Identification of RNA Binding Partners of CRISPR-Cas Proteins in Prokaryotes Using RIP-Seq

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Abstract

CRISPR-Cas systems consist of a complex ribonucleoprotein (RNP) machinery encoded in prokaryotic genomes to confer adaptive immunity against foreign mobile genetic elements. Of these, especially the class 2, Type II CRISPR-Cas9 RNA-guided systems with single protein effector modules have recently received much attention for their application as programmable DNA scissors that can be used for genome editing in eukaryotes. While many studies have concentrated their efforts on improving RNA-mediated DNA targeting with these Type II systems, little is known about the factors that modulate processing or binding of the CRISPR RNA (crRNA) guides and the *trans*-activating tracrRNA to the nuclease protein Cas9, and whether Cas9 can also potentially interact with other endogenous RNAs encoded within the host genome. Here, we describe RIP-seq as a method to globally identify the direct RNA binding partners of CRISPR-Cas RNPs using the Cas9 nuclease as an example. RIP-seq combines co-immunoprecipitation (coIP) of an epitope-tagged Cas9 followed by isolation and deep sequencing analysis of its co-purified bound RNAs. This method can not only be used to study interactions of Cas9 with its known interaction partners, crRNAs and tracrRNA in native systems, but also to reveal potential additional RNA substrates of Cas9. For example, in RIP-seq analysis of Cas9 from the foodborne pathogen *Campylobacter jejuni* (CjeCas9), we recently identified several endogenous RNAs bound to CjeCas9 RNP in a crRNA-dependent manner, leading to the discovery of PAM-independent RNA cleavage activity of CjeCas9 as well as non-canonical crRNAs. RIP-seq can be easily adapted to any other effector RNP of choice from other CRISPR-Cas systems, allowing for the identification of target RNAs. Deciphering novel RNA-protein interactions for CRISPR-Cas proteins within host bacterial genomes will lead to a better understanding of the molecular mechanisms and functions of these systems and enable us to use the *in vivo* identified interaction rules as design principles for nucleic acid-targeting applications, fitted to each nuclease of interest.

Key words CRISPR-Cas, Prokaryotes, *Campylobacter jejuni*, Cas9, RNA-immunoprecipitation, Next-generation sequencing

1 Introduction

Post-transcriptional regulation represents an important layer of gene expression control in uni- and multicellular organisms. Hereby, RNA molecules (rRNA, mRNA, tRNA, and non-coding

RNAs) as well as RNA-binding proteins (RBPs) and ribonucleases (RNases) are central players in mediating and regulating gene expression. Most RNA molecules associate with proteins for exerting this control and co-exist as ribonucleoprotein (RNP) complexes. RNP complexes can execute numerous cellular functions including gene regulation, RNA modifications, mRNA translation, and RNA stability control [1, 2]. Studying these RNPs enables us to understand the role of RBPs and RNases in cellular physiology. Moreover, with the advent of numerous RNA-sequencing (RNA-seq) approaches using deep sequencing technologies it has become possible to study and analyze these RNPs on a transcriptome-wide scale in diverse organisms, including bacteria, and dissect their complex biological tasks [3, 4].

One diverse and widespread group of RNP complexes used by prokaryotes are the CRISPR-Cas (Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated) systems. These RNA-based prokaryotic immune systems are present in approximately half of bacterial and 85% archeal genomes, which have evolved over the course of many years to create an immunological memory, guided by the incorporated nucleic acids, forming the basis of an organism's adaptive immunity [5–7]. Such CRISPR-Cas adaptive immune systems have been extensively studied over the last decade for their role as defense systems in prokaryotes to protect against foreign mobile genetic elements such as phages and plasmids and more recently as tools for enabling precise genome editing in various organisms [8–10]. CRISPR-Cas systems primarily function in the form of active RNPs with Cas nuclease effector complexes, which are guided by CRISPR RNA (crRNA) guides encoded in CRISPR repeat-spacer arrays to target and destroy specific DNA/RNA sequences. CRISPR-Cas systems are divided into two broad classes, with class I systems being composed of multiple Cas proteins, which form the core effector module, whereas class II systems comprise a single, multidomain effector nuclease [11]. Each class of CRISPR-Cas is further divided into three types each, i.e., types I, III, and IV forming class 1, and types II, V, and VI in class 2. Each type further contains subtypes based on variations either in structure or function of CRISPR-Cas genes. CRISPR-Cas immune systems work by incorporating foreign DNA fragments, known as spacers into the host genome between specific repeat regions in the genome—the CRISPR locus. Once the spacers are acquired into these repeats, they are transcribed and processed into individual crRNAs, which interact with Cas proteins and mediate RNA-guided genome defense [12, 13]. Hereby, the sequence of the crRNAs defines the specificity of the adaptive immune response, both for the host and its progeny. This adaptive immune response can be achieved by targeting either DNA or RNA. While RNA targeting is specific to Type III and VI systems, DNA targeting is mediated by Type I, II, and V systems. Type III

CRISPR-Cas systems are the only exception that can target invading nucleic acids at the level of both DNA and RNA.

