6x His-tagged SpyCas9. After incubation, the bead-protein complexes were loaded onto a gravity flow column, washed with buffer containing 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 80 mM Imidazole. Bound proteins were eluted with buffer containing 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 250 mM Imidazole. Eluted samples were analyzed by SDS-PAGE and visualized by Coomassie Brilliant Blue staining.

Mutagenesis

Site-directed mutagenesis was performed utilizing a QuickChange kit obtained from Stratagene (San Diego, CA, USA), following the manufacturer's instructions. Primer sequences used for mutagenesis are listed in Supplementary Data 1. The resulting mutations were subjected to sequencing analysis to verify the precision of the introduced alterations. This step ensured the fidelity of the modifications. Following successful validation, all mutant protein variants were produced and purified by applying the identical methodology previously described for the wild-type protein⁴⁸.

Electrophoretic mobility shift assay with polyacrylamide gel (EMSA-P)

Each purified target DNA (20 nM), sgRNAs (150 nM), crRNA (150 nM), type II-A tracrRNA (150 nM), type II-C tracrRNA (150 nM), structured single strand (Sss) RNA (20 nM), tracrRNA variants (150 nM) were incubated with AcrIIA7 proteins at room temperature (25°C) for 1 hour. The samples were then separated by gel electrophoresis at 100 V on a 10 % native 0.5 × TBE (Tris-Borate EDTA) polyacrylamide gel. After electrophoresis, gels were stained with SYBR Gold (Invitrogen, Waltham, MA, USA) and visualized according to the manufacturer's instructions.

For the gRNA formation, synthesized crRNA_repeat (300 nM), and anti-repeat_tracrRNA (300 nM) were incubated in a buffer containing 10 mM Tris pH 8.0, 50 mM NaCl, 1 mM EDTA at 37°C for 2 hours. The formed gRNA complexes were also analyzed by electrophoresis on a 10 % native polyacrylamide gel in 0.5X TBE buffer and visualized using SYBR Gold dye.

In vitro anti-CRISPR activity assay using separately synthesized tracrRNA and crRNA

To further investigate the order-dependent inhibitory activity of AcrIIA7, additional *in vitro* target DNA cleavage assays were performed using crRNA and tracrRNA supplied as separate components. crRNA, tracrRNA, SpyCas9, and AcrIIA7 were combined in various orders, as specified for each experimental condition. Depending on the reaction sequence, RNA-AcrIIA7 binding reactions were incubated at 25 °C for 1 hour, whereas crRNA-tracrRNA annealing reactions were incubated for 2 hours in buffer containing 30 mM Tris-HCl (pH 7.5) and 100 mM potassium acetate. Where indicated, Cas9 was subsequently added and incubated at 25 °C for 30 minutes. For all conditions, target DNA was added as the final step in the presence of NEBuffer r3.1 (NEB), followed by incubation at 37 °C for 40 minutes. Reactions were terminated by the addition of Proteinase K and incubation for 10 minutes. All reaction products were subjected to loading and separation through electrophoresis on 4% polyacrylamide gels. The visualization of DNA cleavage products was accomplished by staining the gels with SYBR GOLD. The resulting gel images were analyzed to evaluate the inhibitory impact of the wild-type AcrIIA7 and the various mutant forms on Cas9-mediated target cleavage.

Native Polyacrylamide Gel Electrophoresis (Native-PAGE) assay

The binding of AcrIIA7 to cyclic nucleotides was analyzed using Native-PAGE assay. The cyclic nucleotides used in this study were cGG(c-di-2'-dGMP) and 1''-2'-gcADPR(2'-cADPR). These cyclic nucleotides (5, 10, 20, and 50µM) were incubated with 20µM AcrIIA7 at 18°C for 30min. The reaction mixtures were then loaded onto 5% native polyacrylamide gels and electrophoresed at 120V. After electrophoresis, the gels were visualized by Coomassie Brilliant Blue staining.

DATA AVAILABILITY

The data supporting the findings of this study are available from the corresponding authors upon request. The coordinate and structural factors have been deposited into the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB) under the PDB code 9KZR. Source data are provided with this paper.

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AUTHOR CONTRIBUTIONS STATEMENT

H.H.P. designed and supervised the project. S.Y.L. performed all the experiments, including collecting the biochemical and structural data and solving the structure. H.H.P. and S.Y.L. wrote the manuscript. Both authors discussed the results and commented on the manuscript.

CONFLICTS OF INTERESTS STATEMENT

The authors declare no competing interests.

FIGURE LEGENDS

FIGURE 1. Structural analysis of AcrIIA7 derived from *Phocaeicola dorei*.

(A) Size-exclusion chromatography profile of AcrIIA7, along with an SDS-PAGE gel showing the protein fractions eluted at the peak position. M indicates the size marker. Experiments were independently repeated three times with similar results. (B) Cartoon representation of the four AcrIIA7 molecules arranged within the asymmetric unit. (C) Rainbow-colored cartoon representation of monomeric AcrIIA7, where the chain gradually transitions from blue (N-terminus) to red (C-terminus). (D) Topological diagram illustrating the secondary structure of AcrIIA7. (E) Superimposition of the four molecules in one asymmetric unit. (F, G) Enlarged views of regions with significant structural divergence, highlighting $\alpha 1$ - $\alpha 2$ (F) and $\alpha 7$ (G). (H) Surface electrostatic potential map of AcrIIA7, ranging from -6.9 kT/e (red) to +6.9 kT/e (blue). (I) B-factor distribution represented using a putty model, with colors transitioning from red to violet to indicate varying B-factor values. (J) Structural similarity analysis conducted via the DALI server. (K) Structural comparison of the experimentally determined crystal structure (magenta) with the predicted structure (yellow) modeled using AlphaFold2. Source data are provided as a Source Data file.

FIGURE 2. Tetrameric structure of AcrIIA7 and analysis of its interface and conformation.

(A) Multi-angle light scattering (MALS) profile for the SEC-eluted AcrIIA7 peak. The red line represents the molecular mass determined experimentally using MALS. (B) Summary table of interaction details for each interface type, analyzed using the PISA server. CSS denotes the Complex Formation Significance Score. (C) The tetrameric structure of AcrIIA7 at the initial rotation stage is depicted on the left as a cartoon representation. (D, E, F) Magnified views of protein-protein interactions (PPIs) for the AB dimer (D), the BC dimer (E), and the BD dimer (F).

FIGURE 3. AcrIIA7 inhibits SpyCas9-mediated DNA cleavage only in the absence of the head domain and specifically when added before Cas9-RNP formation.

(A) *In vitro* anti-CRISPR activity assay showing the reaction order-dependent inhibition of SpyCas9 activity by full-length pdAcrIIA7. The numbers indicate the sequence in which components were added to the reaction. The experiment was repeated three times, yielding consistent results. (B) Sequence alignment of AcrIIA7 proteins derived from *Phocaeicola dorei* and metagenomic sources. Fully conserved residues are highlighted in red, while partially conserved residues are shown in blue. Most residues are conserved, except for 48 residues at the N-terminus of pdAcrIIA7 and two residues. (C) A schematic diagram depicting the domain organization of AcrIIA7. The domain organization of Δ N48AcrIIA7 is indicated by double arrow-line. (D, E) Cartoon representations of pdAcrIIA7 lacking the head domain: monomeric structure (D) and tetrameric structure (E). (F) SEC-MALS profiles comparing the elution peak positions of wild-type pdAcrIIA7 (black), Δ N48AcrIIA7 (blue), and meAcrIIA7 (green). (G) Table summarizing the results of SEC-MALS. (H) *In vitro* anti-CRISPR activity assay illustrating the reaction order-dependent inhibition of SpyCas9 activity by meAcrIIA7. (I) *In vitro* anti-CRISPR activity assay demonstrating the reaction order-dependent inhibition of SpyCas9 activity by the Δ N48AcrIIA7 mutant. (J) *In vitro* anti-CRISPR activity assay evaluating the concentration-dependent inhibition of SpyCas9 activity by the Δ N48AcrIIA7 mutant. The assays were performed under two conditions: preincubation of sgRNA with AcrIIA7 versus preincubation of sgRNA with SpyCas9. Experiments were independently repeated three times with similar results. Source data are provided as a Source Data file.

FIGURE 4. Interaction analysis between SpyCas9 and pdAcrIIA7 or Δ N48AcrIIA7 or meAcrIIA7 using SEC.

(A) Interaction analysis between pdAcrIIA7 and SpyCas9 using SEC. SEC profiles for pdAcrIIA7 WT (black), SpyCas9 (blue), and their mixtures (red) are displayed. (B) Interaction analysis between Δ N48AcrIIA7 and SpyCas9 using SEC. (C) Interaction analysis between meAcrIIA7 and SpyCas9 using SEC. The SpyCas9 sample exhibited partial heterogeneity in SEC, likely due to minor aggregation observed under these conditions. Experiments were independently repeated three times with similar results. Source data are provided as a Source Data file.

FIGURE 5. AcrIIA7 exhibits specific binding to the tracrRNA.

(A) Surface electrostatic potential of tetrameric pdAcrIIA7, with a scale ranging from -7.7 kT/e (red) to +7.7 kT/e (blue). (B) EMSAs demonstrating the specific interaction of AcrIIA7 with sgRNA. B represents the “sgRNA in Buffer”. (C) EMSA assay performed with AcrIIA7 and single-stranded DNA (ssDNA). (D) EMSA assay performed with AcrIIA7 and double-stranded DNA (dsDNA). (E) Schematic representation of SpyCas9 sgRNA, synthesized and used for our assay, comprising crRNA, repeat: anti-repeat, and tracrRNA regions. (F) EMSA assay performed with AcrIIA7 and crRNA. B represents the “sgRNA in Buffer” (G) EMSA assay performed with AcrIIA7 and tracrRNA. B represents the “sgRNA in Buffer”. The bands shifted due to the interaction between tracrRNA and AcrIIA7 are indicated with red arrows. Experiments were independently repeated three times with similar results. Source data are provided as a Source Data file.

FIGURE 6. Structure dependent binding of AcrIIA7 to nucleic acids.

(A) EMSA analyzing the concentration-dependent binding of AcrIIA7 to HpaCas9 tracrRNA. The left panel shows the predicted secondary structure and sequence of tracrRNA of the type II-C HpaCas9. (B) Schematic representation of RNA secondary structures with progressive deletions of stem-loop elements in the gRNA scaffold. SL123 represents the full structured gRNA scaffold, SL12 lacks stem loop 3, SL1 lacks stem loops 2 and 3, and SL0 lacks all stem-loop structures. (C) EMSA comparing the binding affinities of RNAs with different stem-loop configurations in the presence of 10 μ M meAcrIIA7. (D) EMSA comparing the binding affinities of RNAs with different stem-loop configurations in the presence of 10 μ M Δ N48AcrIIA7. Experiments were independently repeated three times with similar results. Source data are provided as a Source Data file.

FIGURE 7. Mechanism of tracrRNA recognition by tetrameric AcrIIA7.

(A) Cartoon representation of monomeric pdAcrIIA7, with amino acid conservation levels depicted using colors generated by the ConSurf server. (B) Cartoon representation of tetrameric pdAcrIIA7, similarly colored to indicate amino acid conservation levels based on ConSurf analysis. (C) Amino acid sequence of pdAcrIIA7, color-coded by conservation levels as determined by ConSurf analysis. (D) Key residues potentially involved in RNA binding are highlighted in red and labelled. The predicted deep groove for RNA binding is marked with black dots. (E) Predicted structure of tracrRNA-pdAcrIIA7 complex generated using Alphafold3. (F) EMSA showing the interaction between sgRNA and generated mutants of Δ N48AcrIIA7, including K58D, K66W, K71W, K93W, and R101W. DW, distilled water control; Pd, pdAcrIIA7; Me, MeAcrIIA7. (G) *In vitro* target DNA cleavage assay using SpyCas9 and various Δ N48AcrIIA7 mutants. In the assay, “+” and “-” indicate the presence or absence of the respective component in the reaction. Experiments were independently repeated three times with similar results. Source data are provided as a Source Data file.

FIGURE 8. Inhibition of gRNA formation by AcrIIA7.

(A) Concentration-dependent inhibition of gRNA formation by pdAcrIIA7. *In vitro* gRNA formation reactions were performed by incubating tracrRNA and crRNA in the presence of increasing concentrations of purified pdAcrIIA7 (0.5–20 μ M, indicated by black triangle). Formation of mature gRNA was analyzed by native PAGE. (B) Concentration-dependent inhibition of gRNA formation by meAcrIIA7. Similar to (A), increasing concentrations of meAcrIIA7 (0.05–2 μ M, indicated by black triangle) were tested in gRNA formation assays. (C) Order-of-addition assay assessing pdAcrIIA7-mediated inhibition of gRNA formation. Components were added sequentially to assess whether pdAcrIIA7 inhibits gRNA formation in an order-dependent manner. The numbers explicitly indicate the sequential order of component addition, where '1' represents the components that were incubated first, followed by '2' and '3' for subsequent additions. Thus, two components labeled with '1' indicate that they were co-incubated during the initial step. (D) Order-of-addition assay for meAcrIIA7-mediated inhibition. Reactions were performed with 2 μ M meAcrIIA7 following different component addition sequences, as described in (B). Inhibition patterns reveal the stage at which meAcrIIA7 interferes with gRNA complex formation. Experiments were independently repeated three times with similar results. Source data are provided as a Source Data file.

