Molecular Cell, Volume *45*

Supplemental Information

Essential Features and Rational Design of CRISPR RNAs that Function with the Cas RAMP Module Complex to Cleave RNAs

Caryn R. Hale, Sonali Majumdar, Joshua Elmore, Neil Pfister, Mark Compton, Sara Olson, Alissa M. Resch, Claiborne V.C. Glover III, Brenton R. Graveley, Rebecca M. Terns, and Michael P. Terns

INVENTORY OF SUPPLEMENTAL INFORMATION

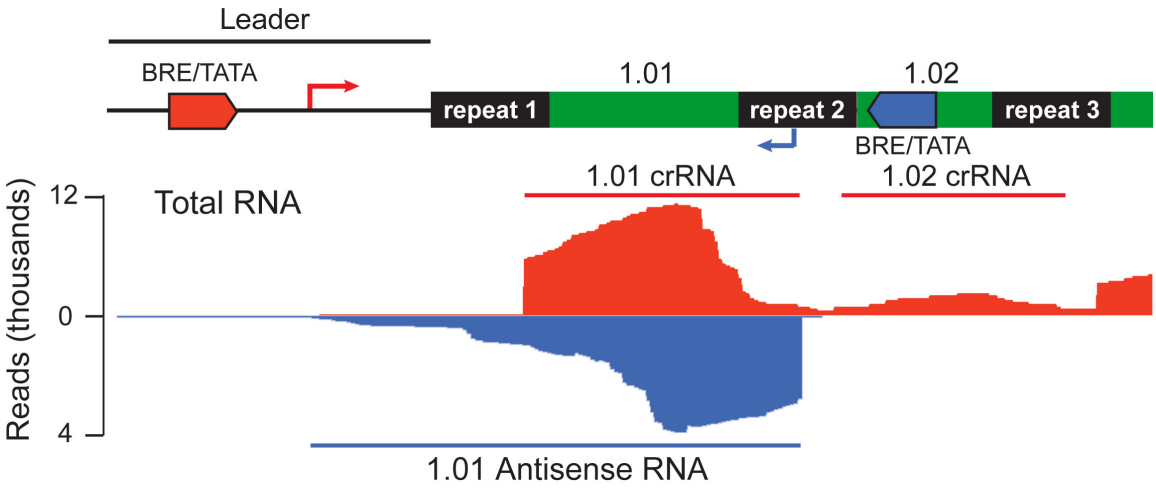

Figure S1, related to Figure 4. **Antisense RNA complementary to crRNA 1.01 in *P. furiosus*.** Deep sequencing profiles from total *P. furiosus* RNA in the region encoding crRNA 1.01 are shown. Red indicates RNAs transcribed from the leader region, and blue corresponds to reads from the opposite strand. The positions of CRISPR repeats (black), guide sequences (green) and BRE/TATA promoters (red and blue) are indicated. A blue line represents the apparent full-length 1.01 antisense RNA.