Of the six types of CRISPR-Cas systems, Cas9 nucleases from class 2, Type II are currently the most widely used for RNA-guided genome editing and various technological applications in bacterial and eukaryotic genomes [8, 14, 15]. This system uses a single Cas9 nuclease for targeting double-stranded DNA, using a crRNA guide and endonuclease activity of Cas9 via its RuvC and HNH nuclease domains [16, 17]. Type II-A, II-B, and II-C systems differ based on the size of their Cas9 protein and the presence of associated additional *cas* genes [18]. In addition to crRNA guides, Type II systems also require the presence of a *trans*-activating crRNA (tracrRNA) [19]. The tracrRNA has base-pairing complementarity to the repeat region of the crRNA guide and mediates processing and maturation of crRNAs via processing by the host factor RNase III. Moreover, tracrRNA is also required for Cas9 function as the mature tracrRNA-crRNA duplex is bound by Cas9, thereby guiding the Cas9:crRNA:tracrRNA RNP to a protospacer in the target DNA flanked by a protospacer-adjacent motif (PAM) [20]. For the purpose of genome editing, a chimeric RNA formed by the fusion of crRNA and tracrRNA called the single-guide RNA (sgRNA) is used, thus simplifying the system further [16].

Type II systems are widespread in pathogenic and commensal bacteria and there is emerging evidence that they could have additional functions beyond genome defense [21]. These unconventional functions are not restricted to Type II systems alone and involve regulation of bacterial virulence, group behavior dynamics, genome remodeling, DNA repair, antisense RNAs, and self-targeting mechanisms in numerous CRISPR-Cas types [22–29]. It is well known that Cas9:crRNA:tracrRNA RNP surveils the cytoplasm and targets DNA from invading genomes, but whether Cas9 has additional RNA partners involved in regulating host gene expression, remains unclear. Recently, the Cas9 homolog in *Francisella novicida* (FnoCas9) has been identified to target its own DNA with the help of a small CRISPR/Cas associated RNA termed scaRNA, leading to RNA-directed transcriptional repression [30]. Moreover, recent studies also indicate that certain members of the Cas9 family are capable of targeting RNA [14]. For example, using RNA co-immunoprecipitation combined with RNA-sequencing (RIP-seq) of epitope-tagged Cas9 from *Campylobacter jejuni* (CjeCas9), we recently uncovered that the CjeCas9 nuclease is capable of binding and cleaving endogenous RNAs in vivo, in addition to binding its canonical crRNA:tracrRNA pairs [31]. This crRNA-dependent targeting of endogenous RNAs is PAM-independent. Also, other Type II Cas9 family members from *Neisseria meningitidis* (NmeCas9) and *Staphylococcus aureus* (SauCas9) were shown to target single-stranded RNAs independently of a PAM in vitro [32, 33]. Moreover, Cas9 from

Streptococcus pyogenes (SpyCas9) can target RNA after addition of single-stranded DNA oligonucleotides with a PAM sequence in vitro and has been applied in human cells for RNA tracking and localization by replacing the active enzyme with catalytically dead SpyCas9 in vivo [34, 35]. These studies showed that even within the Type II system, Cas9 homologs from different species can have a variety of molecular activities. Moreover, while a lot of studies have concentrated on RNA-mediated DNA targeting with Type II Cas9 systems and its tremendous technological applications, very little is known about how these systems regulate activity of the RNA-guided effector nucleases and about their potential interactions with other RNAs encoded within the host genomes. With the exception of CjeCas9 and FnoCas9, most studies have described CRISPR-Cas Type II proteins in vitro or expressed exogenously in eukaryotic cells or other bacteria, which while providing valuable insights also limits the mechanistic understanding of these effector molecules in bacteria natively harboring these systems.

Here, we describe the so-called RIP-seq approach to identify the direct RNA substrates of CRISPR-Cas RNPs in prokaryotes (Fig. 1). RIP-seq combines co-immunoprecipitation (coIP) of an epitope-tagged RBP followed by isolation and deep sequencing analysis of its co-purified bound RNAs. Prior to the development of high-throughput sequencing technologies, co-purified transcripts from coIPs of RBPs were typically analyzed with either high-density microarrays or conventional low-throughput RNomeics using direct RNA-sequencing or Sanger sequencing to identify, e.g., the targetome of the RNA chaperone Hfq in *Escherichia coli*, *Listeria monocytogenes*, and *Pseudomonas aeruginosa*, respectively [36–38]. The combination of coIP with RNA-seq for RIP-seq analyses of Hfq then allowed for identifying its small RNA (sRNA) and messenger RNA binding partners in *Salmonella* on a transcriptome-wide scale and with single-nucleotide resolution [39]. RIP-seq has also been successfully adapted and applied to study the regulons of a number of other RNA-binding proteins in bacterial species such as *E. coli*, *C. jejuni*, *Helicobacter pylori*, and *N. meningitidis* [40–47]. Using a similar coIP approach combined with deep sequencing in organisms harboring Type III CRISPR-Cas systems identified mature crRNAs bound to the effector proteins, which shed light into the mechanism governing targeting of both host and foreign RNAs in those systems [23, 48].

Based on RIP-seq to examine the direct RNA binding partners of the Cas9 nuclease of the foodborne pathogen *C. jejuni*, we had identified the abovementioned RNA-targeting activity of CjeCas9 and more recently also discovered non-canonical crRNAs [31, 67]. Hereby, we built on a RIP-seq protocol previously adapted for the use in Epsilonproteobacteria [40–47]. Using a genetically modified strain of *C. jejuni* expressing a 3xFLAG epitope at the C-terminus of CjeCas9 (CjeCas9-3xFLAG) at the