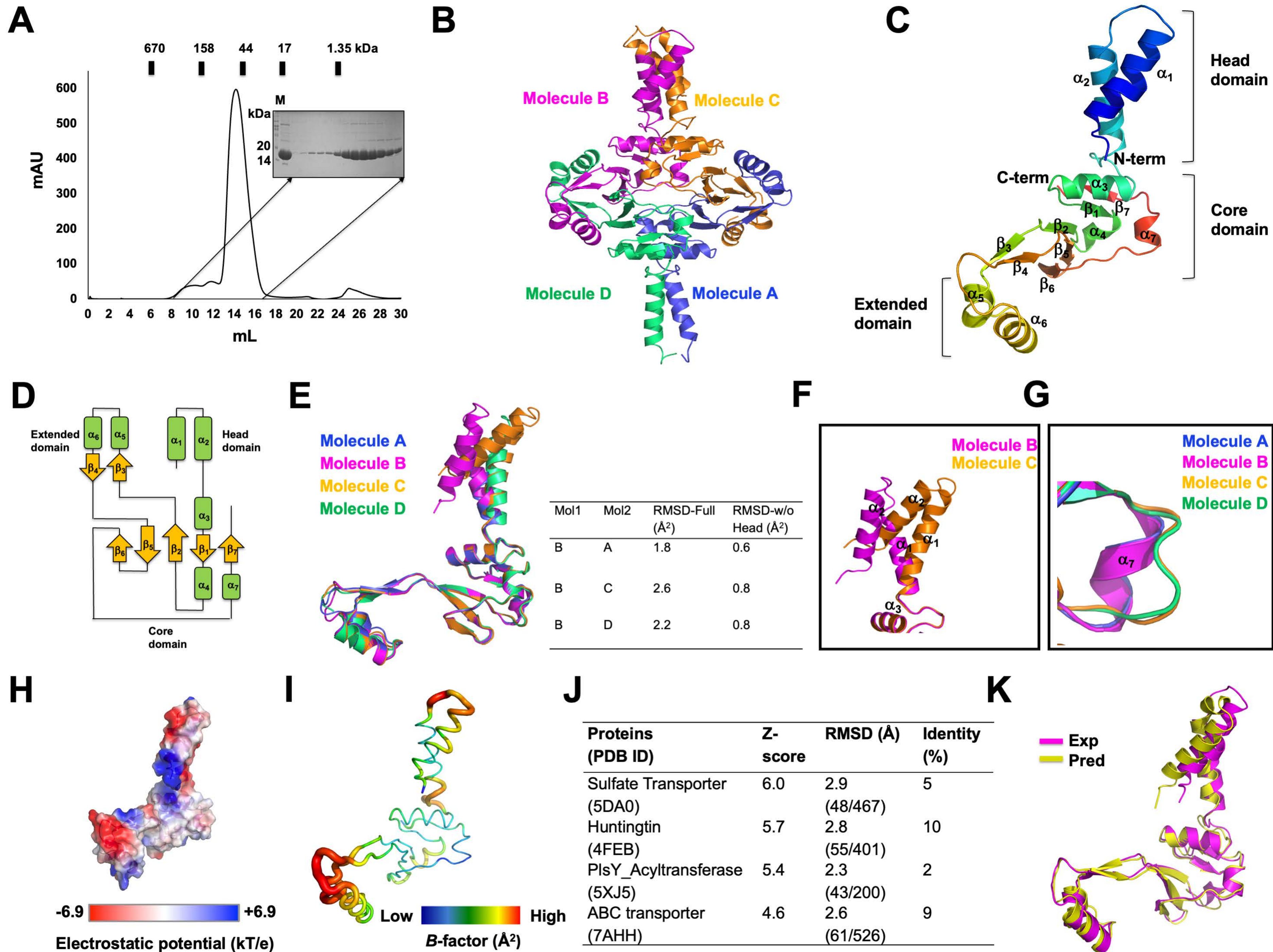
FIGURE 9. Proposed model of Cas9 inhibition by AcrIIA7.

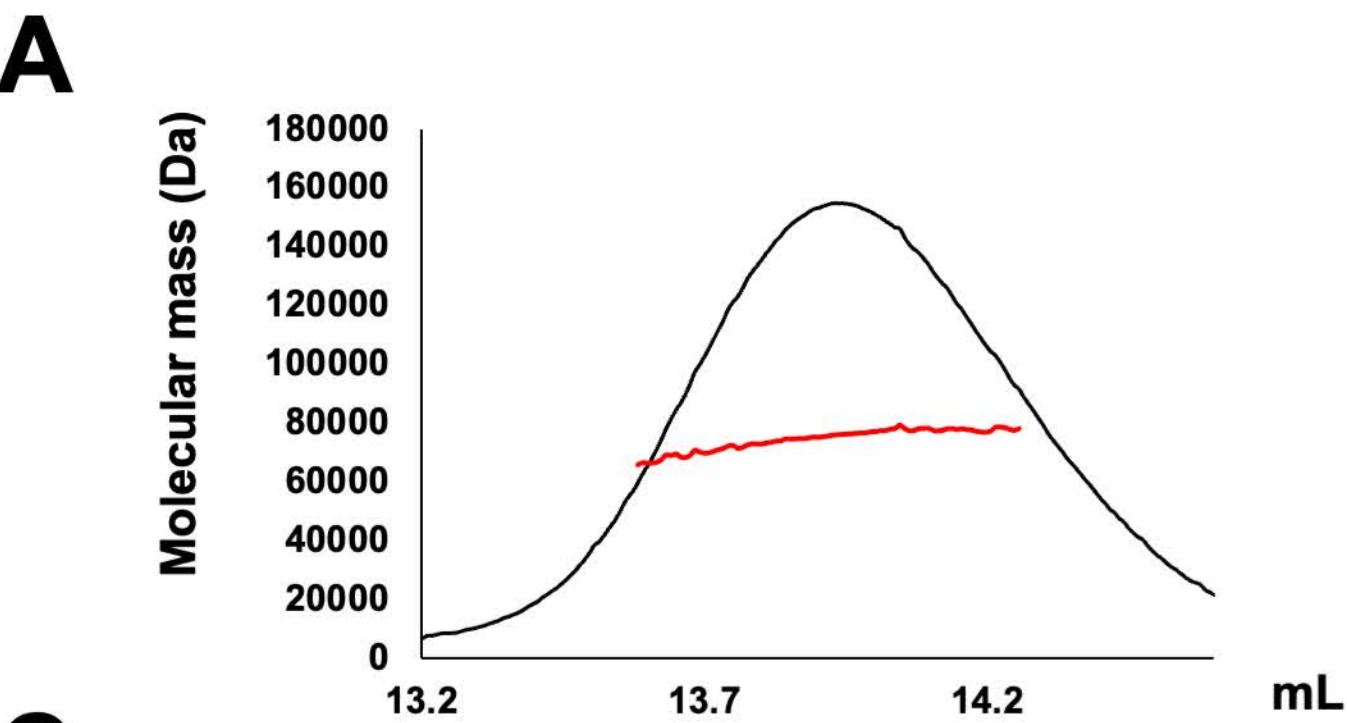
(A) The schematic figure of CRISPR-Cas9 immune system: Upon phage infection, bacterial CRISPR loci are transcribed into pre-crRNA, which are processed into mature crRNAs. The tracrRNA facilitates crRNA processing into sgRNA, which guides Cas9 to recognize and cleave complementary phage DNA, enabling cleavage and destruction of the invading phage genome. This immune response effectively neutralizes phage infection. (B) The proposed model of AcrIIA7 as an anti-CRISPR protein: AcrIIA7 counteracts the CRISPR-Cas9 system through two possible mechanisms. First, AcrIIA7 specifically binds to the tracrRNA, forming an AcrIIA7-tracrRNA complex, which hijacks the RNA and inhibits sgRNA biogenesis. Second, AcrIIA7 directly interacts with the tracrRNA of gRNA to form an AcrIIA7-gRNA complex, preventing the assembly of the Cas9-RNP complex. This disruption blocks the loading of target DNA into Cas9, thereby inhibiting DNA cleavage activity. As a result, the phage evades bacterial immune defense, enabling lysogeny or successful lytic replication. Created in BioRender. Lee, S. Y. (2026) <https://BioRender.com/fhubnyr>

Editorial Summary:

CRISPR–Cas9 immunity relies on forming a Cas9–crRNA–tracrRNA ribonucleoprotein complex. Here, the authors show that AcrIIA7 blocks this process by binding tracrRNA, preventing its pairing with crRNA.

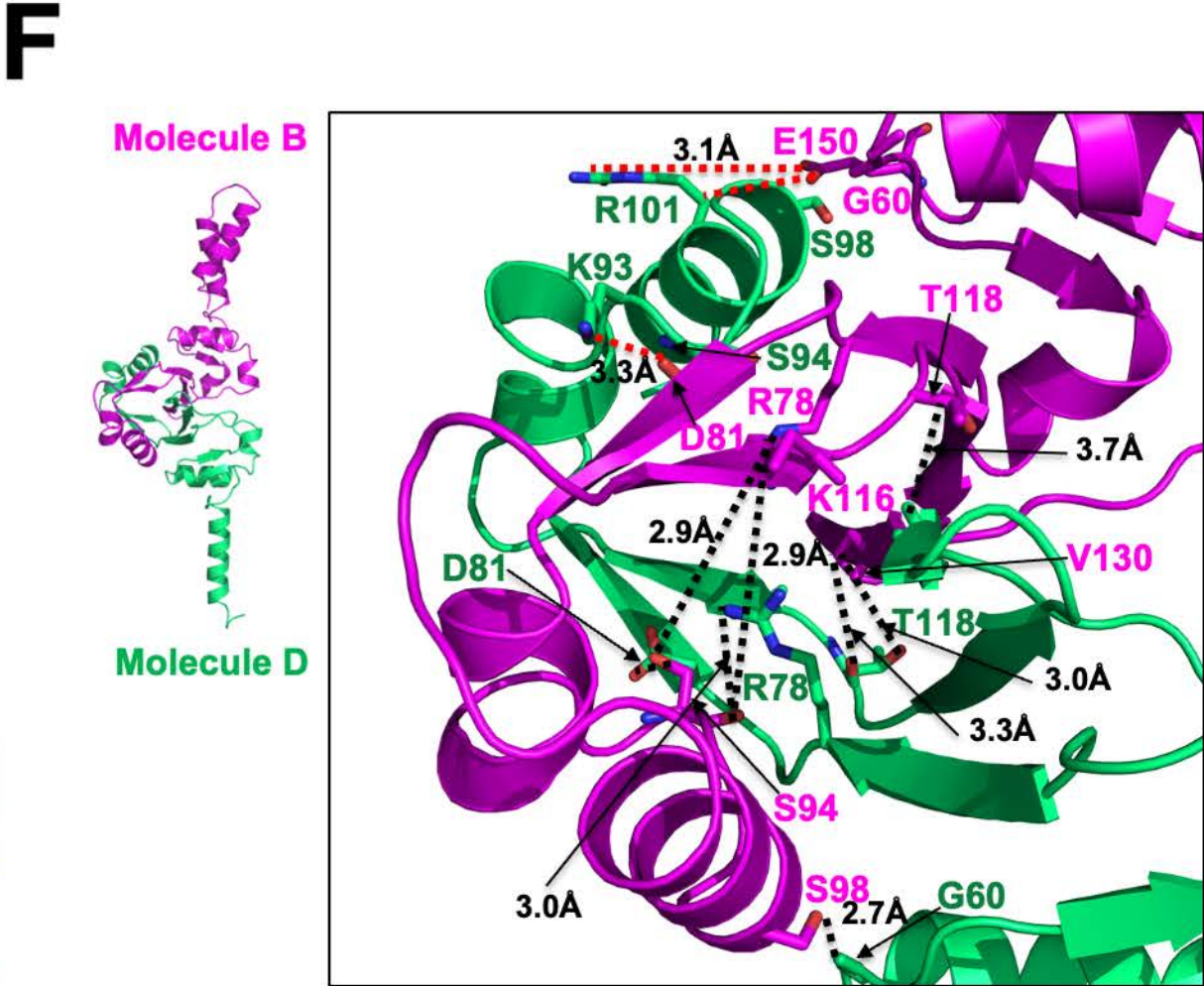
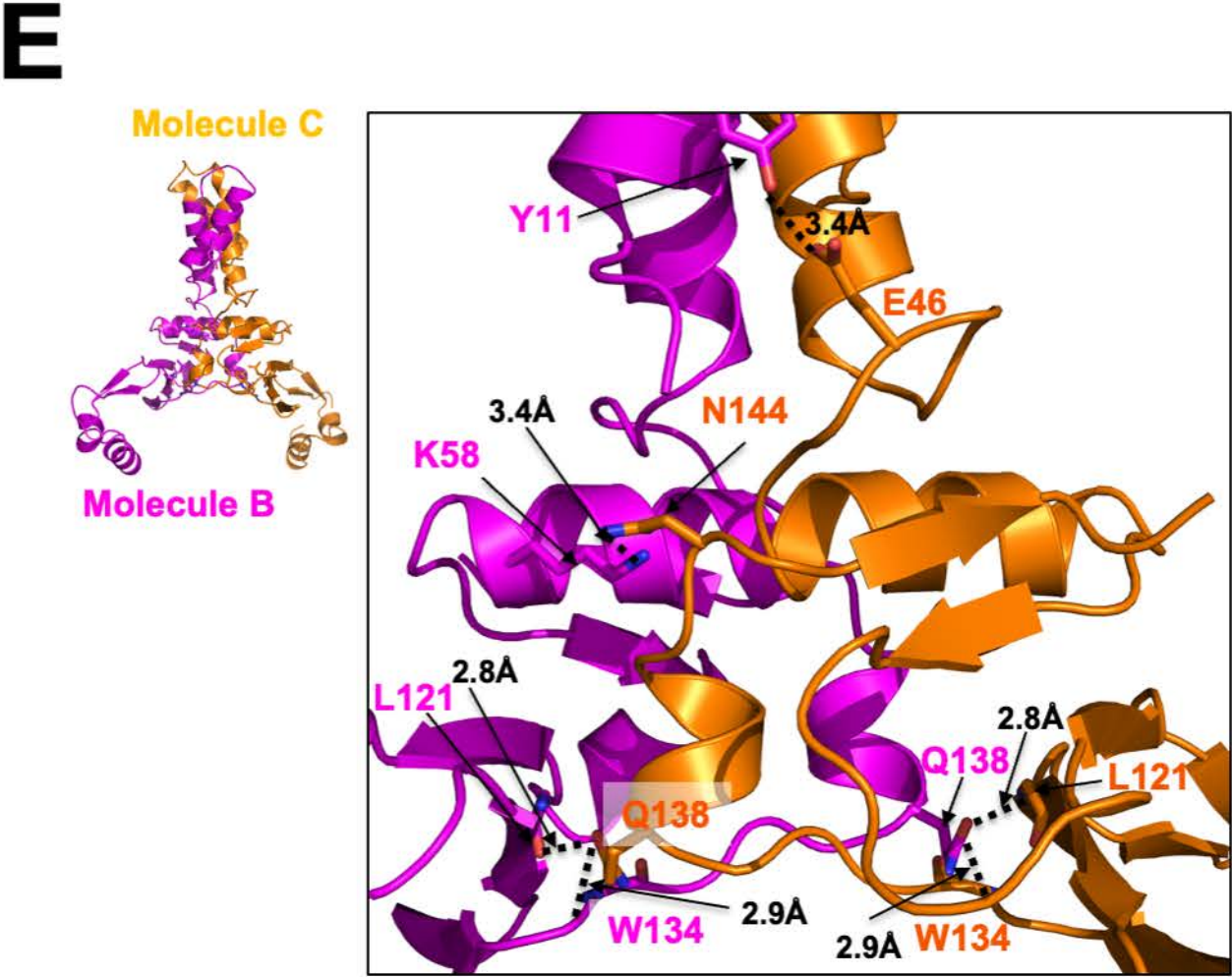
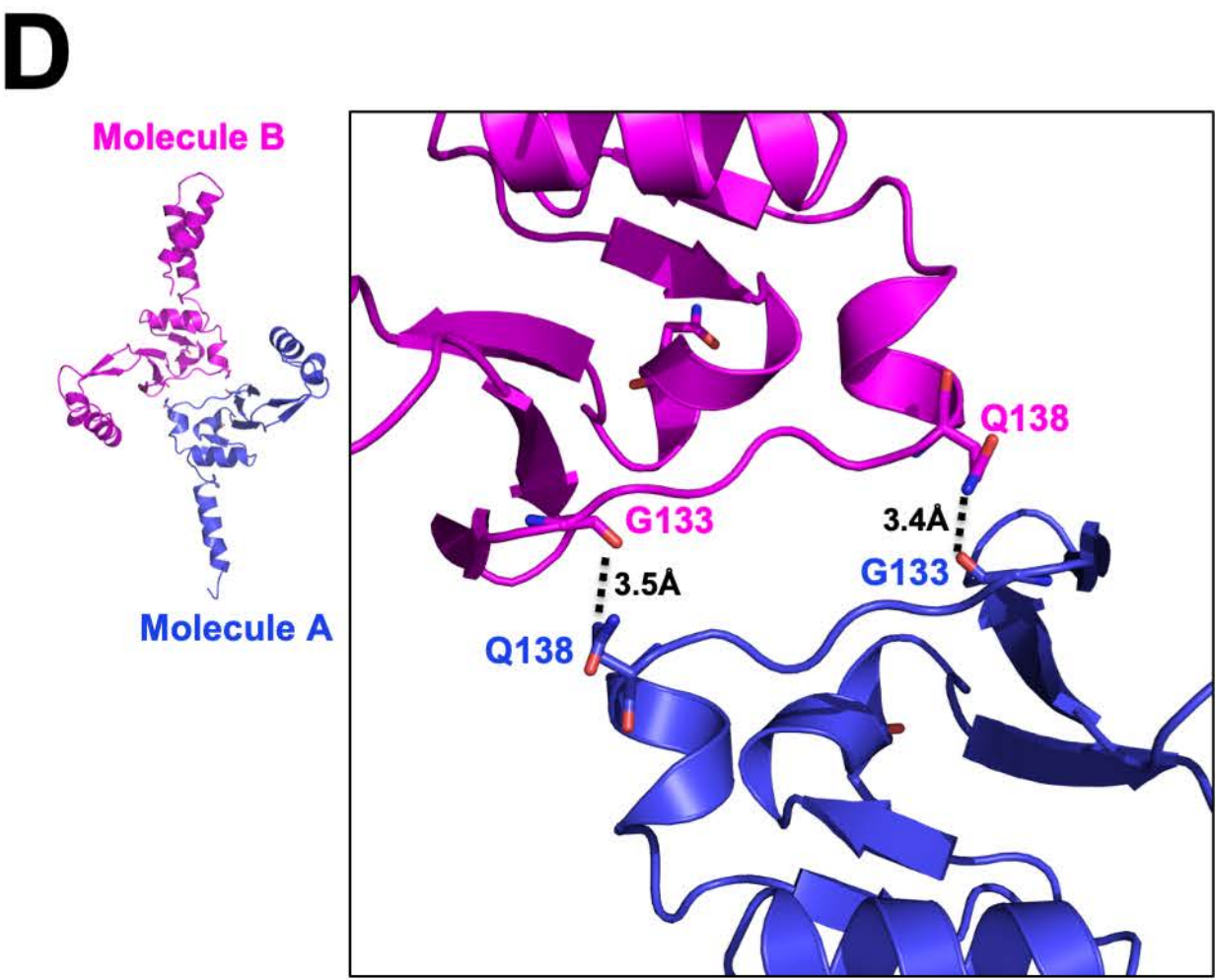
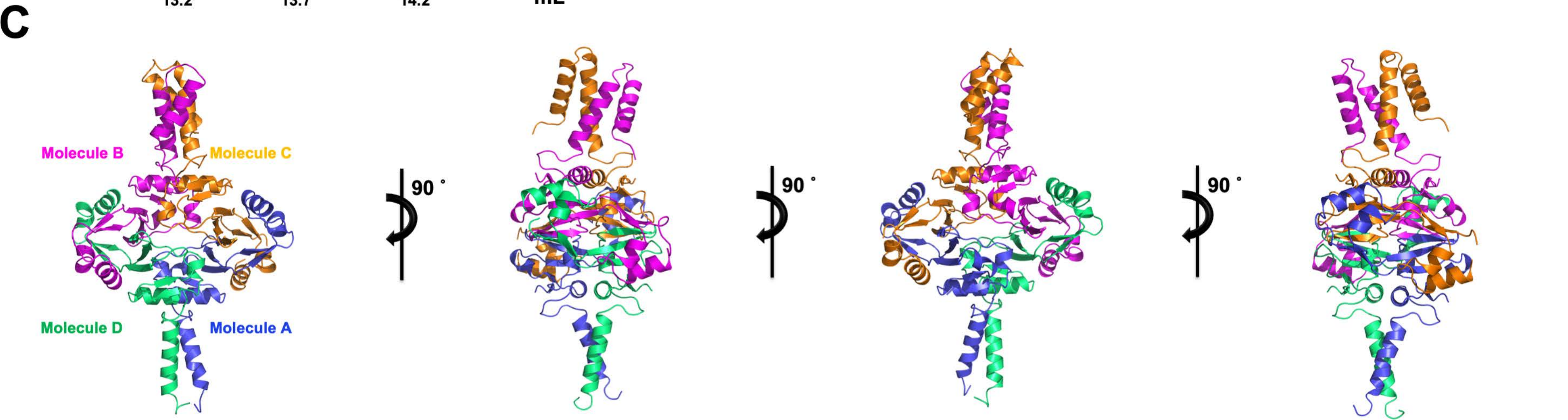
Peer review information: *Nature Communications* thanks Yue Feng, and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. A peer review file is available.