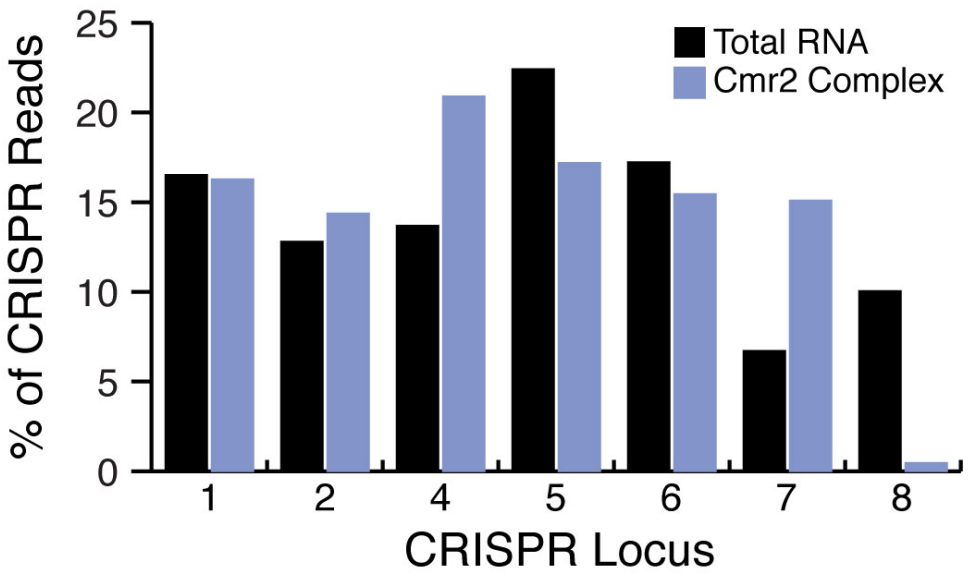

Figure S2, related to Figure 5. **crRNAs from CRISPR 8 are underrepresented in the Cmr complex.** Graph indicates the percentage of CRISPR-derived deep sequencing reads that map to each CRISPR locus in total RNA (black) and Cmr-associated RNAs (blue)

Table S1. RNA and DNA primers used in this study.

	Sequence	Figure(s)
RNA oligos		
7.01 target	CUGAAGUGCUCUCAGCCGCAAGGACCGCAUACUACAA	1C, 5B-D
7.01 crRNA (45)	AUUGAAAGUUGUAGUAUGCGGUCCUUGCGGCUGAGAGC ACUUCAG	5B-D
7.01 - tag crRNA (45)	UUGUAGUAUGCGGUCCUUGCGGCUGAGAGCACUUCAG	5B
UAACUUUC tag 7.01 crRNA	UAACUUUCUUGUAGUAUGCGGUCCUUGCGGCUGAGAGC ACUUCAG	5C
X crRNA	AUUGAAAGCUGAAGUGCUCUCAGCCGCAAGGACCGCAUA CUACAA	6A
X' target	UUGUAGUAUGCGGUCCUUGCGGCUGAGAGCACUUCAG	6A
bla target	AUGAGUAUUCAACAUUUCCGUGUCGCCCUUAUUCCCU	6A
5' bla crRNA	AUUGAAAGAGGGAAUAAGGGCGACACGGAAAUGUUGAAU ACUCAU	6A-B
internal bla crRNA	AUUGAAAGCGUCAACACGGGAUAAUACCGCGCCACAUAG	6B
Northern probes		
7.01 sense	GCTCTCAGCCGCAAGGACCGCATAC	1B
1.01 Antisense	AAGCAATCTGATCTGACCAGAGCTGGTTCC	4A
1.01 sense	GATTGCTTAAGACAAGAAATGAATCATATAACTTTCAAT	4A
Annealed IVT Templates		
1.01 sense template 5' (T7)	TAATACGACTCACTATAGGGTTATATGATTCATTTCTTGTCT TAAGCAATCTGATCTGACCAGAGCTG	4B
1.01 sense template 3' (T7)	CAGCTCTGGTCAGATCAGATTGCTTAAGACAAGAAATGAAT CATATAACCCTATAGTGAGTCGTATTA	4B
GAUUGAAAG tag 7.01 crRNA (45) 5' (SP6)	ATTTAGGTGACACTATAGATTGAAAGTTGTAGTATGCGGTC CTTGCGGCTGAGAGCACTTCAG	5C
GAUUGAAAG tag 7.01 crRNA (45) 3' (SP6)	CTGAAGTGCTCTCAGCCGCAAGGACCGCATACTACAACTT TCAATCTATAGTGTCACCTAAAT	5C
GAUGAAAG tag 7.01 crRNA (45) 5' (SP6)	ATTTAGGTGACACTATAGATGAAAGTTGTAGTATGCGGTCC TTGCGGCTGAGAGCACTTCAG	5C
GAUGAAAG tag 7.01 crRNA (45) 3' (SP6)	CTGAAGTGCTCTCAGCCGCAAGGACCGCATACTACAACTT TCATCTATAGTGTCACCTAAAT	5C
tag complementary 7.01 target 5' (SP6)	ATTTAGGTGACACTATAGACTGAAGTGCTCTCAGCCGCAA GGACCGCATACTACAACTTTCAAT	5D
tag complementary 7.01 target 3' (SP6)	ATTGAAAGTTGTAGTATGCGGTCCTTGCGGCTGAGAGCAC TTCAGTCTATAGTGTCACCTAAAT	5D
PCR amplified IVT templates		
1.01 AS PCR 5' (T7)	TAATACGACTCACTATAGGGCTTATTGGAACCAGCTCTGGT CAG	4B
1.01 AS PCR 3' (T7)	CGAGTGATATAGTTACTCCGTAGG	4B
bla mRNA 5' (T7)	TAATACGACTCACTATAGGGATGAGTATTCAACATTTCCGT GTCGCC	6B
bla mRNA 3' (T7)	TTACCAATGCTTAATCAGTGAGGCACC	6B
Small RNA Cloning		
3' adapter	rAppTCGTATGCCGTCTTCTGCTTGTddC	2-5
5' adapter	invdTGTTCrArGrArGrUrUrCrUrArGrUrCrCrGrArCrGrArUrC	2-5
RT primer	CAAGCAGAAGACGGCATACGA	2-5
PCR primer	AATGATACGGCGACCACCGACAGGTTCAGAGTTCTACAGT CCGA	2-5