Identifying RNA substrates of CjeCas9 by RIP-seq

Fig. 1 RIP-seq analysis to identify the direct RNA substrates of Cas9 in *C. jejuni*. (Left) Schematic representation of co-immunoprecipitation of 3xFLAG-tagged CjeCas9. (Right) Detailed overview of the RIP-seq protocol used to identify CjeCas9-bound RNAs in *C. jejuni* strain NCTC11168. Bacterial lysates prepared from cell pellets corresponding to a cell number of 60 OD₆₀₀ pellets using WT-untagged and CjeCas9-3xFLAG tagged

native locus and an antibody against the FLAG epitope allowed for immunoprecipitation and isolation of native ribonucleoprotein complexes comprised of CjeCas9-3xFLAG bound crRNA: tracrRNA and other endogenous RNAs. The parallel use of a WT-untagged strain as a negative control allows for discriminating enriched RNAs that are specifically bound to CjeCas9-3xFLAG from RNAs that are nonspecifically bound to the beads or the antibody during the coIP procedure as background noise. The use of an epitope tag allows for immunoprecipitating a protein of interest in the absence of a mono- / polyclonal antibody against the protein itself. Deep sequencing analysis of the transcripts co-purified with CjeCas9-3XFLAG revealed that the majority correspond to tracrRNA and crRNAs, indicating successful pull-down of Cas9-crRNA-tracrRNA complexes. Surprisingly, a fraction of endogenous transcripts, including many mRNAs, were also enriched in the CjeCas9-3xFLAG coIP, and we could demonstrate that CjeCas9 can bind and cleave single-stranded RNAs in a crRNA-dependent manner and that non-canonical crRNA guides can be derived from mRNAs [31, 67]. Similar as for studying RNA binding partners of CjeCas9, RIP-seq can be readily applied to any bacterial species of choice (Gram-positive or Gram-negative), harboring a CRISPR-Cas system and capable of genetic manipulation (chromosomally or via plasmids), for transcriptome-wide identification of RNAs interacting with the CRISPR-Cas protein of interest. A better understanding of the different CRISPR-Cas RNP complexes will facilitate deciphering their underlying molecular principles of function and to use them for further improving the biotechnological application of these systems.

2 Materials

2.1 Bacterial Strains

For the RIP-seq analysis, *C. jejuni* NCTC11168 wildtype (WT) as well as a CjeCas9-3xFLAG strain are used. The *C. jejuni* strain with a 3xFLAG-tagged CjeCas9 at the native locus was constructed using a double-stranded DNA construct and double-crossover homologous recombination, as described previously [31]. Hereby, the *cas9* (Cj1523c) gene was chromosomally tagged at its

Fig. 1 (continued) bacterial cells grown to exponential phase are used as the starting material for co-immunoprecipitation. This is followed by incubation with an anti-FLAG antibody, protein A-sepharose beads and washing to enrich for CjeCas9-3xFLAG RNPs. CjeCas9-3XFLAG protein and bound RNAs are separated using phenol/chloroform/isoamyl alcohol, precipitated, and eluted into protein and RNA fractions. Lysate, supernatant 1 (SN1), supernatant 2 (SN2), and wash fractions are collected during the course of the experiment along with the final eluate. All samples are used for quality control before proceeding with DNase I digestion and next-generation sequencing of eluted RNAs

C-terminus by introducing overlap PCR products containing 500 bp homologous ends encompassing a 3xFLAG sequence (DY KDHDGDYKDHDIDYKDDDDK) and a kanamycin resistance marker using electroporation into the *C. jejuni* NCTC11168 WT strain.

2.2 Bacterial Culture and Collection

1. Müller–Hinton agar plates and Brucella broth (BB), both supplemented with 10 µg/mL vancomycin. The agar was further supplemented with marker-selective antibiotics (50 µg/mL kanamycin) when required.
2. Resuspension buffer (Buffer A): 20 mM Tris–HCl, pH 8.0, 150 mM KCl, 1 mM MgCl₂ with 1 mM Dithiothreitol (DTT) (added freshly each time before use).
3. 1× Protein loading buffer (1× PLB): 62.5 mM Tris–HCl, pH 6.8, 100 mM DTT, 10% (v/v) Glycerol, 2% (w/v) SDS, 0.01% (w/v) Bromophenol blue.

2.3 RNA Co-immunoprecipitation

1. Buffer A.
2. Lysis buffer (per mL): 967 µL Buffer A (without DTT), 1 µL 1 mM DTT, 10 µL 0.1 M Phenylmethylsulfonylfluoride (PMSF), 2 µL Triton X-100, 20 µL DNase I, and 10 µL Suprase-In RNase Inhibitor.
3. 5× Protein loading buffer.
4. Eppendorf tubes (2 mL and 1.5 mL).
5. Fast-prep tubes with lysing Matrix B.
6. FastPrep-24™ benchtop Homogenizer (MP Bio).
7. Monoclonal anti-FLAG M2 antibody (1 mg/mL).
8. Protein A-sepharose beads.
9. GlycoBlue™.
10. Roti-Aqua-P/C/I (phenol/chloroform/isoamyl alcohol).
11. RNA precipitation mix (30:1): Ethanol:3M NaOAc, pH 6.5.
12. 70% Ethanol (stored at –20 °C).
13. Acetone (stored at –20 °C).
14. RNase-free water.

2.4 Quality Control and DNase I Treatment

1. Antibodies: Monoclonal anti-FLAG antibody (1:1000 #F1804 Sigma-Aldrich) and anti-GroEL (1:10,000 #G6532 Sigma-Aldrich). Horseradish peroxidase-coupled anti-mouse IgG secondary antibody (1:10,000) and anti-rabbit IgG secondary antibody (1:10,000).
2. SDS-Polyacrylamide gels and electrophoresis equipment and buffers for western blots.