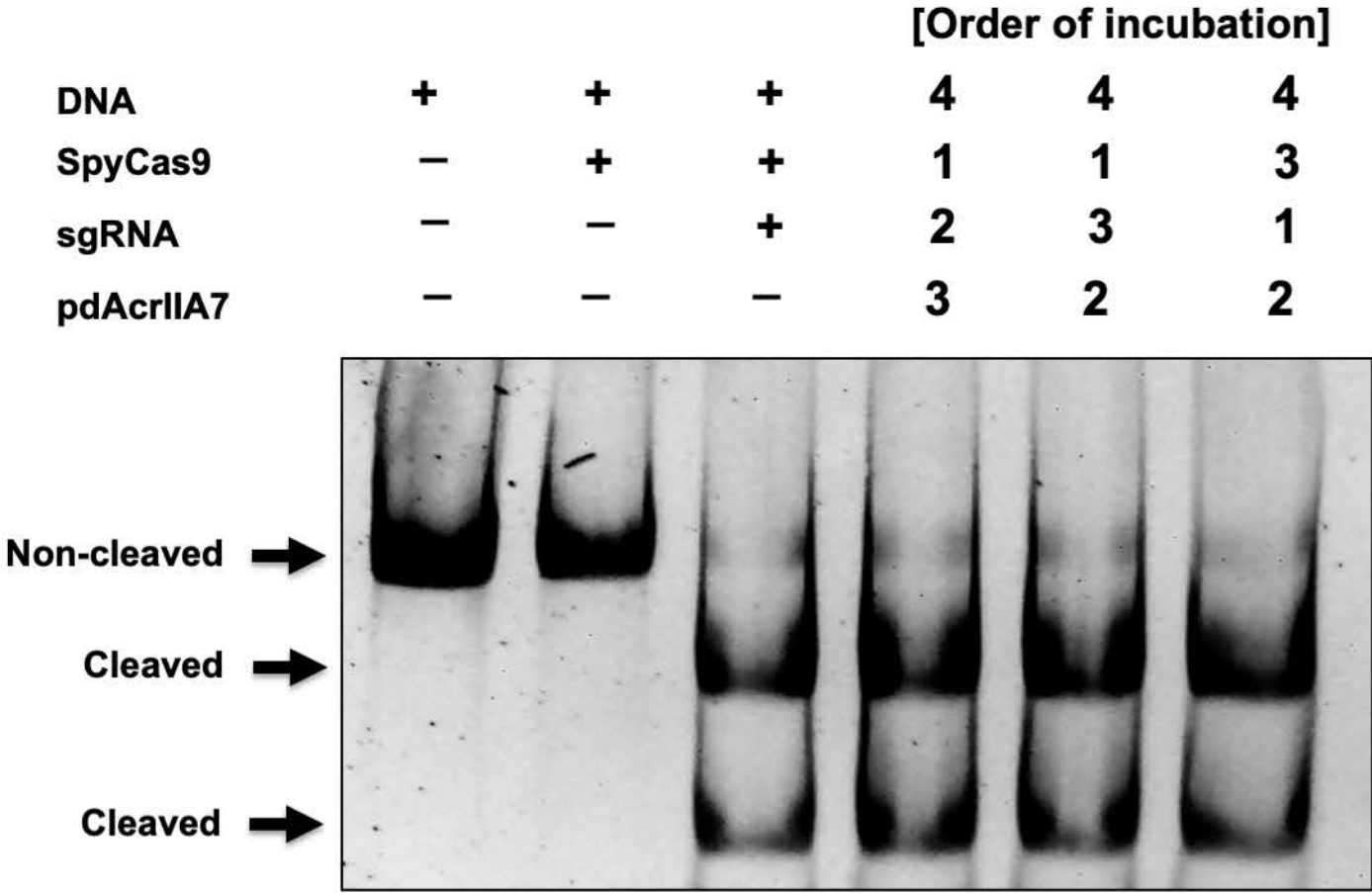


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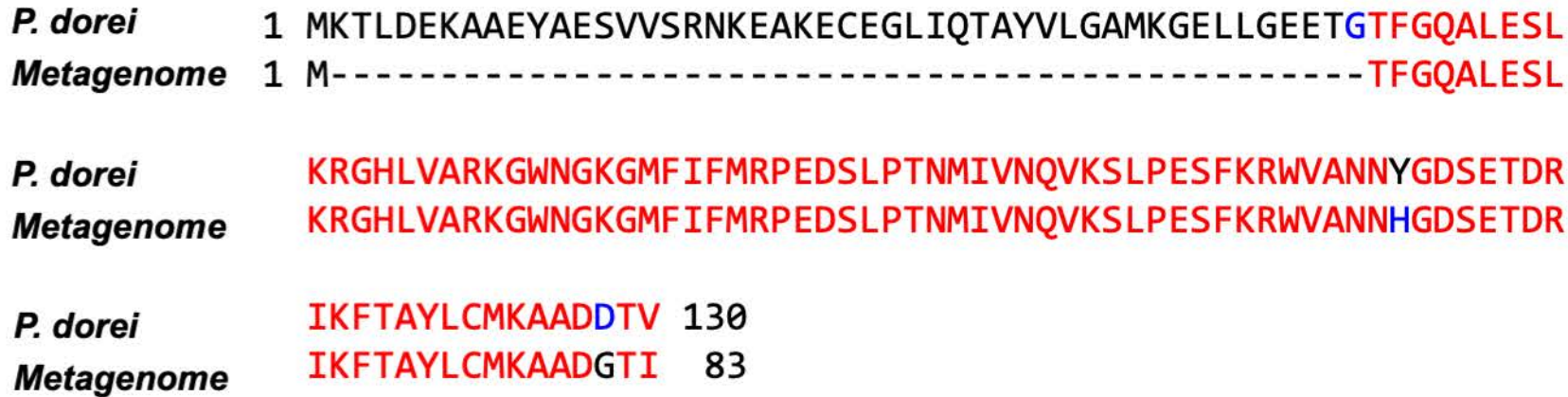
Mol 1	Mol2	Interface area (Å ²)	interface residue	ΔG (kcal/mol)	Hydrophobic P-value	# of HB	# of SB	PISA score
B (D)	A (C)	271.1 (3.2%)	12/13 (8.2/10.2%)	-4.3	0.343	2	0	0.000
B (A)	C (D)	1565.6 (15.5%)	46/48 (30.9/32.7%)	-23.9	0.186	6	0	0.100
B (A)	D (C)	1470.8 (14.2%)	42/41 (28.2/32.3%)	-16.6	0.493	14	6	1.000