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Antibody preparation and co-immunoprecipitation reactions

IgY antibodies were prepared in *Gallus gallus* against recombinant Cmr2 from *Pyrococcus furiosus* as described (Carte et al., 2010). For co-immunoprecipitations (co-IPs) to be followed by Northern analysis, end analysis and small RNA sequencing, 80 μg of immune or pre-immune antibodies were coupled to 30 μl anti-IgY conjugated agarose beads (Gallus Immunotech) per the manufacturer's protocol. For Western analysis, 625 μg of antibodies were coupled directly to 125 μl CarboLink beads (Pierce) per the manufacturer's recommendations. Conjugation was confirmed by A260 readings. *P. furiosus* S100 extract was prepared as described (Hale et al., 2009). In the co-IP reactions, 200 μl of *P. furiosus* cell extract (~15 mg/mL total protein) was added to 30 μl of the conjugated beads, and the volume was brought to 1 mL with IPP-300 (10 mM Tris, pH 8.0, 300 mM NaCl, 0.05% Igepal) supplemented with 1X proteinase inhibitor (Mini EDTA-free proteinase inhibitor cocktail, Roche) and 50 U SUPERas•In (Ambion). The samples were rotated end-over-end for 2 hours at room temperature. Beads were collected by centrifugation at 3,000 rpm (960 x g) for 2 minutes, followed by washing with 1 mL IPP-300 four times. The pelleted material was resuspended in IPP-300 and used for further analysis.

Co-IP/Western analysis

For Western analysis, 1/3 of a co-IP reaction was resuspended in 3X non-reducing loading dye (62.7 mM Tris, 2.3% SDS, 10% glycerol, 0.05% bromophenol blue). The co-IP samples were eluted at 60°C for 5 minutes, centrifuged briefly, and loaded onto 12.5% SDS-PAGE gels. 1 μl of S100 extract (~15 μg of total protein) was loaded for comparison (T). S100 sample was boiled in reducing loading dye (supplemented with 240 mM β–mercaptoethanol). The resulting gels were subject to Western blotting using standard procedures. 0.45 μm nitrocellulose membranes were used, and blotting was performed using the Trans-Blot SD Semi-Dry Cell (Bio-Rad) at a constant current of 0.25 A for 1 hour. Blots were blocked in 5% blotting grade non-fat dry milk (Bio-Rad)

overnight at 4°C and washed twice in TBST (20 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween-20). Pf Cmr2 antibody was used for primary antibody incubation at a concentration of 0.48 μg/ml in TBST for 1 hour at room temperature. The blots were washed three times with TBST and incubated with HRP-conjugated anti-IgY antibody (0.2 μg/ml in TBST) (Gallus Immunotech). Blots were visualized using blue film (Denville Scientific) and the ECL plus detection kit (GE Healthcare) as recommended. Optimum developing time was around 1 minute.