3. Polyacrylamide/Urea gels and electrophoresis equipment and buffers for northern blots.
4. RNA loading buffer II: 95% Formamide, 18 mM Ethylenediaminetetraacetic acid (EDTA), 0.025% w/v each of SDS, Xylene Cyanol and Bromophenol Blue.
5. DNase I.
6. 10× DNase I buffer containing MgCl₂.
7. Superase-In RNase Inhibitor.
8. Phase-lock tubes.

3 Methods

3.1 Bacterial Culture and Pellet Collection

1. Inoculate bacterial cultures of *C. jejuni* NCTC11168 WT control and the corresponding CjeCas9-3xFLAG strain in 100 mL (50 mL × 2 flasks) Brucella broth containing 10 mg/mL vancomycin to the selected growth phase, e.g., mid-exponential growth (OD₆₀₀ = 0.5–0.6) at 37 °C under microaerobic (10% CO₂, 5% O₂) conditions as previously described [31] (see Notes 1–4).
2. Harvest cells by centrifugation at 6000 × *g* at 4 °C for 15 min using a refrigerated benchtop centrifuge. Resuspend cell pellets in 1 mL Buffer A (ice-cold), transfer to 1.5 mL eppendorf tube, and centrifuge at 11,000 × *g*, 4 °C for 3 min using a refrigerated microcentrifuge. Aspirate the supernatant as cleanly as possible and immediately snap-freeze the pellets in liquid nitrogen. Store the pellets at –80 °C (see Note 5).

3.2 Cell Lysis and Incubation with Antibody/Protein A-Sepharose Beads

1. Thaw frozen pellets (see Subheading 3.1, step 2) on ice and resuspend in 1 mL Lysis buffer by gentle pipetting. Transfer the 1 mL lysate from each sample onto one fast-prep tube for lysis (see Note 6). Place tubes on ice and avoid RNase contamination.
2. Perform lysis with FastPrep-24TM benchtop homogenizer (MP Bio) at settings of 4 *M/s*, 15 *s* and clear the lysate in the fast-prep tube by centrifugation at 15,000 × *g*, 4 °C for 10 min to remove cellular debris generated during mechanical lysis (see Note 7).
3. Transfer the cleared lysate into a pre-chilled 2 mL eppendorf tube without disturbing the debris at the bottom and place the tube on ice. Measure the volume of the lysate collected and aliquot volumes equivalent to 1 and 5 OD₆₀₀ for protein and RNA, respectively. As an example, for a lysate volume of approximately 700 µL collected from 60 OD₆₀₀ culture, 11.5 µL (1/60 OD₆₀₀) for protein and 58 µL (5/60 OD₆₀₀)

volume for RNA are transferred to chilled 2 mL eppendorf tubes. For the protein aliquot, add 38.5 μL buffer A and 50 μL of 5 $\times$ Protein loading buffer to adjust to a final volume of 100 μL or 0.01 OD₆₀₀/ μL . For the RNA aliquot, adjust the volume to 500 μL with Buffer A and store the tubes on ice (Fig. 1). These aliquots represent the amount of protein and RNA present in the sample after lysis and will be needed for quality control of the immunoprecipitation experiment (*see Note 8*).

4. Take the remaining lysate and add 35 μL anti-FLAG monoclonal antibody per tube. Incubate for 30–60 min at 4 °C, gently tumbling the tube (Fig. 1). While the antibody is incubated with the lysate, pre-wash 75 μL Protein A-Sepharose beads per sample with 500 μL of Buffer A by gently inverting tubes and centrifugation at 15,000 $\times g$ for 1 min at 4 °C. Wash at least three times to saturate the beads with Buffer A (*see Note 9*).
5. After 30–60 min incubation with the anti-FLAG antibody, aliquot volumes equivalent to 1 and 5 OD₆₀₀ for protein and RNA, respectively (*see Subheading 3.2, step 3*). These aliquots are labeled as supernatant 1 (SN1) and stored at –20 °C for later use (Fig. 1).
6. After aliquoting SN1, transfer the rest of the supernatant to a fresh 2 mL tube containing 75 μL of pre-washed Protein A-sepharose beads per sample and incubate with gentle tumbling for 30–60 min at 4 °C (Fig. 1) (*see Note 10*).
7. After 30–60 min incubation, centrifuge at 15,000 $\times g$, 4 °C for 1 min to spin down the beads and transfer the supernatant, now referred to as supernatant 2 (SN2) into fresh 2 mL tubes, without losing any beads (Fig. 1). From SN2, take an aliquot equivalent to 1 and 5 OD₆₀₀ for protein and RNA quality control, respectively (*see Note 11*).
8. Wash the 2 mL eppendorf tube containing the beads with 500 μL Buffer A. Mix gently by inverting the tube (3–5 times) and centrifuge at 15,000 $\times g$, 4 °C for 1 min. Discard the first wash fraction and repeat this step four times (Fig. 1). Collect fractions after each washing step into one tube (approx. 2 mL collected from four washes) for aliquoting protein and RNA samples equivalent to 1 and 5 OD₆₀₀, respectively (*see Note 12*).
9. After the last washing step, add 500 μL Buffer A to the 2 mL eppendorf tube containing the beads. Subsequently, add 500 μL phenol/chloroform/isoamyl alcohol (P/C/I) to separate protein and RNA fractions from the beads into aqueous and organic phase (Fig. 1). This tube is labeled as the elution fraction (*see Note 13*).