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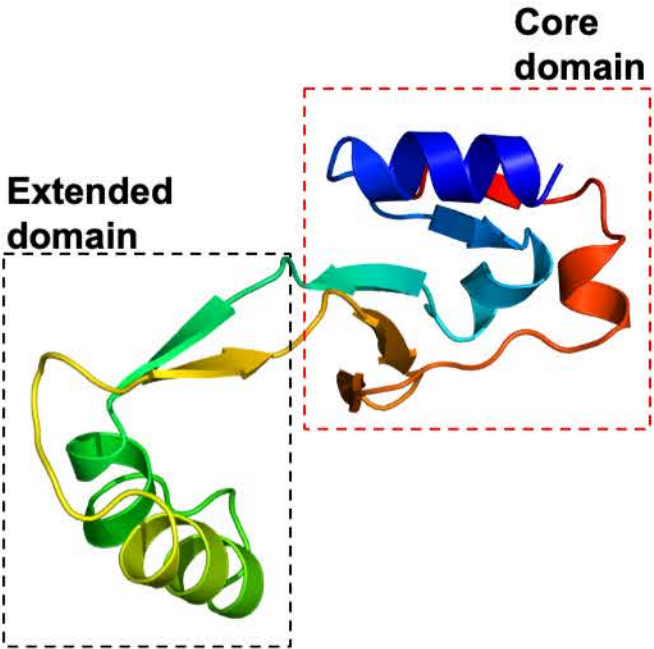
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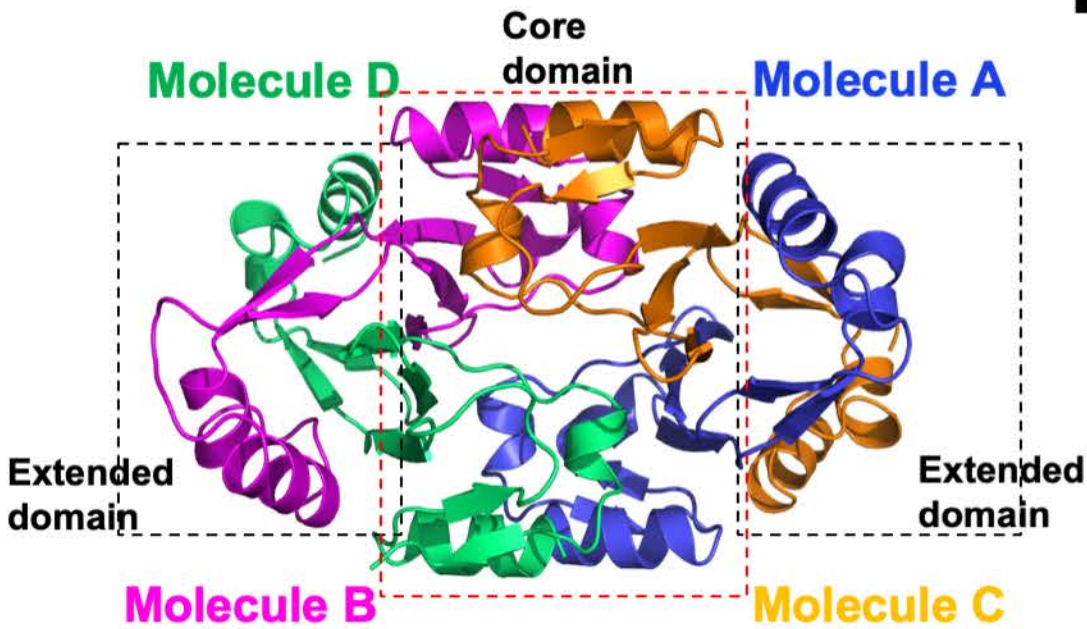
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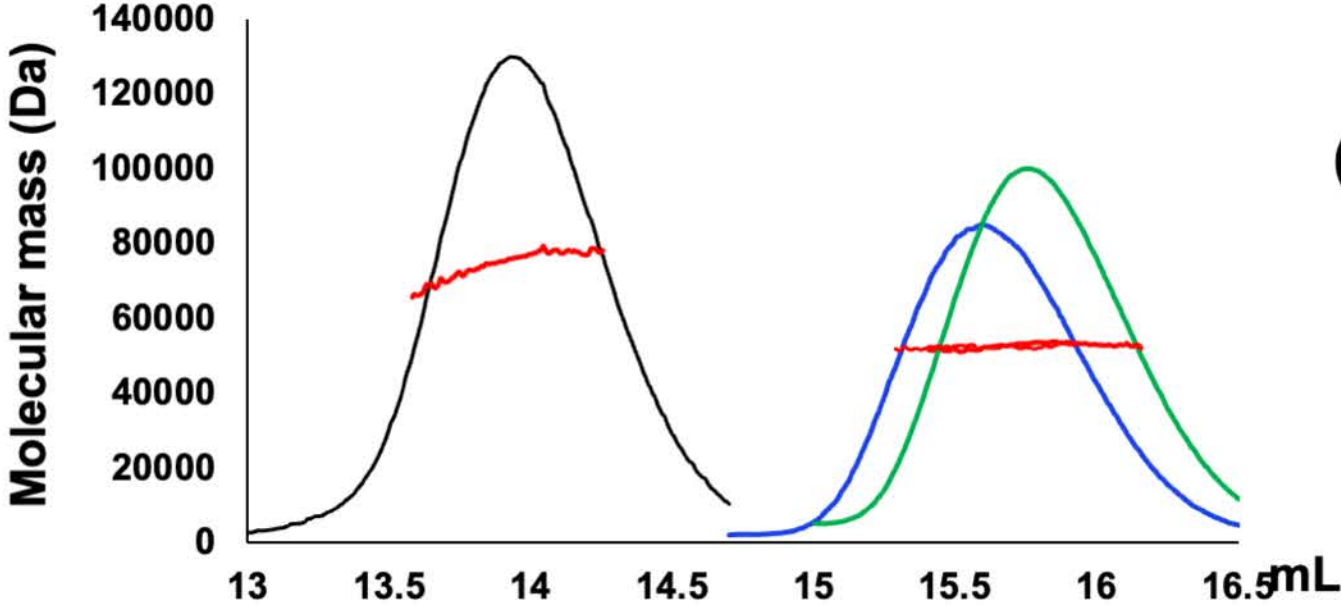
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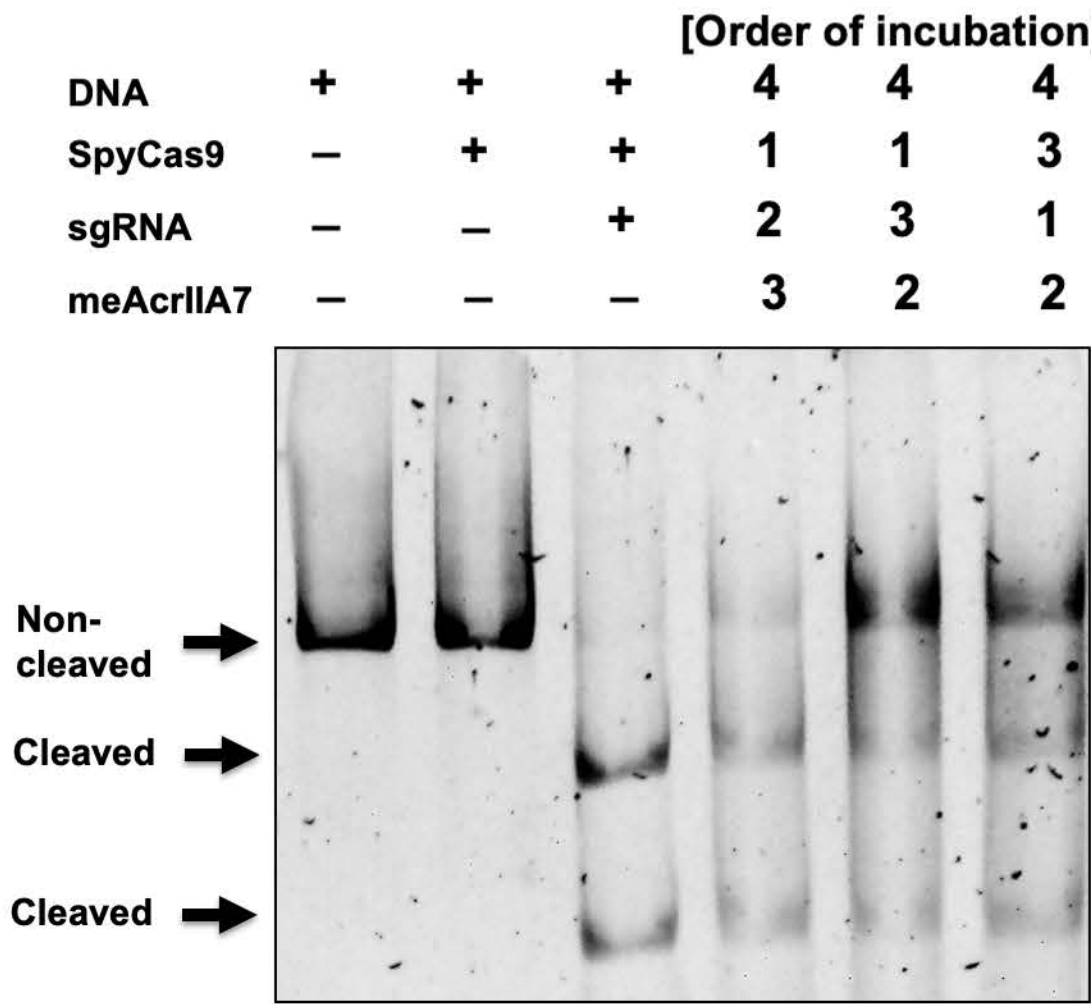
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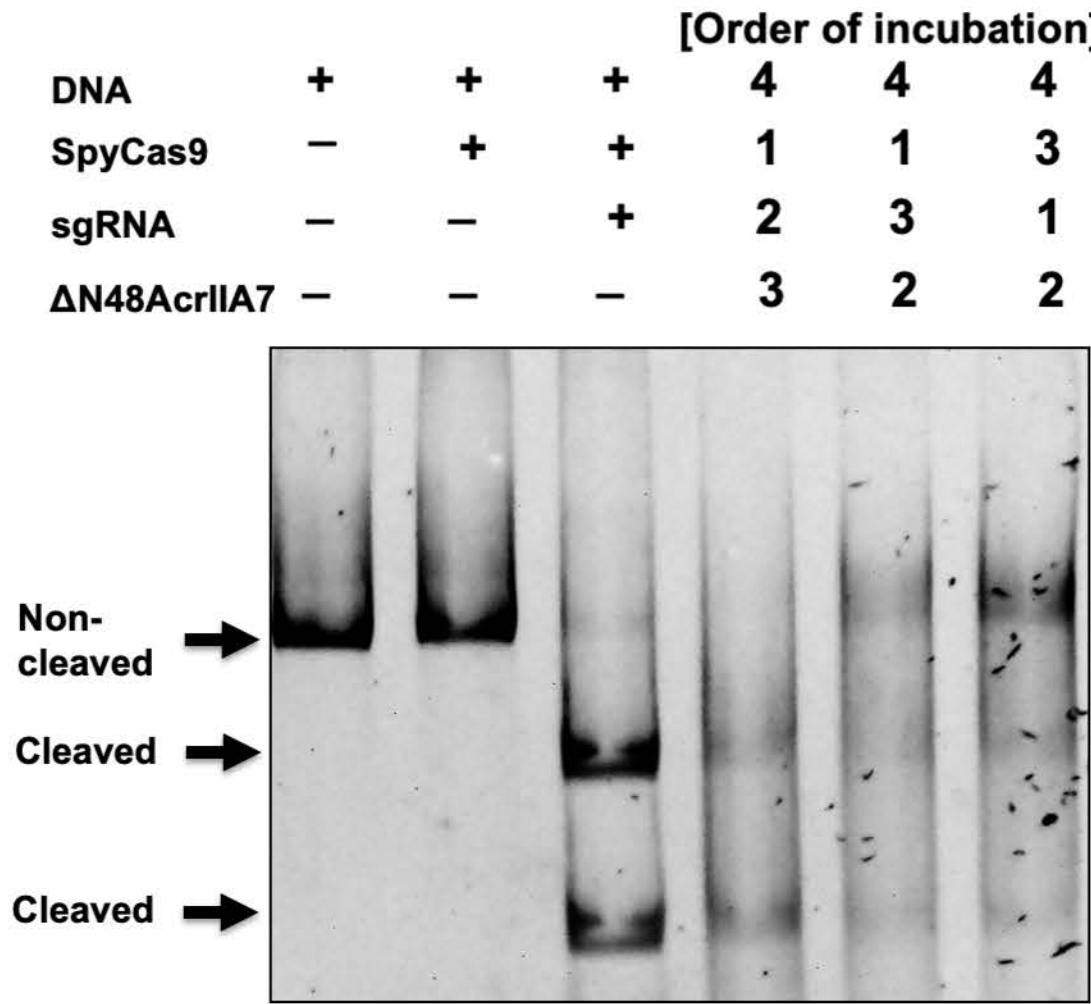
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Protein	Theoretical MW (kDa)	Experimental MW (kDa)
pdAcrIIA7	17.81	74.86 (±1.1%)
delN48AcrIIA7	12.76	52.46 (±5.6%)
meAcrIIA7	12.70	52.79 (±6.2%)

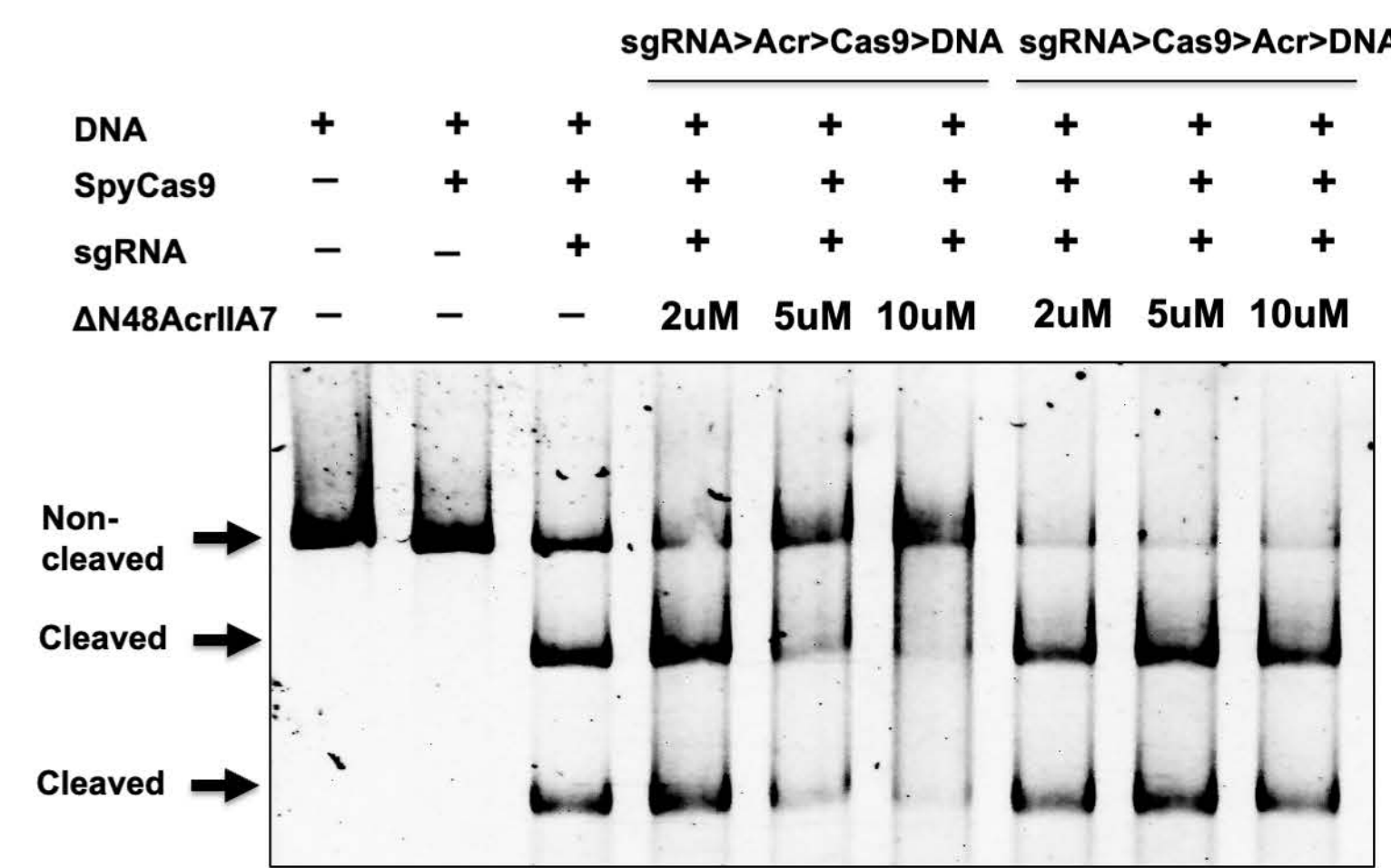
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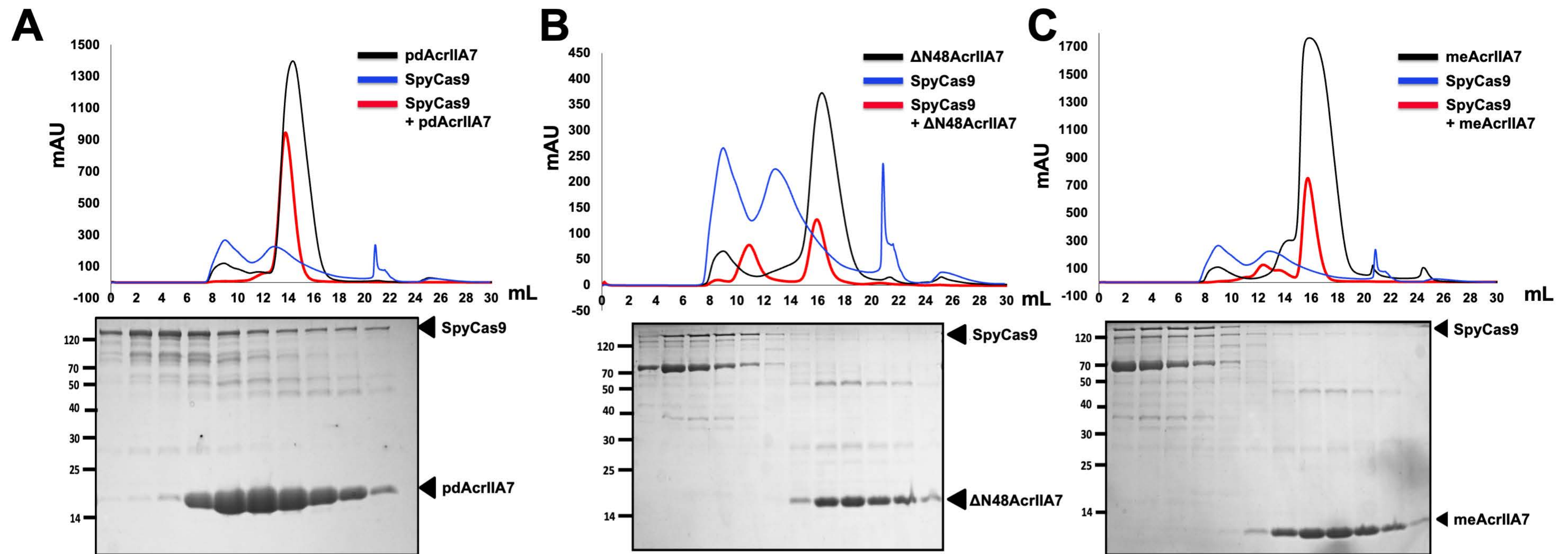