Co-IP/Northern analysis

For Northern analysis of co-immunopurified RNAs, Trizol LS reagent (Invitrogen) was used to isolate RNAs directly from the co-IP sample. For Trizol LS, ~50 mg of *P. furiosus* cells were resuspended in 250 μl of water followed by RNA purification as recommended. For Figure 1B, one half of a co-IP sample was used for each Northern lane. RNA isolated from 20 μl of S100 (300 mg total protein) was used for the total RNA (T) lanes for all Northerns. For Figure 4A, 2/3 and a full co-IP sample were used for the 1.01 antisense and 1.01 crRNA Northern, respectively. Northern analysis was performed as described previously (Hale et al., 2008) with the following modifications. RNAs were separated on standard 7M urea TBE 15% polyacrylamide gels. All Northerns were performed using hybridization buffer containing 5X SSC, 7% SDS, 20 mM sodium phosphate (pH 7.0), and 1X Denhardt's solution. Probe sequences can be found in Supplemental Table 1.

Co-IP/Activity assays

For Cmr activity assays, co-IP beads were washed into 40 mM Hepes pH 7.0, 500 mM KCl, and resuspended to a 50% slurry. 10 μl of the slurry (1/6 of a co-IP reaction) was added to a 20 μl reaction containing 10% glycerol, 1.5 mM $MgCl_2$, 5 mM DTT, 5 μg of *E. coli* tRNA, 1 U SUPERas•In (Ambion) and approximately 5,000 cpm of radiolabled target RNA (see details of RNA preparation below). Reactions were incubated for 3 hours at 70°C, with mixing to resuspend beads every 1 hour. Subsequently, 1 μg of proteinase K was added and incubated for 15 minutes at 37°C, followed by phenol

extraction and ethanol precipitation. The resulting RNAs were separated on 7M urea 15% polyacrylamide gels and visualized on phosphor imaging screens.

Small RNA library preparation

For preparation of total and Cmr-associated small RNA libraries, Trizol was used to extract RNAs directly from *P. furiosus* cells or from 30 μl of co-IP beads (one co-IP reaction), respectively. 50 μg of total RNA was used for total small RNA library preparation. RNAs were separated on 10% denaturing gels, the gels were stained briefly with SYBR Gold (Invitrogen), and regions containing small RNAs (~20 – 70 nucleotides) were excised with sterile razor blades. The gel slices were crushed in 300 mM NaCl, and RNAs were eluted by end-over-end rotation at 4°C overnight. RNAs were collected by phenol extraction and ethanol precipitation. The RNAs were treated with 1 U of TSAP in 1X Multi-Core Buffer (Promega) for 30 minutes at 37°C, followed by heat inactivation at 74°C for 15 minutes. The RNAs were collected by phenol extraction and ethanol precipitation, and ligated to a 3' adapter (50 pmol) (see Supplemental Table 1 for sequences). For 3' ligation, the RNAs were incubated in 50 mM Tris pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 15% PEG 3350, for 30 seconds at 90°C and on ice for 20 seconds. 200 U T4 RNA Ligase 2, truncated (NEB) and 20 U SUPERas•In (Ambion) were added, and the reaction was incubated at 37°C for 1 hour. The RNAs were isolated and separated by denaturing gel electrophoresis, and the ligated RNA bands were excised and gel purified as described above. The gel-purified RNAs were treated with 10 U T4 Polynucleotide Kinase (Ambion) in 50 mM Tris pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, and 100 μM ATP for 1 hour at 37°C, and then isolated by phenol extraction and ethanol precipitation. Next, the treated RNAs were ligated to 5' adapter (5 pmol, see Supplemental Table 1) using T4 ssRNA Ligase 1 (NEB) in 50 mM Tris pH 7.5, 10 mM $MgCl_2$, 5 mM DTT and 1 mM ATP, 15% PEG 3350, and 10% DMSO at 22°C for 6 hours. The ligated RNAs were isolated and subjected to reverse transcription by Superscript III reverse transcriptase (Invitrogen) and RNase H (NEB) treatment following the manufacturer's protocol (Invitrogen). PCR was performed with Crimson

Taq (NEB) as recommended, 2 minutes at 94°C, followed by 12-16 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 45 s. Extension was performed for 7 minutes at 72°C.