10. Similarly, add 500 μL P/C/I to all 2 mL eppendorf tubes on ice containing RNA aliquots collected during the course of the experiment, i.e., Lysate, SN1, SN2, and Wash aliquots (Fig. 1). The protein fractions collected during the course of the experiment in the $5\times$ Protein loading buffer can be transferred to $-20\text{ }^{\circ}\text{C}$ for storage and later use.
11. Shake all tubes containing P/C/I vigorously for 15 s and incubate for 5 min at room temperature (Fig. 1).
12. Centrifuge at $15,000 \times g$, $4\text{ }^{\circ}\text{C}$ for 30 min.
13. After separation of the aqueous and organic phase, transfer the aqueous phase (on top) from all aliquots into fresh 2 mL eppendorf tubes containing 1 mL of 30:1 mix of Ethanol:3 M sodium acetate and 1 μL GlycoBlue™. Store the tubes overnight at $-20\text{ }^{\circ}\text{C}$ for RNA precipitation (Fig. 1).
14. To the tube containing the Protein A-sepharose beads in organic phase (at bottom), add 1.4 mL acetone (ice-cold) and incubate overnight at $-20\text{ }^{\circ}\text{C}$ for protein precipitation.

RNA extraction:

15. On the next day, centrifuge the tubes containing the aqueous phases with 30:1 mix of Ethanol:3M sodium acetate at $15,000 \times g$, $4\text{ }^{\circ}\text{C}$ for 30 min.
16. Carefully remove supernatant with a pipette without disturbing the pellet and wash once with 500 μL of ice-cold 70% EtOH. Centrifuge at $15,000 \times g$, $4\text{ }^{\circ}\text{C}$ for 10 min. Carefully aspirate EtOH without touching the pellet (*see* **Note 14**).
17. Air-dry the pellets at room temperature in a laminar hood and resuspend them in 30 μL RNase-free water. Dissolve the pellet by heating the samples at $65\text{ }^{\circ}\text{C}$ for 5 min on a heat block with constant shaking at 800 rpm and then store at $-20\text{ }^{\circ}\text{C}$ (Fig. 1). Use 5 μL for verification of successful coIP via northern blot analysis.

Protein extraction:

18. On the next day, centrifuge the tubes containing the beads and acetone at maximum speed $\sim 21,000 \times g$, $4\text{ }^{\circ}\text{C}$ for 60 min.
19. Carefully remove the supernatant and wash twice with 1 mL ice-cold acetone at maximum speed at $4\text{ }^{\circ}\text{C}$ for 30 min (*see* **Note 15**).
20. Remove supernatant and air-dry the pellets at room temperature. Afterwards, resuspend in 120 μL of $1\times$ Protein loading buffer. Boil the elution samples for 8 min at $95\text{ }^{\circ}\text{C}$ on a heat block with constant shaking at 800 rpm and store at $-20\text{ }^{\circ}\text{C}$ (Fig. 1).

3.3 Quality Control for Verification of Successful CjeCas9-3xFLAG Immunoprecipitation

Prior to DNase I digestion, cDNA synthesis, and RNA-sequencing, the amount of immunoprecipitated CjeCas9-3xFLAG protein as well as co-purified crRNA and tracrRNA can be visualized by western and northern blot analysis, respectively (Fig. 2). This allows for quality control before proceeding with cDNA library preparation and next-generation sequencing (*see* **Note 16**).

3.3.1 Western Blot

1. Boil protein samples stored at -20°C from all collected aliquots at 95°C using a heat block with constant shaking at 800 rpm for 5–8 min.
2. 20 μL volume each from lysate, SN1, SN2, wash, and eluate fractions (corresponding to 0.2 OD_{600} for L, SN1, SN2, W, and 10 OD_{600} for eluate, i.e., 1/6 of a final of 120 μL lysate derived from 60 OD_{600} starting material) is used for western blot analysis after resolving on 10% SDS-PAGE. The blots are probed using monoclonal anti-FLAG and anti-GroEL antibodies (Fig. 2a). For western blot, a protocol can be found here [49].

3.3.2 Northern Blot

1. Transfer 5 μL volume of RNA from a total of 30 μL collected per fraction into a fresh 1.5 mL eppendorf tube, i.e., one each for Lysate, SN1, SN2, wash, and eluate.
2. Add 5 μL of RNA loading buffer II to all the tubes containing 5 μL RNA, for both WT control and CjeCas9-3xFLAG tagged samples.
3. Denature the mix at 95°C on a heat block for 2 min and separate RNA samples by gel electrophoresis using a 6% PAA/7 M urea gel.
4. Transfer RNA from the PAA gel to a Hybond-XL membrane (GE-Healthcare) by electroblotting. After blotting, cross-link RNA to the membrane using UV irradiation and proceed with northern blotting to detect tracrRNA and crRNA3 in the eluted fractions as described previously (Fig. 2b) [31]. A protocol for northern blotting can be found here [50, 51].

3.4 DNase I Treatment

Prior to cDNA library preparations and after quality control, the remaining RNA samples must be treated with DNase I to remove any residual genomic DNA.

1. Denature 25 μL of the remaining RNA eluate samples at 65°C on a heat block for 5 min. Afterwards, transfer the tube to ice for another 5 min.
2. Add 5 μL of $10\times$ DNase I buffer containing MgCl_2 , 0.5 μL Suprase-In RNase Inhibitor, and 5 μL DNase I. Fill the total reaction volume to 50 μL by adding 14.5 μL of RNase-free water.