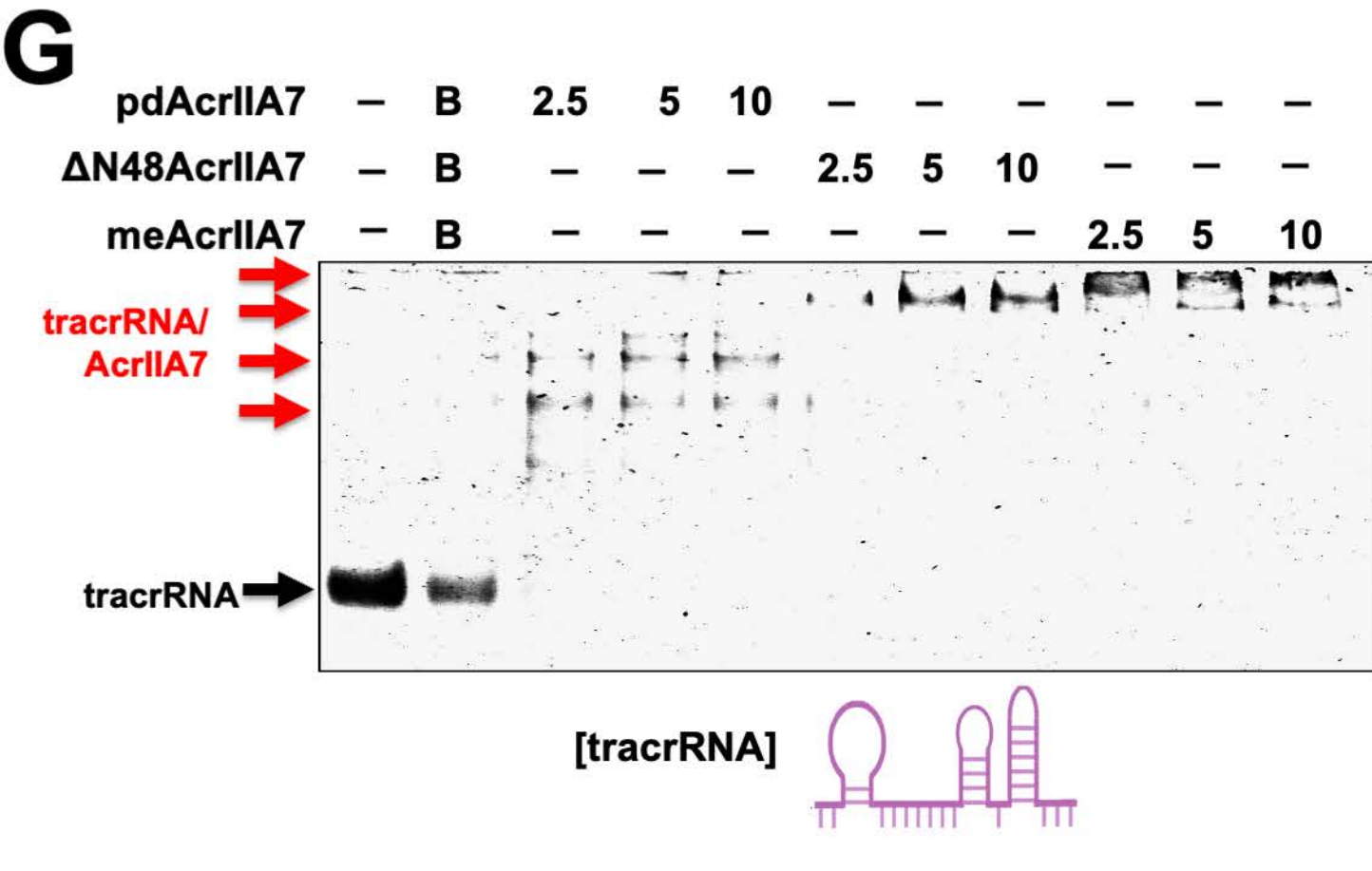
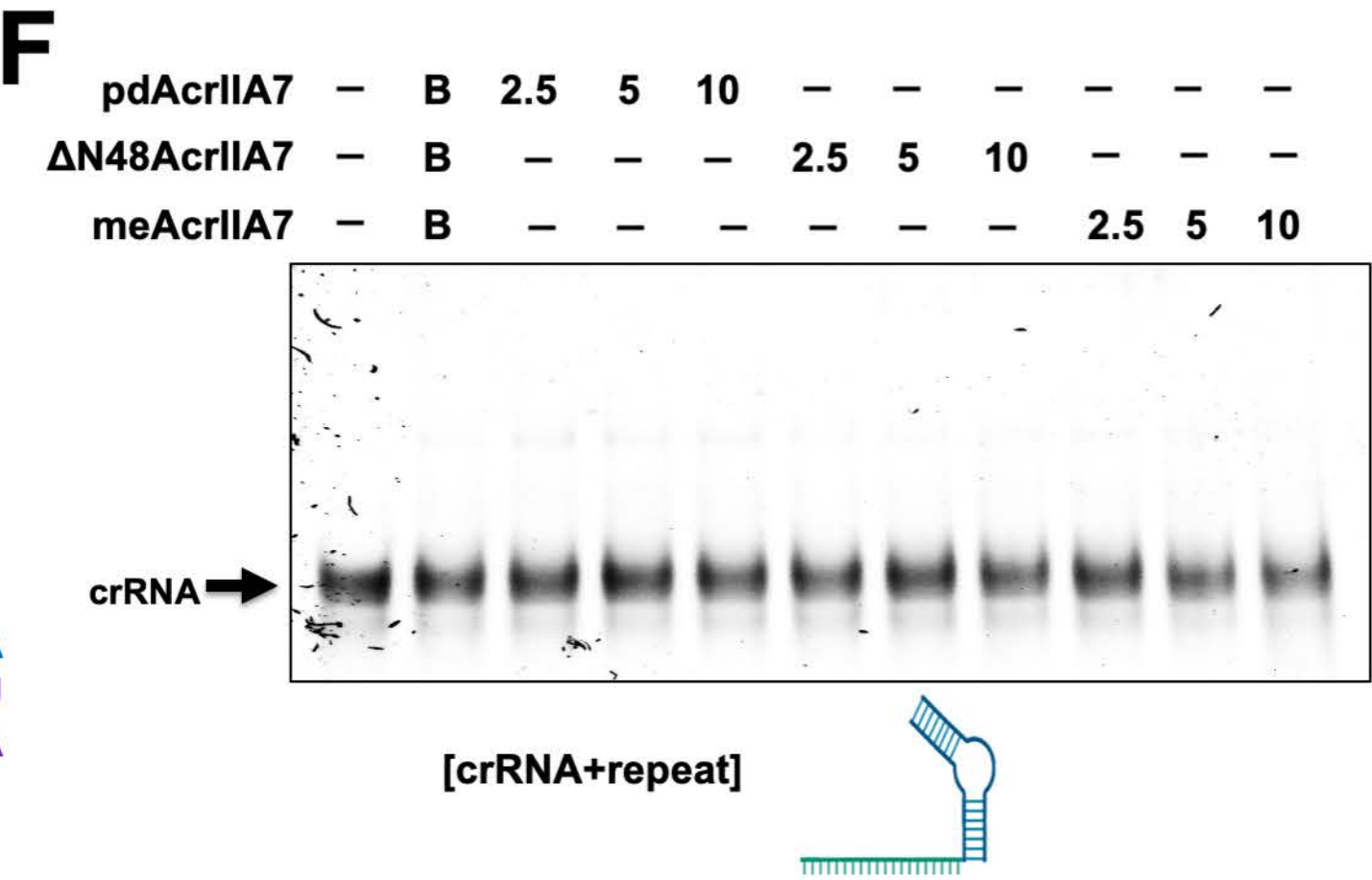
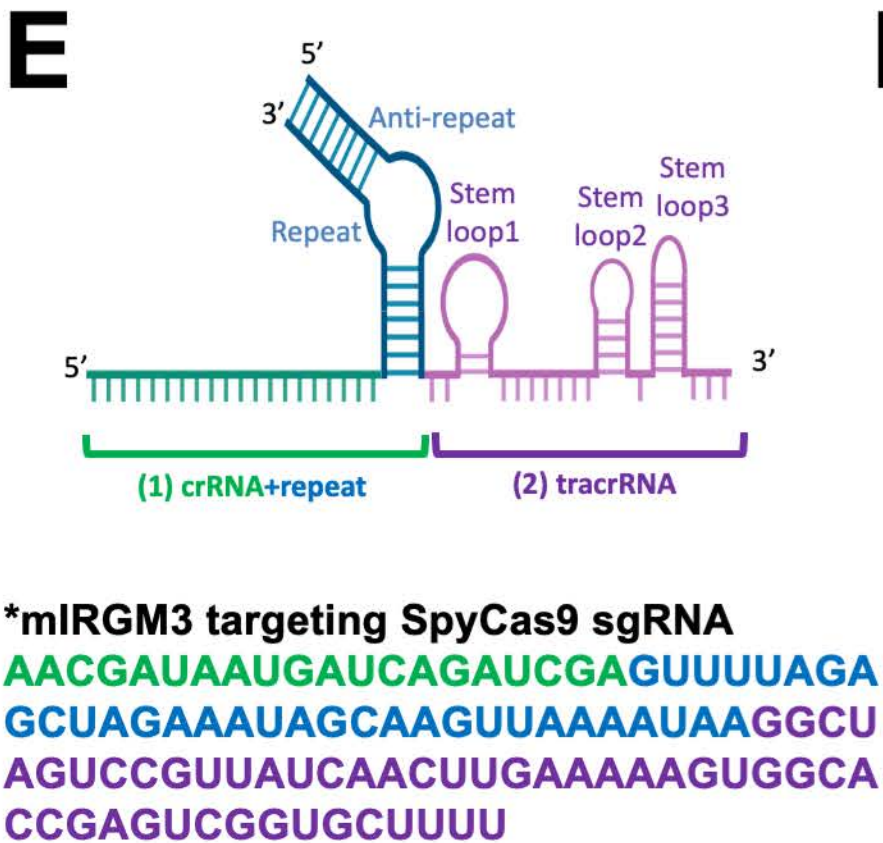
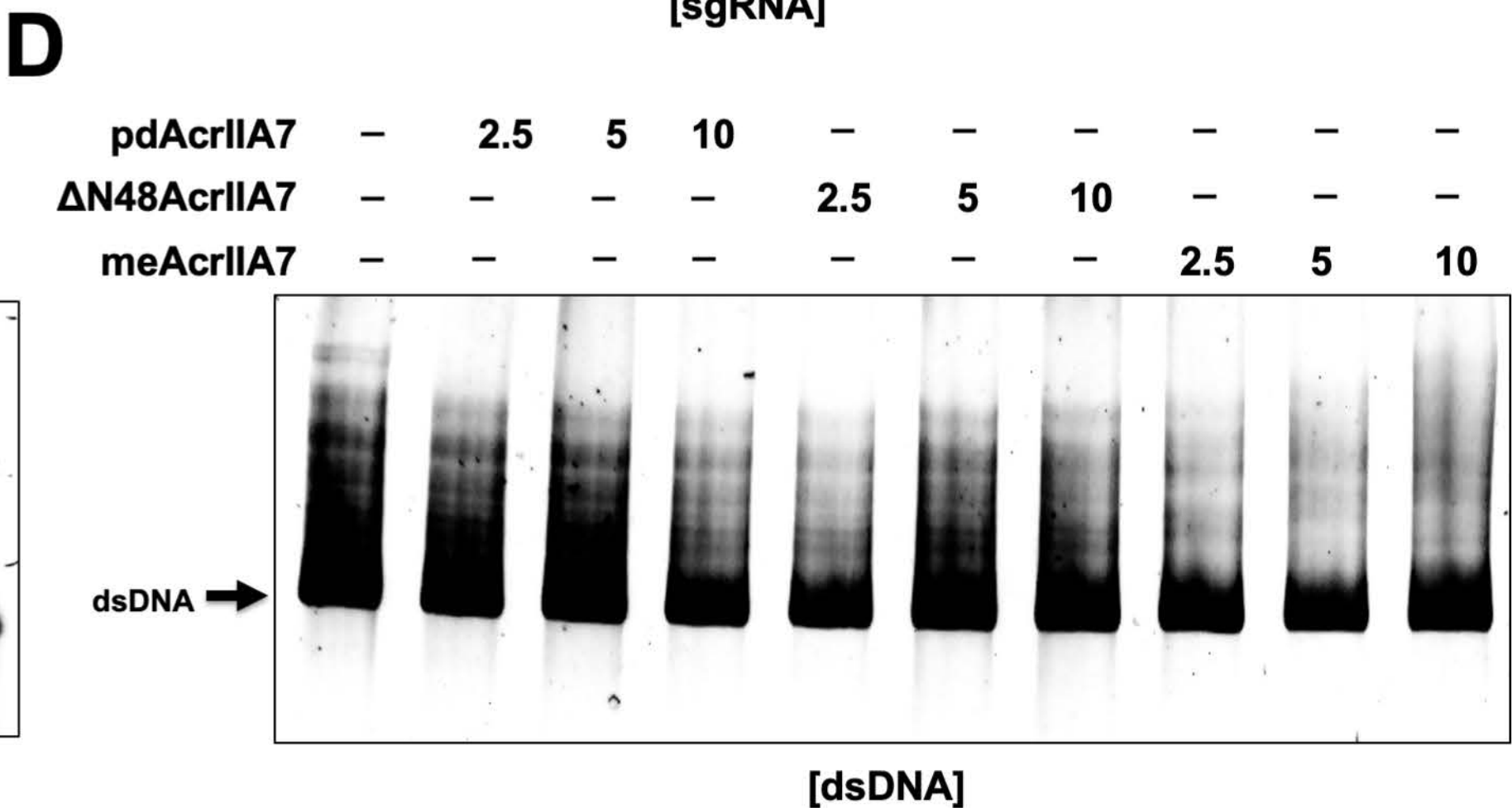
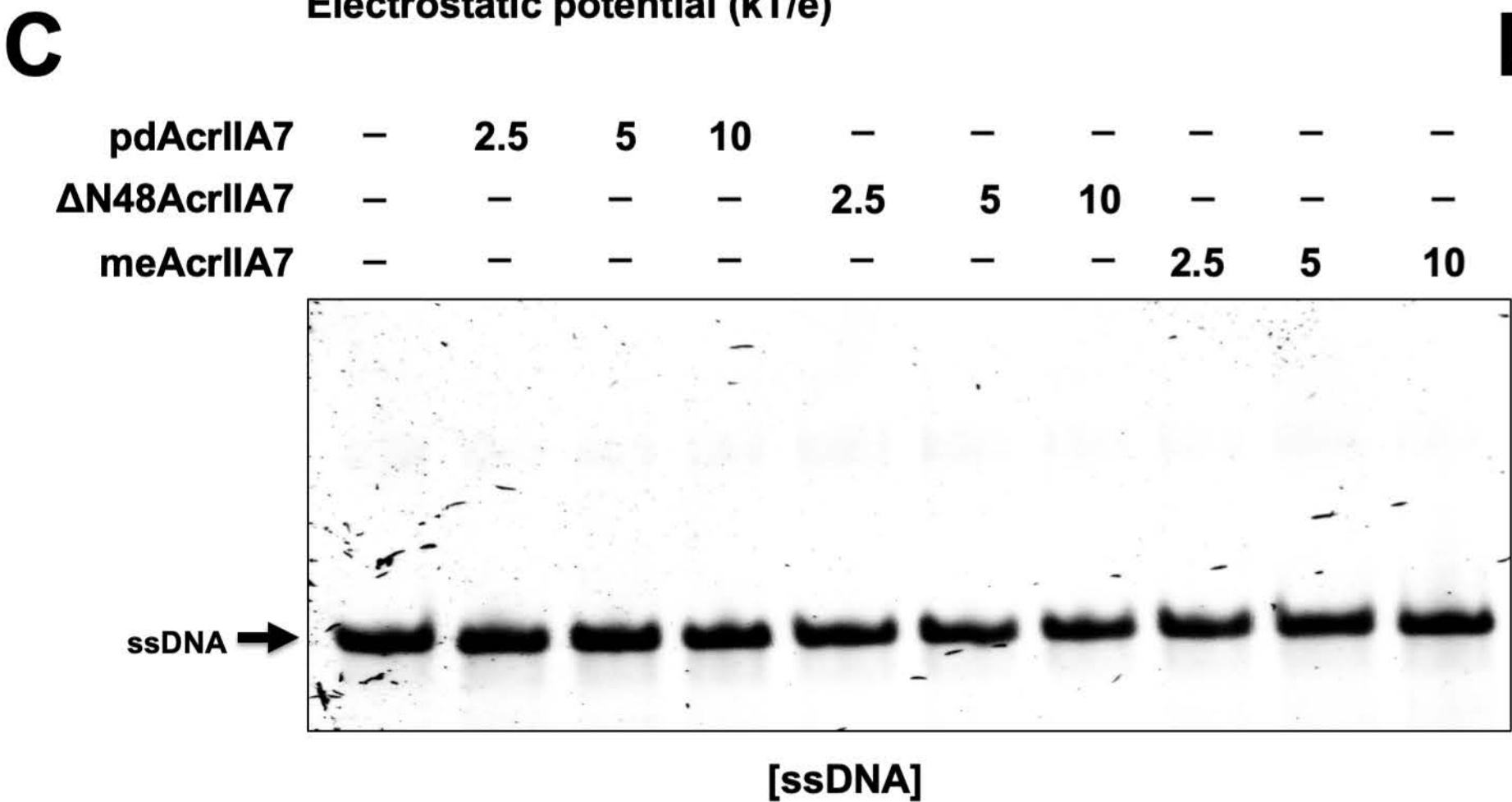
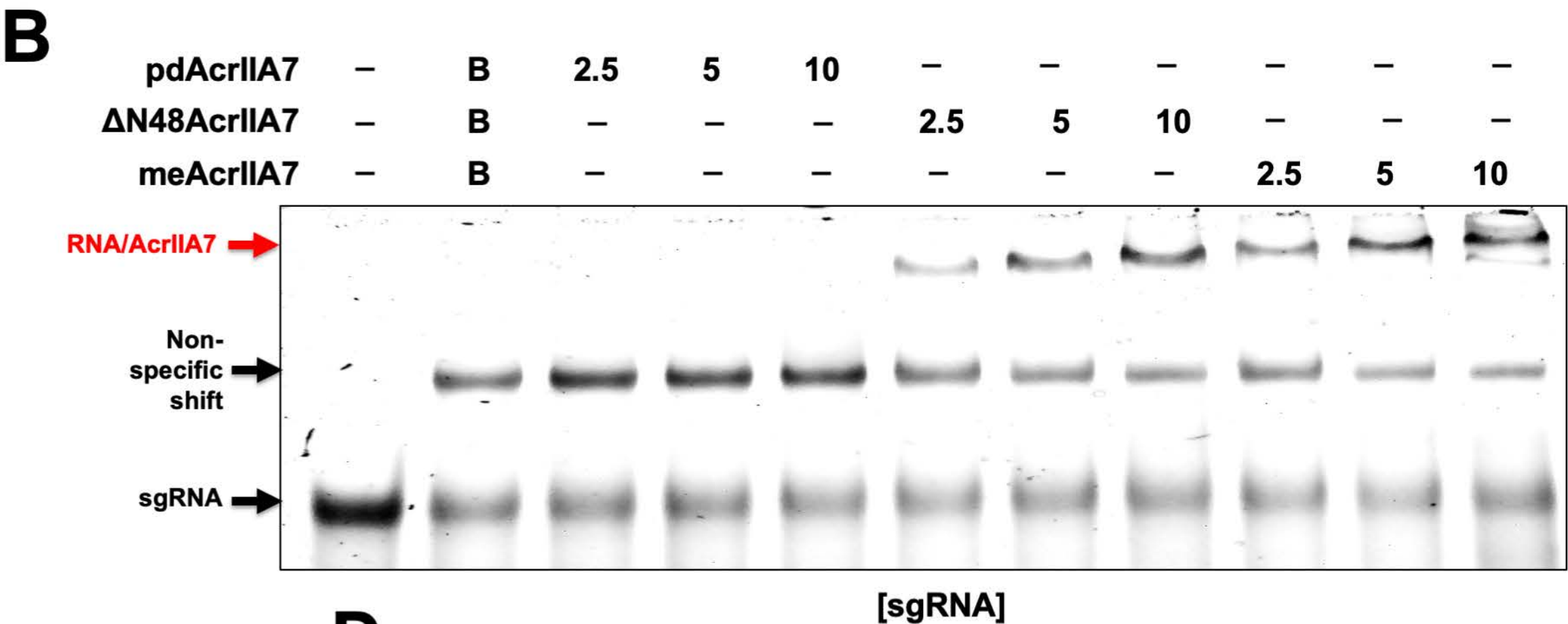