Recombinant Cmr protein production and activity assays

Expression and purification of recombinant Cmr proteins was performed as described (Hale et al., 2009) with the following modifications. PF1130 was cloned into pET101D to provide a C-terminal 6X histidine tag. Culture sizes varied from 200 mL to 2 L. Cells were resuspended in sodium phosphate buffer containing 5 mM imidazole. After sonication, the samples were subject to thermal precipitation (20 minutes at 75°C with occasional mixing) before centrifugation at 14,000 rpm (20,817 x g) for 20 minutes. Purification was performed in batch using either Ni-NTA agarose beads (Qiagen, PF1125, 1126 and 1129) or Talon Cobalt Resin (Clonetech, PF1124, 1128, 1130). Sequential elutions were performed using phosphate buffer containing 50, 100, 200 and 500 mM imidazole. Select elutions were dialyzed into 40 mM HEPES pH 7.0, 500 mM KCl using Slide-a-lyzer MINI dialysis cassettes (ThermoScientific) for use in recombinant assays. Recombinant Cmr assays using 500 nM of each Cmr protein were performed as described (Hale et al., 2009). Briefly, recombinant proteins were pre-incubated with unlabeled crRNA (0.05 pmol) prior to addition of 5' radiolabeled target RNAs (5,000 cpm). Reactions were incubated for 1-3 hours at 70°C. Following incubation, reactions were subject to proteinase K treatment, PCI and ethanol precipitation, and separated by denaturing gel electrophoresis. The gels were dried and visualized on phosphor imaging screens (Amersham).

RNA preparation

Sequences of RNA/DNA oligos used for RNA preparation are found in Supplemetal Table 1. Synthetic RNA oligos and oligos used for small RNA cloning were purchased from Integrated DNA Technologies (IDT). In some cases, RNA oligos were 5' end labeled with T4 Polynucleotide Kinase as described (Carte et al., 2008). DNA oligos used for generation of in vitro transcription (IVT) templates were purchased from Eurofins MWG Operon. Annealing of IVT template oligos was performed by mixing

equimolar amounts of each oligo in 30 mM HEPES pH 7.4, 100 mM potassium acetate and 2 mM magnesium acetate, and incubating 1 minute at 95°C followed by 1 hour at 37°C. Annealing was confirmed by native gel electrophoresis. For PCR-generated IVT templates, PCR was performed using the High Fidelity PCR system (Roche) as recommended by the manufacturer. The 1.01 antisense RNA template was amplified from *P. furiosus* genomic DNA, and the bla mRNA was amplified from a pET21D-based construct (Clontech). PCR products were subject to PCR cleanup (Wizard SV Gel and PCR Clean-Up system, Promega) and quantitated by gel analysis and nano-drop spectrophotometry (ThermoScientific). IVT was performed using the MEGAshortscript T7 or MAXIscript SP6 large-scale IVT kits (Ambion). For removal of 5' phosphates (either for the purpose of 5' end labeling of IVT products or for use as crRNAs) the KinaseMax kit (1.01 antisense RNA, Bla mRNA, 1.01 sense RNA, Ambion) or TSAP treatment (tag complementary target, GAUUGAAAG tag 7.01 crRNA (45) and GAUGAAAG tag 7.01 crRNA (45), Promega) was used as recommended by the manufacturers. Removal of phosphates from crRNAs was confirmed by the ability to 5' end-label the resulting RNAs.