Fig. 2 Quality control for colP of CjeCas9-3XFLAG and co-purified RNAs using western and northern blotting. (a) Protein eluates collected post IP are subjected to 10% SDS-polyacrylamide gel electrophoresis. 20 μ L sample from lysate, SN1, SN2, wash, and eluate fractions from the untagged WT control strain and the CjeCas9-3xFLAG tagged strain samples were probed with anti-FLAG and anti-GroEL (control) antibodies using the same blot. CjeCas9 is specifically enriched in the CjeCas9-3xFLAG samples and absent in untagged WT samples, confirming successful immunoprecipitation. The presence of IgG heavy (Hc) and light (Lc) chains in both WT and CjeCas9-3xFLAG elution samples indicates successful capture of the FLAG antibody by protein-A beads. (b) RNA eluates collected post immunoprecipitation are subjected to 6% PAA/7M urea gel electrophoresis followed by northern blotting. 5 μ L RNA sample each from lysate, SN1, SN2, wash, and eluate fractions of WT and CjeCas9-3xFLAG were used for northern blotting and blots were probed with ATP(γ -32P) labeled DNA oligonucleotides complementary to tracrRNA and crRNA3 to confirm their enrichment in CjeCas9-3xFLAG tagged samples. Representative western and northern blots are shown

3. Incubate the reaction mix for 45 min at 37 °C. Then add 1 µL DNase I to the tube, mix and spin down the reaction mix. Incubate at 37 °C for further 15 min.
4. After 15 min, add 50 µL RNase-free water to adjust the reaction volume to a total of 100 µL.
5. Add 100 µL P/C/I to stop the reaction for all samples and transfer the total volume to PLG tubes. Make sure to spin down the PLG tubes before use. Mix by rigorous shaking and afterwards centrifuge at $15,000 \times g$, 15 °C for 12 min.
6. Transfer the aqueous phase to a fresh 2 mL Eppendorf tube and add 2.5 volumes (~ 200 µL) of 30:1 mix of Ethanol:3M sodium acetate. Incubate overnight at -20 °C for RNA precipitation.
7. On the next day, centrifuge at $15,000 \times g$, 4 °C for 30 min. Carefully remove supernatant and wash the pellet with 350 µL of 70% Ethanol (stored at -20 °C). Centrifuge at $15,000 \times g$, 4 °C for 10 min.
8. Remove the supernatant and air-dry the RNA pellet in a laminar hood.
9. Resuspend the pellet in 30 µL RNase-free water, incubate at 65 °C on a heat block with constant shaking at 800 rpm for 5 min, and store at -20 °C for use later. Confirm successful depletion of genomic DNA using 1 µL of DNase I treated RNA as a template for a control PCR to amplify a gene of choice, with genomic DNA template serving as a positive control. If the product is still amplified in DNase I treated RNA sample, repeat DNase treatment for the sample(s).

3.5 cDNA Library Preparation, Sequencing, and Analysis

All RNA samples post DNase I treatment can be directly used for cDNA synthesis for Illumina sequencing without prior size-selection, fragmentation, or rRNA depletion. Methodological details of cDNA sequencing and analysis are beyond the scope of this chapter. An example protocol for cDNA library preparation has been previously described [31]. Other cDNA library preparation protocols can also be used based on instructions provided by the manufacturer. Here we describe the downstream data analysis workflow using our recently published data from our CjeCas9 RIP-seq analysis [31].

Briefly, RIP-seq analysis of CjeCas9 aims to identify enriched RNAs specifically bound to the protein. Sequencing of cDNA fragments generated from CjeCas9 co-purified RNAs leads to identification of individual reads, each of which can be computationally mapped to the reference genome. After sequencing and quality control steps, coverage plots are generated representing the number of mapped reads per nucleotide which can be used for visualization in a genome browser such as the Integrated Genome

Fig. 3 Visualizing RIP-seq results and downstream analysis. (a) RIP-seq results for Cas9 from *C. jejuni* strain NCTC11168 [31]. (Upper panel) Screenshot of cDNA coverage plots of normalized RNA-seq reads from untagged WT control (black) and CjeCas9-3xFLAG colP (green) libraries mapped to the *C. jejuni* NCTC11168 genome (both strands) and visualized using the Integrated Genome Browser. (Lower panel) A zoomed-in screenshot of the normalized RNA-seq reads from WT and CjeCas9-3xFLAG colP libraries mapped to the CRISPR locus is shown to highlight the enrichment of crRNAs and tracrRNA in the CjeCas9-3xFLAG tagged

Browser (IGB) or Integrative Genomics Viewer (IGV) [52, 53]. This enables a direct visual comparison of relative cDNA reads for WT-untagged and epitope-tagged libraries mapped to a particular locus of choice, as shown for CRISPR locus, allowing for better interpretation of the data (Fig. 3a). Transcript quantification can then be performed between the CjeCas9-3xFLAG and WT coIP libraries and the resulting count data can be used for differential expression analysis via DESeq2 [55] to generate enrichment scores, thereby identifying RNA targets specifically bound to CjeCas9. Furthermore, the co-purified transcripts can be grouped according to functional classes based on the annotations. In the CjeCas9 RIP-seq example, the most abundantly co-purified RNA classes in the WT and CjeCas9-3xFLAG coIP library reads belong to ribosomal RNA (rRNA), transfer RNA (tRNA), and the house-keeping RNAs (hkRNAs). This is not surprising as rRNAs and tRNAs constitute more than 95% of the total cellular RNA pool and can be nonspecifically bound during the coIP approach. However, they are typically not enriched in the library of the tagged RBP vs the WT control and thus can be excluded as potential binding partners. On the other hand, a strong enrichment of the tracrRNA and all four crRNAs only in CjeCas9-3xFLAG coIP library indicates specific binding to CjeCas9. Moreover, a striking observation during the RIP-seq analysis of CjeCas9 was the enrichment of RNAs aside from tracrRNA and crRNAs in the CjeCas9 coIP library (Fig. 3b). Most of the additionally enriched RNAs corresponded to regions from open reading frames (ORFs), indicating co-purification of messenger RNAs with CjeCas9. Some of them