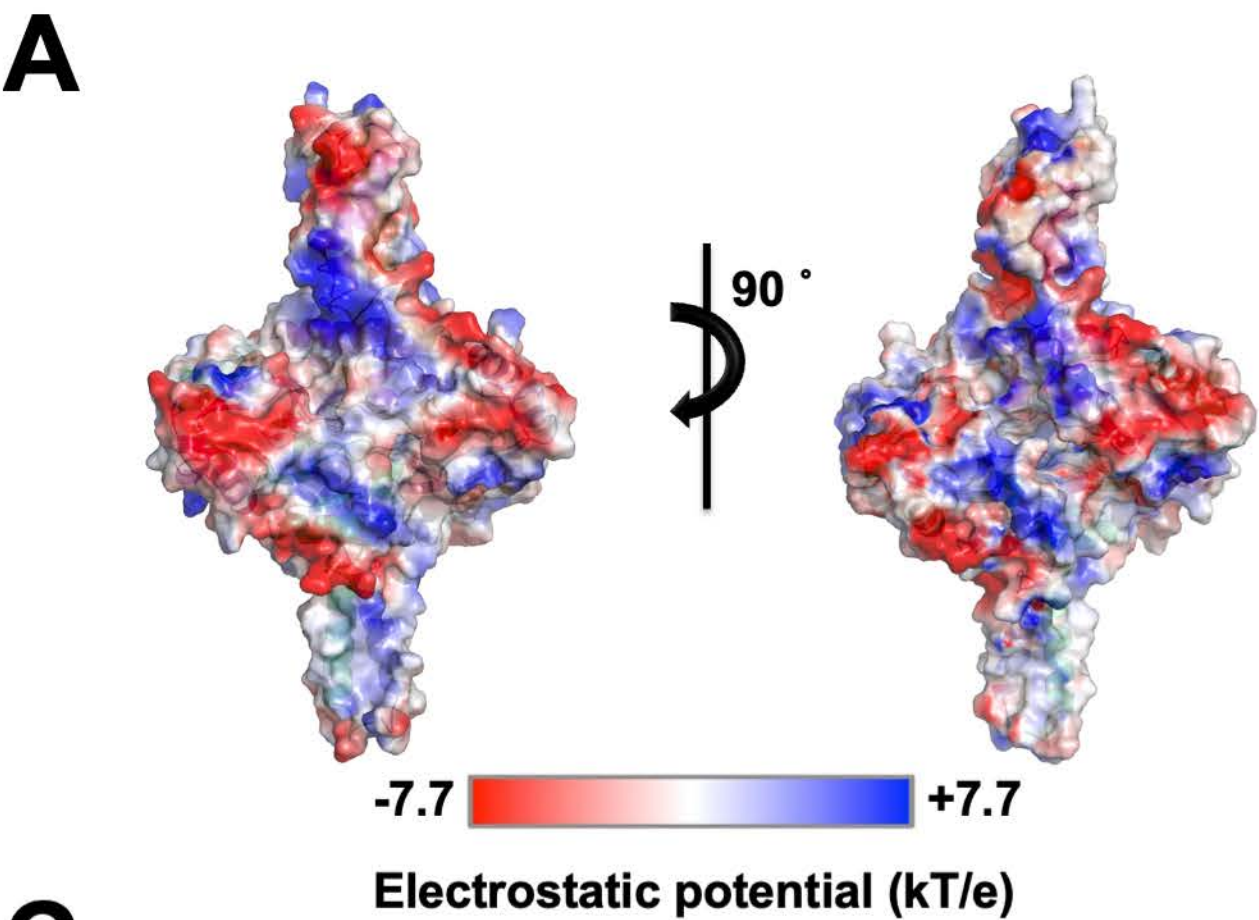
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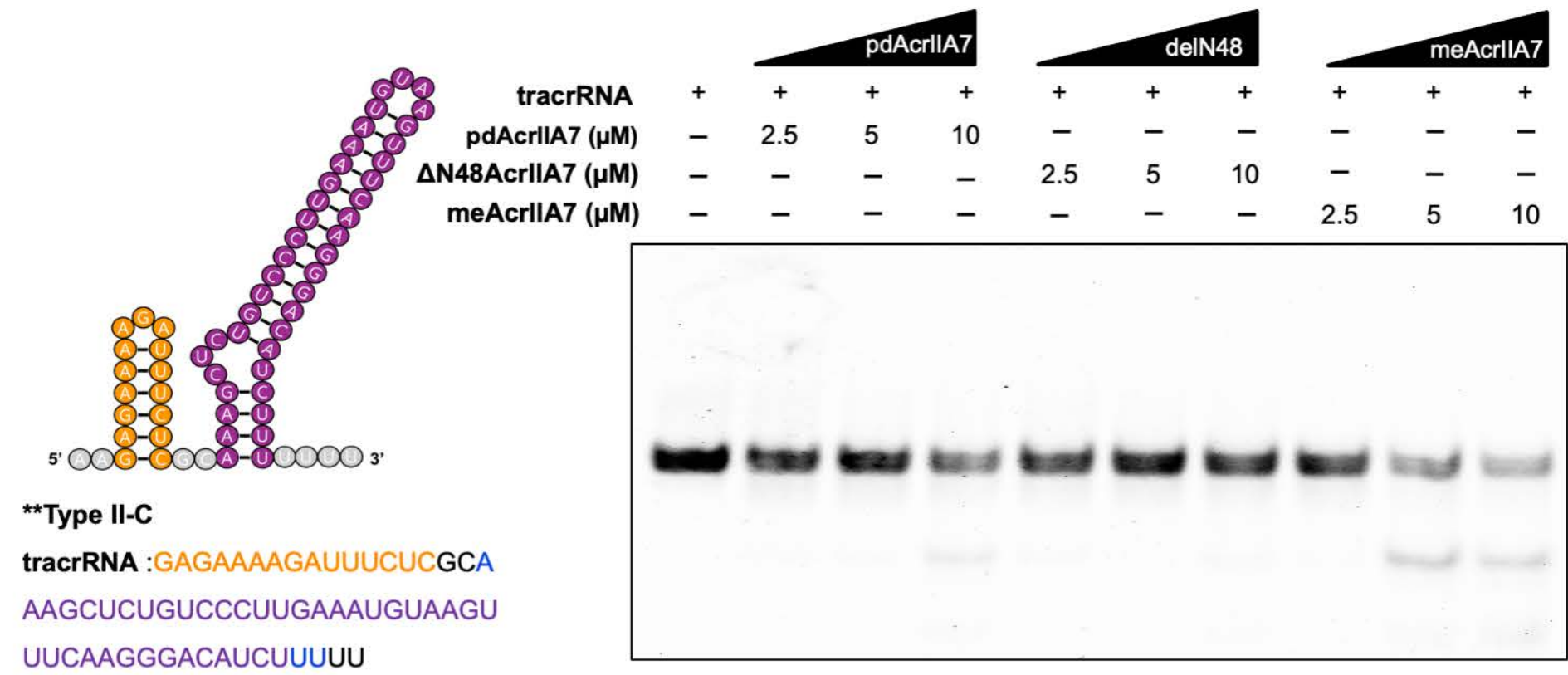
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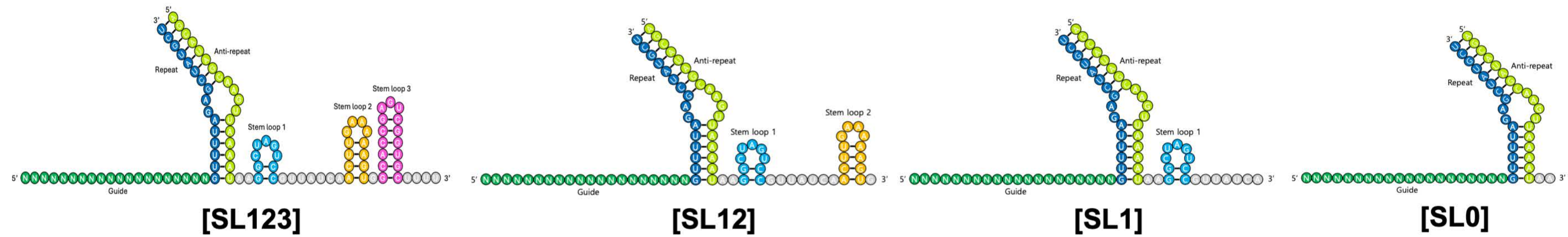




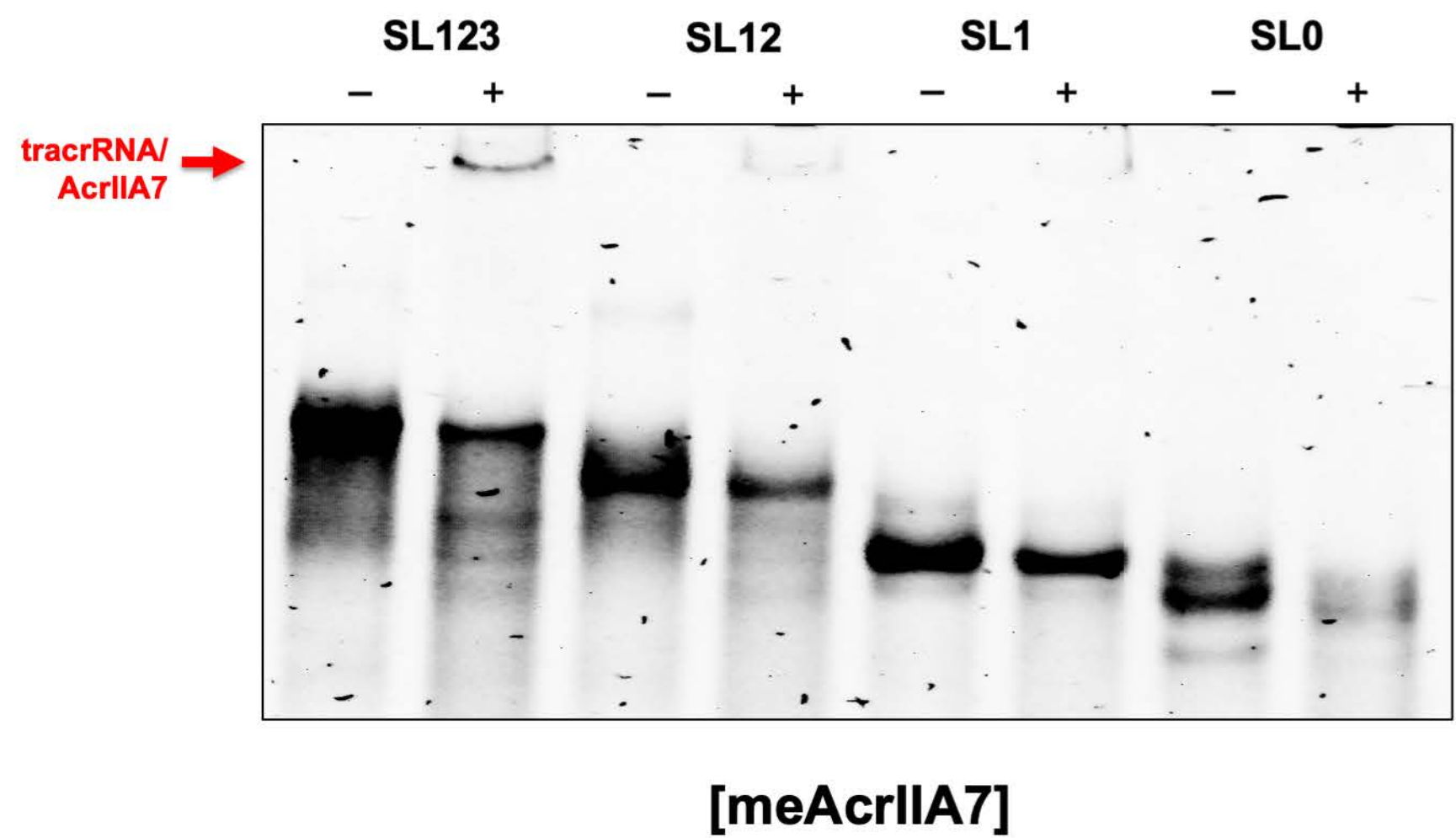
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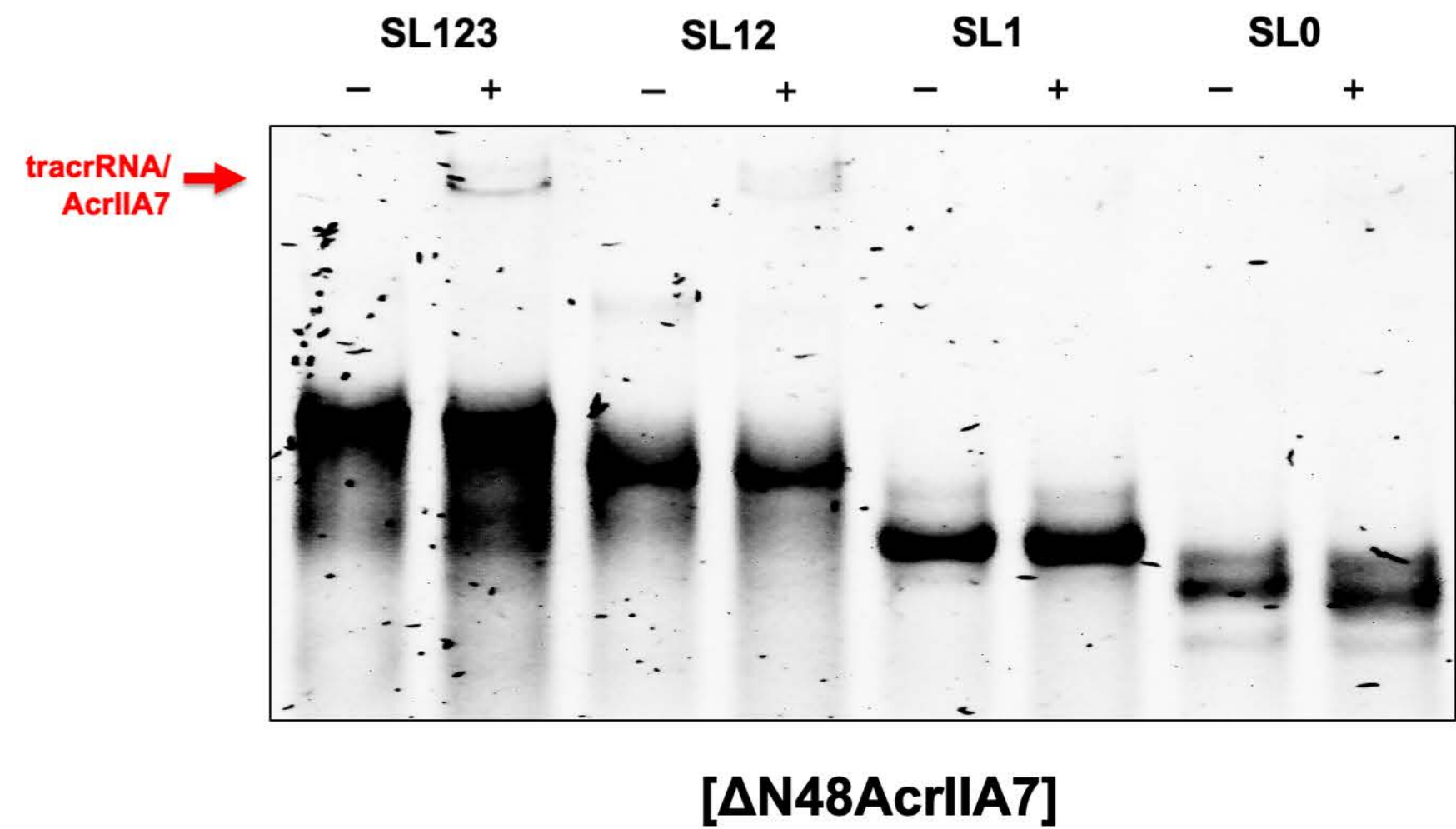
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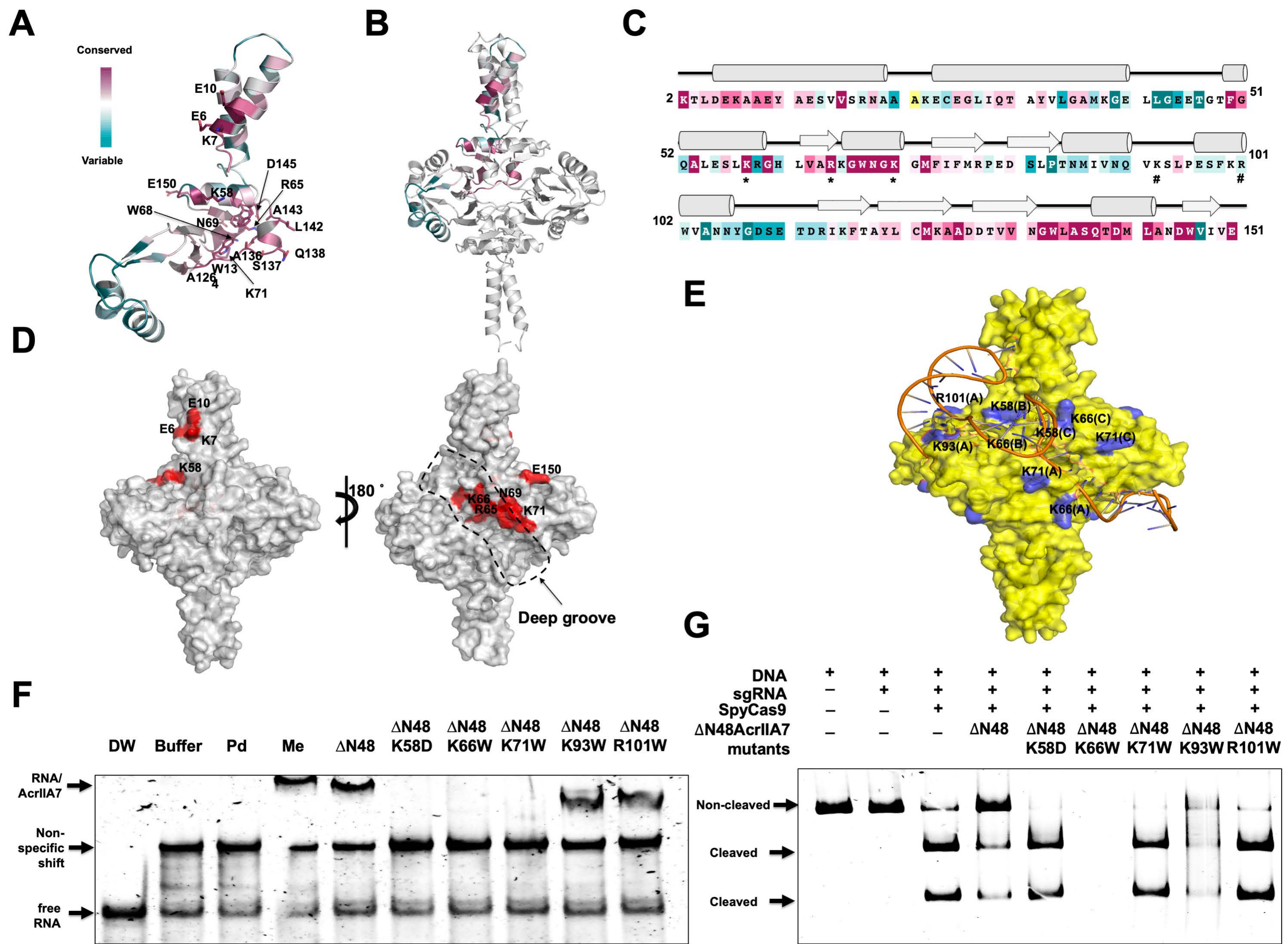