Fig. 3 (continued) versus WT coIP sample. Genomic coordinates and relative number of cDNA reads are indicated next to each screenshot. **(b)** Read distribution of different RNA classes depicted as doughnut charts showing the relative proportions of mapped cDNA reads in the CjeCas9-3xFLAG and WT coIP libraries. The RNA classes include ribosomal RNAs (rRNAs), messenger RNAs (ORFs), 5' untranslated regions (5' UTRs), pseudogenes, house-keeping RNAs (hkRNAs), transfer RNAs (tRNAs), small RNAs (sRNAs), the tracrRNA, and the four crRNAs of *C. jejuni* strain NCTC11168. ORFs (red), crRNAs (violet), and tracrRNA (green) were highly enriched while other RNA classes were depleted following immunoprecipitation and sequencing of co-purified RNAs from CjeCas9-3xFLAG coIP library versus the untagged WT coIP library. **(c)** Workflow for peak detection and motif discovery used for CjeCas9-3xFLAG bound co-purified RNAs. Normalized coverage files generated post cDNA sequencing of the CjeCas9 coIP vs. WT control coIP libraries were subjected to a peak-detection algorithm based on a sliding-window approach [31]. The resulting peaks were used as input for MEME analysis [54] and revealed the presence of two distinct motifs in several of the co-purified transcripts. These motifs were further analyzed regarding sequence and base-pairing probability to identify potential RNA:RNA interactions within the CjeCas9-bound interactome. Motif 1 and 2 displayed complementarity to the guide portion of crRNA3 and crRNA4 encoded in *C. jejuni* NCTC11168, indicating that these transcripts are bound by CjCas9 in a crRNA-dependent manner [31]

showed defined enriched peaks in the Cas9-3xFLAG coIP compared to the WT coIP sample.

Besides a manual annotation of such enriched peaks across the genome, numerous peak-detection tools are available for analyzing RIP-seq data such as RIP-seeker [56], Piranha [57], and our in-house peak-detection algorithm based on a sliding-window approach [31]. The resulting peaks enriched in the CjeCas9-3XFLAG coIP library can then be used to identify the presence of distinct motifs using MEME analysis [54]. In our study, MEME led to the identification of two motifs in most of the co-purified mRNAs. These motifs showed base-pairing complementarity to the sequences of the crRNA3 and crRNA4 guides encoded naturally in the host system, hinting at direct RNA:RNA interactions and association with the CjeCas9:crRNA:tracrRNA RNP (Fig. 3c). Depending on the RNA-binding protein used for coIP, the motifs could also indicate the binding site specificity of the RBP [40]. Bioinformatic programs such as IntaRNA or NUPACK can be used to examine base-pairing probabilities between the crRNAs and additional co-purified transcripts [58, 59]. Once computationally identified, it is important to confirm and validate the identified RNA:RNA interactions via other methods such as genetic manipulation of identified RNAs or direct biochemical assays which confer specificity to the identified RNAs and associated RNPs in the studied organism. For example, using deletion strains of either individual crRNAs [31] or the whole CRISPR array for performing RIP-seq could help validate direct crRNA-dependent RNA:RNA interactions and identify other potential crRNA-independent interactions with CRISPR-Cas proteins of interest, respectively. In addition, RIP-seq data can be analyzed together with additional available transcriptome data such as differential RNA-sequencing (dRNA-seq) data to distinguish between the enriched primary transcripts marked by a 5' tri-phosphate (5'PPP) and processed RNAs with a 5' monophosphate (5'P) group [60, 61]. dRNA-seq has facilitated the identification of transcriptional start sites and small RNAs and helped to decipher processing of CRISPR-derived RNAs in the human pathogen *Streptococcus pyogenes* via tracrRNA [19]. The same approach also helped to uncover distinct crRNA biogenesis mechanisms, leading to the discovery of a new subtype C of Type II CRISPR-Cas systems in *N. meningitidis* and *C. jejuni* [62, 63].

3.6 Outlook

Molecular mechanisms underlying CRISPR-Cas adaptive immunity have been extensively studied over the past decade. While a lot has been uncovered for individual CRISPR systems, networks governing regulation of these systems within the host are yet unknown. Most organisms typically contain a single CRISPR system, while some employ more than one. The interplay between different CRISPR defense systems in varied hosts is crucial for their survival under native conditions. Among all the differences

between the CRISPR-Cas classes, types, and subtypes, there lies one commonality: all use RNA-guided ribonucleoprotein complexes. Thus, it is important to know how the RNA guides of these complexes look like and whether RNAs other than system specific crRNAs or tracrRNAs can also be associated with these complexes. A method to study RNP complexes on a transcriptome-wide scale like RIP-seq can help to identify such RNAs. Depending on what is known regarding the CRISPR-Cas protein of interest, other RNA-seq based global pull-down approaches such as CLIP-seq, RIL-seq, or CLASH could also be employed [64–66]. In CLIP-seq, the protein of interest is cross-linked to its interacting RNAs by ultraviolet light in vivo, immunoprecipitated, and bound RNAs are sequenced. This allows for refinement of precise binding sites on RNA as the crosslinking is detected by a change in the nucleotide sequence. The RIL-seq and CLASH approaches capture RNA-RNA pairs by adding a ligation step while the RNAs are in close proximity to the protein of interest post crosslinking and immunoprecipitation [3]. Application of such techniques can help ascertain whether the RNAs are bound directly or indirectly via RNA–RNA interactions to different Cas proteins in varied CRISPR systems and would help shed light on host-mediated regulation and other possible roles for these effector proteins and adaptive immune systems.