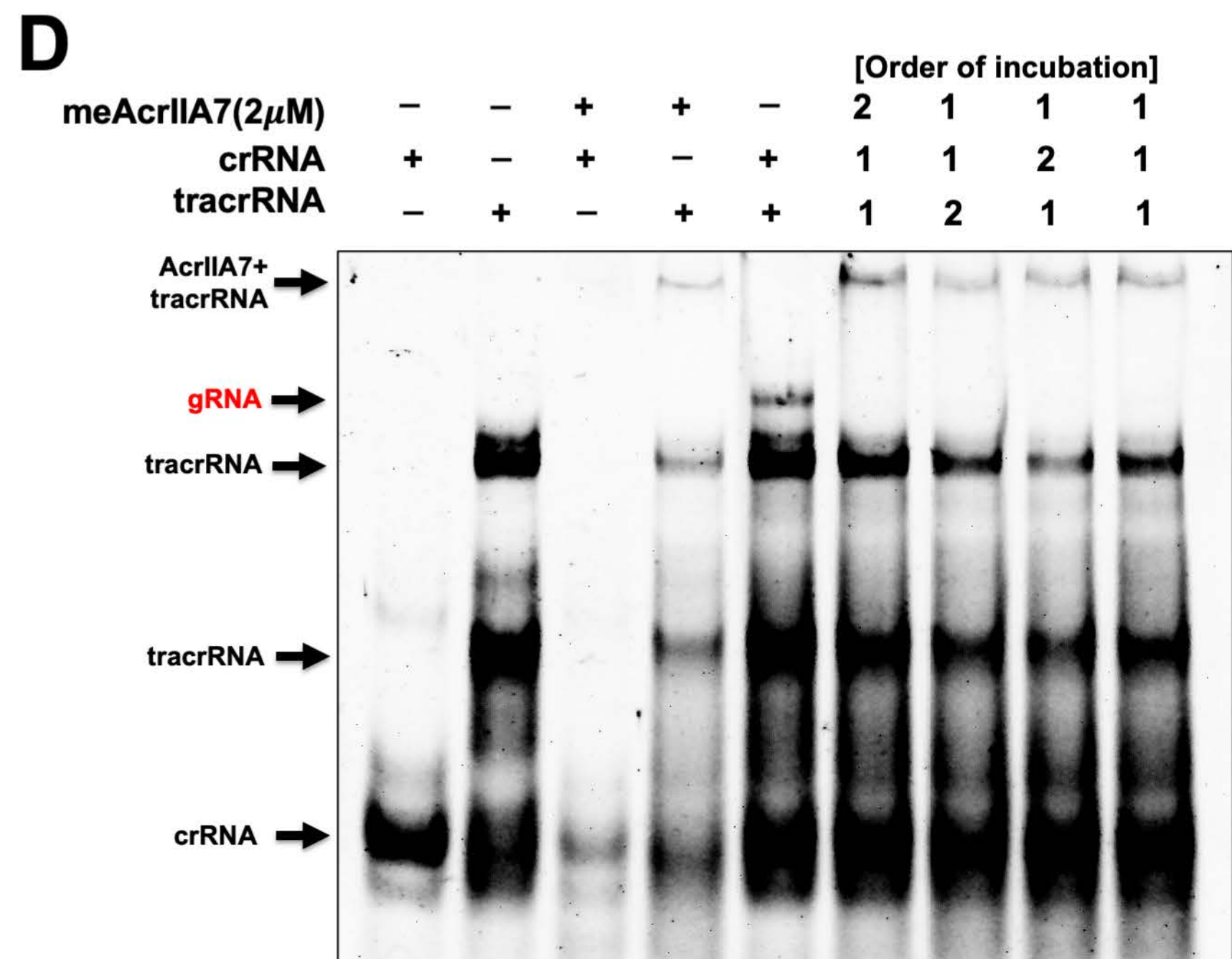
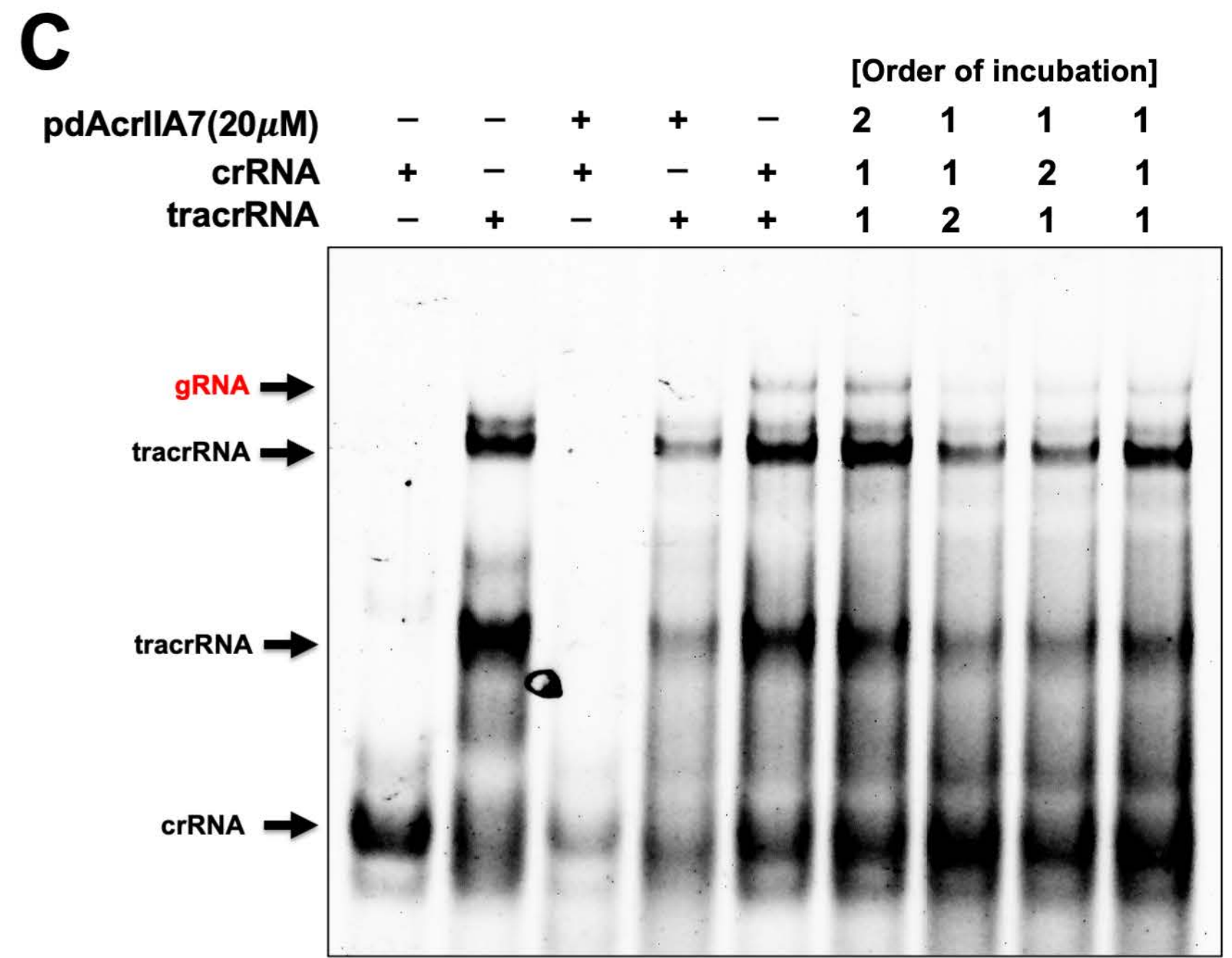
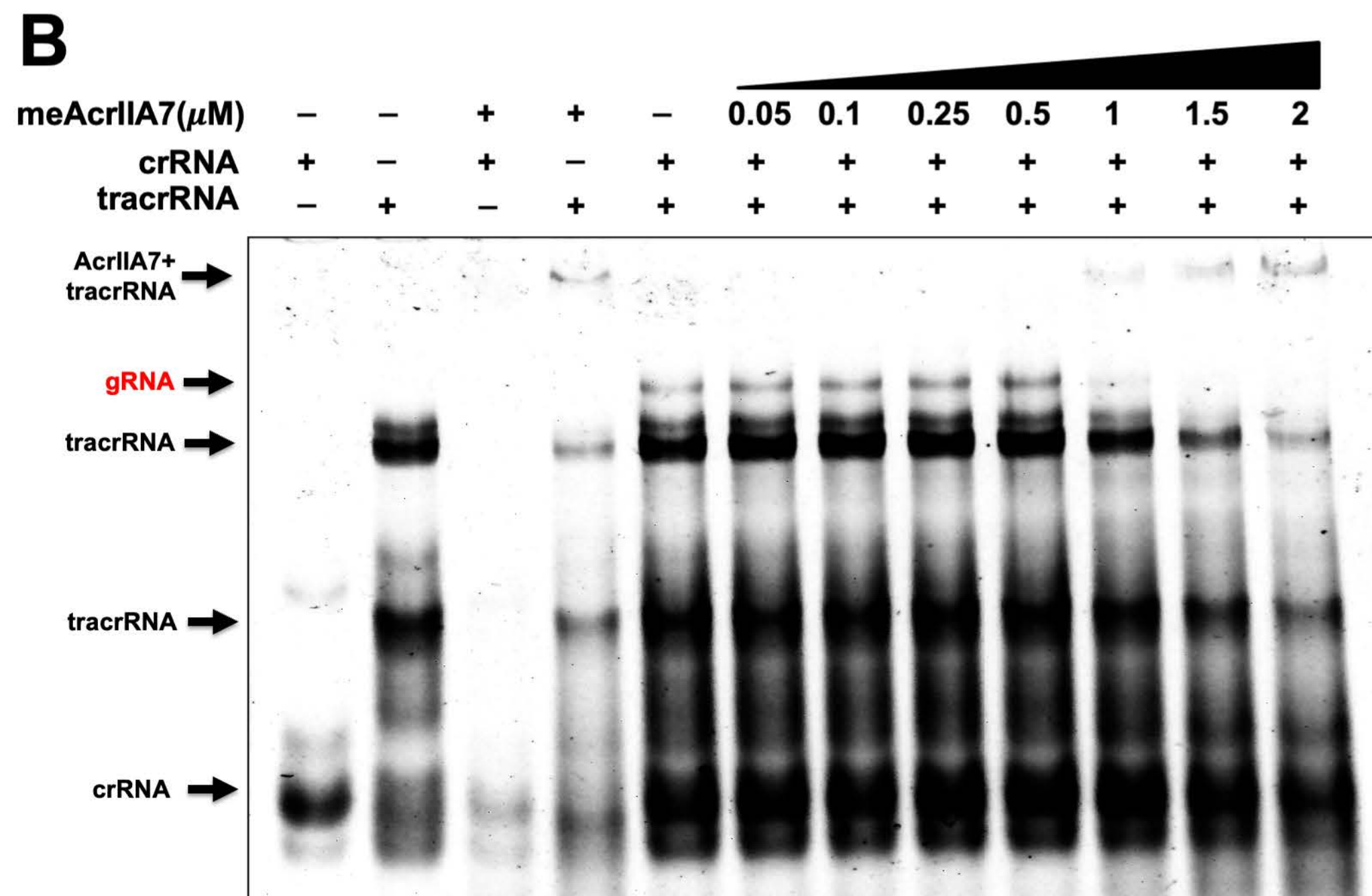
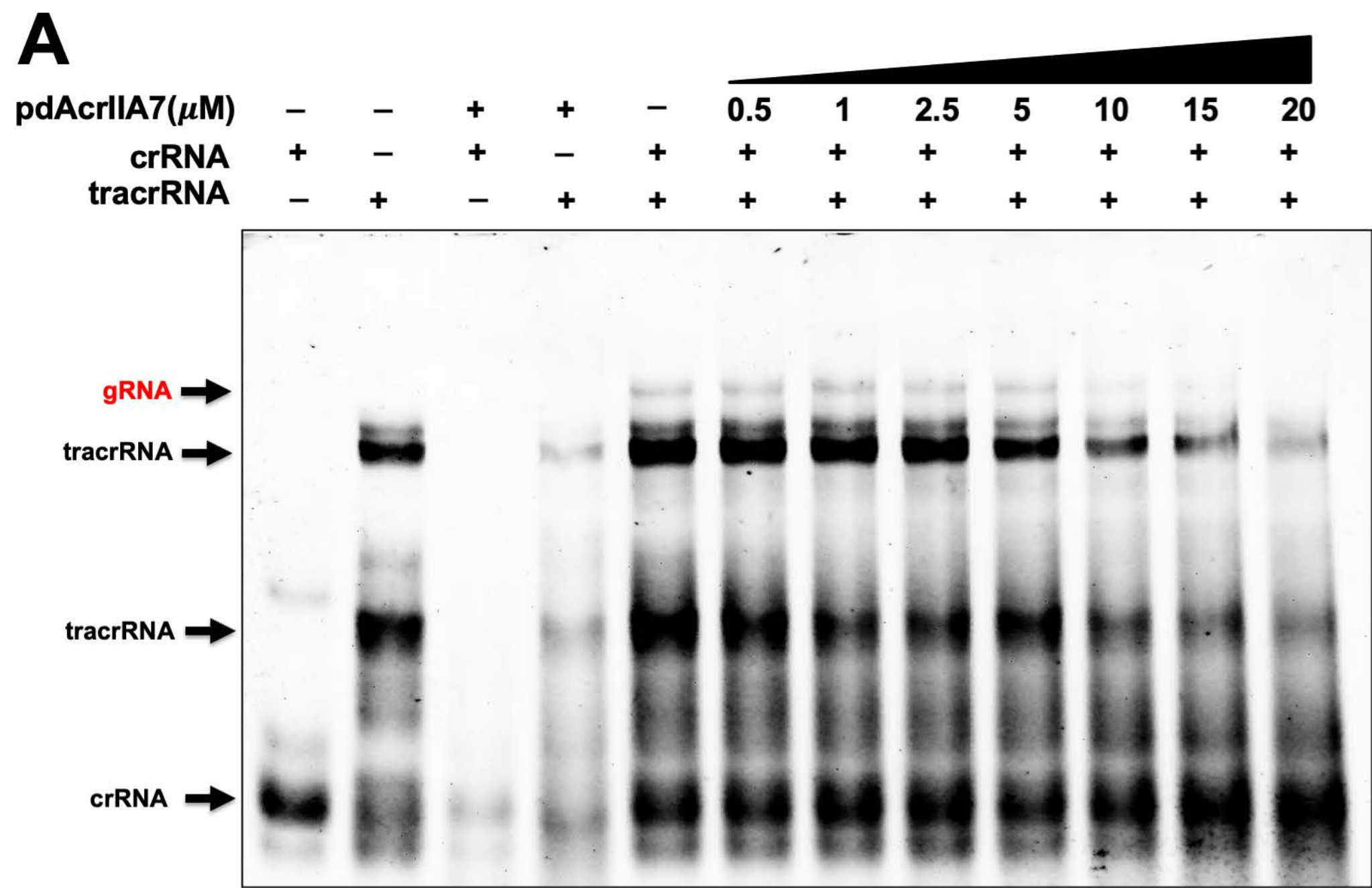
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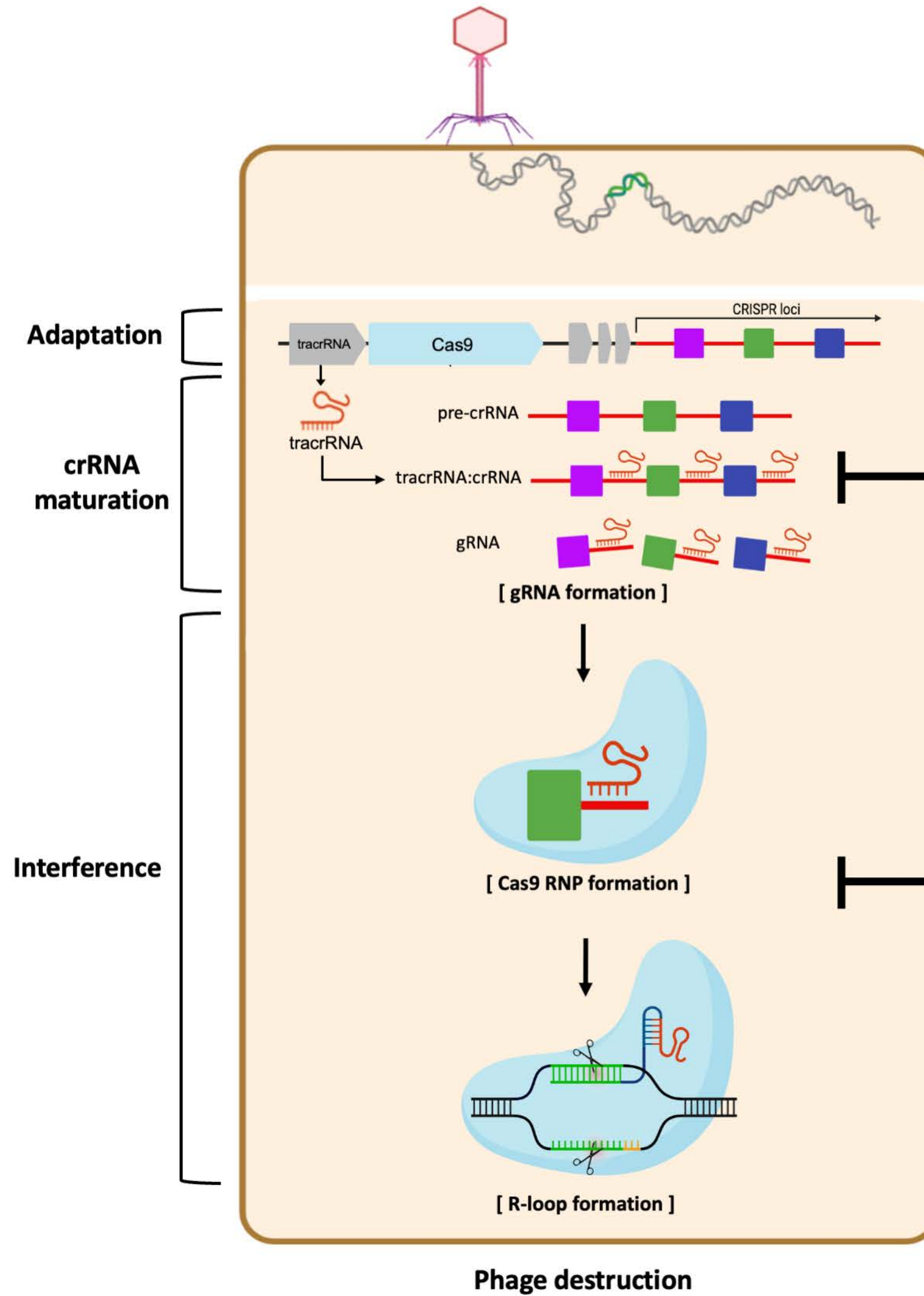


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A**CRISPR-Cas9 immune system****B****Anti-CRISPR mechanisms**