4 Notes

1. RIP-seq experiments can be performed in Gram-positive or Gram-negative bacteria to identify RNA substrates of a CRISPR-Cas protein-of-interest (POI). The amount of required cell starting material can vary between 20 and 60 OD₆₀₀ depending on the expression of the POI and chosen growth condition.
2. To keep expression levels of the POI to physiological levels, it is recommended that the POI is chromosomally tagged using a 3xFLAG tag or any other epitope of choice. It is preferred, if a monoclonal antibody is available against the epitope, else the pull-down can be performed using a polyclonal antibody/anti-serum. In organisms where genome manipulation is difficult, plasmids can also be used to express the POI. It is important to note that expressing a POI on a plasmid will generate an additional copy of the protein and both genome and plasmid-encoded variants will compete for the same RNAs, leading to an enrichment of plasmid-encoded POI-bound RNAs after sequencing. The copy number of the plasmid should be chosen as to generate levels of the POI in the range of physiological levels and growth kinetics should be monitored to detect any

phenotypic changes upon overexpression of the POI before proceeding with the RIP-seq experiment.

3. Ideally, the POI can be tagged either at its N- or C-terminus. It is advisable to check for expression and activity of the POI post epitope tagging at either end. Once this is verified, strains harboring the epitope-tagged POI should be compared with the untagged WT strain to rule out potential growth defects.
4. If possible, western blotting should be performed following growth curve analysis of the untagged and epitope-tagged strain. Samples collected at different growth phases (lag, early-log, mid-log, late-log/early stationary, and stationary phase) and analyzed on a western blot will help to identify the ideal growth condition, where the expression of the POI is strong enough for immunoprecipitation. Also, initial screening with a western blot helps to confirm the predicted size (kDa) of the tagged POI.
5. The volume of resuspension Buffer A can vary depending on the pellet. This step is performed to wash the cells off the remaining medium and any metabolic waste products generated by the cells, which might affect the subsequent steps of immunoprecipitation.
6. It is important to adjust the volume of the Lysis buffer depending on the amount of bacterial culture pelleted. The volume of the Lysis buffer should be just enough to resuspend the pellet but not dilute it. This should be standardized based on the model organism and the pellet collected. Lysis buffer volumes could impact the overall lysis. In case of volumes exceeding 1 mL, the volume must be equally distributed over two to three fast-prep tubes. After lysis and centrifugation, cleared lysates belonging to the same samples can be pooled together for immunoprecipitation.
7. Bacterial cell lysis using a FastPrep-24 machine or other physical disruption methods should be standardized based on the model organism. Before collecting pellets for RIP-seq, parameters such as the amount of bacterial culture and lysis method need to be standardized. This can be done by visualizing the epitope-tagged POI present post-lysis using a western blot. For Gram-positive cells, this might mean increasing the number of cycles and cycle-times on the FastPrep machine. Also, it is important to maintain native conditions for immunoprecipitation of RNA substrates and samples must be kept on ice throughout the protocol. A larger pellet volume does not necessarily lead to better lysis and more protein starting material for immunoprecipitation.
8. The volume of aliquots collected for RNA/protein fractions will vary depending on the starting volume of the Lysis buffer,

culture pellet and the volume of the cleared lysate recovered post-lysis.

9. To aliquot the beads from its stock, it is recommended to use an autoclaved bottom-cropped 1000 μ L tip. To bottom-crop, simply cut 2–3 mm from the bottom of the 1000 μ L tip using sterile scissors.
10. The incubation time of the lysate with the anti-FLAG and protein A-sepharose beads can be standardized for each POI. It should be kept in mind that longer incubation times might be detrimental and thus lead to nonspecific binding which might not reflect physiologically relevant RNA:RNA or RNA: protein interactions.
11. It is recommended to store the rest of the SN2 fraction until quality control of the experiment is completed. If the POI is not enriched in the eluate fraction as visualized using western blot, the leftover SN2 fraction can help ascertain whether the POI was bound to the beads or was lost during washing steps of the immunoprecipitation protocol.
12. Care must be taken while performing the washing steps. Any loss of beads is directly proportional to the loss of bound RNPs, thus affecting the analysis.
13. TRIZOL can also be used to recover RNA at this step, following the instructions provided by the manufacturer. It is advisable to add GlycoBlue™ blue to facilitate RNA precipitation, as the RNA yields obtained from immunoprecipitation can be quite low. GlycoBlue™ acts as a carrier, thus aiding in RNA recovery and visualization of the pellet after centrifugation.
14. Using a combination of a 200 μ L tip on top of a 1000 μ L tip aids in complete removal of the supernatant, without disturbing the pellet. This is a crucial step and any loss of pellets should be avoided.
15. As the protein pellet might be difficult to see among the beads, it is advisable to be cautious while washing and care must be taken to prevent any loss of beads or the protein pellet.
16. Performing a western blot to confirm successful immunoprecipitation of the POI is strongly recommended. This allows to ascertain if the POI was sufficiently enriched in the eluate from the epitope-tagged strain versus the untagged strain. This step also aids in standardization of the coIP protocol by visualizing the tagged protein in other fractions collected (lysate, SN1, SN2, and wash) during the course of the experiment. Performing a northern blot is dependent on the knowledge of previously identified RNAs (acting as positive controls) associated with the POI and can also be excluded if such information is not at hand. In any case, the western blot should

confirm enrichment of the POI prior to proceeding with cDNA library preparation of co-purified transcripts.

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