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Article

A scoutRNA Is Required for Some Type V CRISPR-Cas Systems

Highlights

- scoutRNAs define a new class of noncoding transcript required by Cas12c/d enzymes
- scoutRNAs have minimal base-pairing complementarity with CRISPR RNA
- scoutRNAs enable Cas12c/d-catalyzed CRISPR RNA maturation and DNA cutting

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In Brief

Harrington et al. define scoutRNA as a new class of noncoding RNA that is required for CRISPR-Cas12c/d systems. The scoutRNA assembles with Cas12c/d enzymes and, together with CRISPR RNA, enables RNA-guided DNA binding and cutting.

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A scoutRNA Is Required for Some Type V CRISPR-Cas Systems

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SUMMARY

CRISPR-Cas12c/d proteins share limited homology with Cas12a and Cas9 bacterial CRISPR RNA (crRNA)-guided nucleases used widely for genome editing and DNA detection. However, Cas12c (C2c3)- and Cas12d (CasY)-catalyzed DNA cleavage and genome editing activities have not been directly observed. We show here that a short-complementarity untranslated RNA (scoutRNA), together with crRNA, is required for Cas12d-catalyzed DNA cutting. The scoutRNA differs in secondary structure from previously described tracrRNAs used by CRISPR-Cas9 and some Cas12 enzymes, and in Cas12d-containing systems, scoutRNA includes a conserved five-nucleotide sequence that is essential for activity. In addition to supporting crRNA-directed DNA recognition, biochemical and cell-based experiments establish scoutRNA as an essential cofactor for Cas12c-catalyzed pre-crRNA maturation. These results define scoutRNA as a third type of transcript encoded by a subset of CRISPR-Cas genomic loci and explain how Cas12c/d systems avoid requirements for host factors including ribonuclease III for bacterial RNA-mediated adaptive immunity.

INTRODUCTION

CRISPR-Cas (clustered regularly interspaced short palindromic repeats-CRISPR associated) systems provide bacteria and archaea with adaptive immunity against infectious agents (Barrangou et al., 2007). RNA-guided nucleases are central to these pathways, recognizing and cutting double-stranded DNA (dsDNA) to trigger the degradation of targeted sequences in phage and plasmids (reviewed in Marraffini, 2015; Wright et al., 2016). In addition, the Cas9 and Cas12a enzymes found within type II and type V CRISPR-Cas systems, respectively, are now widely used for genome-editing applications in eukaryotic cells and organisms based on their programmable ability to trigger DNA repair at desired sites (reviewed in Knott and Doudna, 2018; Wu et al., 2018).

Two types of noncoding RNAs have been identified as central components of CRISPR-Cas systems, CRISPR RNA (crRNA) and transactivating CRISPR RNA (tracrRNA). CRISPR RNA is used by all known CRISPR systems, as it provides the sequence-recognition capability of these pathways (Brouns et al., 2008). Produced by transcription and processing of the CRISPR sequence array, which includes direct repeats separated by target-derived spacers, crRNAs guide Cas nucleases to cut DNA with complementarity to a ~20-nt crRNA segment (Bolotin et al., 2005; Brouns et al., 2008; Garneau et al., 2010; Hale et al., 2009; Mojica et al., 2005; Pourcel et al., 2005). A second RNA, tracrRNA, is encoded within type II and some type V CRISPR-Cas genomic loci, where it is necessary for both crRNA maturation (Deltcheva et al., 2011; Chylinski et al., 2013) and CRISPR-Cas9-mediated DNA cleavage (Jinek et al., 2012).

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Extended base-pairing complementarity between tracrRNA and the direct-repeat segment of crRNA creates a dsRNA structure that is a substrate for ribonuclease III-catalyzed processing (Deltcheva et al., 2011). The resulting dual-RNA guide is required for CRISPR-Cas9-catalyzed dsDNA recognition and cleavage (Jinek et al., 2012).

The identification of divergent CRISPR-Cas systems, particularly within metagenomic sequencing datasets, has revealed new enzymes with only limited sequence similarity to known proteins. Among these, the Cas12c and Cas12d enzymes (also known as C2c3 and CasY, respectively) have attracted interest due to their small size and, in the case of Cas12d, predominant occurrence within the compact genomes of candidate phyla radiation (CPR) bacteria (Burstein et al., 2017). However, with the exception of DNA targeting activity detected indirectly for Cas12d (Burstein et al., 2017), Cas12c/d-catalyzed DNA cleavage has not been observed.

We wondered whether Cas12c/d enzymes require additional components, either encoded in the CRISPR-Cas locus or elsewhere in host genomes, for RNA-guided DNA cutting. Here, we show that a third type of CRISPR-Cas-encoded RNA, a short-complementarity untranslated RNA (scoutRNA), assembles with Cas12c/d and crRNA, and as demonstrated for Cas12d, creates a functional DNA-targeting complex. Transcriptomic sequencing data indicate that processing of an initial precursor transcript generates scoutRNA, which includes only a short but highly conserved 3- to 5-nt sequence that is complementary to the repeat sequence in the crRNA. Biochemical experiments reveal that scoutRNA binds directly to Cas12d, where it functions together with crRNA to enable site-specific dsDNA cleavage. We also found that scoutRNA is required for pre-crRNA processing by Cas12c by a mechanism that is distinct from any known crRNA maturation mechanism. These findings explain why Cas12d and, by inference Cas12c, CRISPR systems can exist in the compact genomes of CPR bacteria that lack the ribonuclease III enzyme needed for tracrRNA-mediated crRNA processing. Our results uncover a new category of CRISPR-Cas systems defined by a unique RNA component and activation mechanism, showing how diversification of these pathways could have assisted in their spread among divergent microbial populations.

RESULTS

Cas12c/d Represent Compact CRISPR-Cas Systems Found in Tiny Genomes

Class 2 CRISPR-Cas systems typically include a single large (100–200 kDa) Cas protein that catalyzes the RNA-guided cleavage of DNA or RNA substrates. Searches to identify new class 2 proteins in bacterial metagenomic datasets revealed the existence of proteins classified as Cas12d, defined by proximity to a CRISPR array and the conserved CRISPR-associated gene *cas1* (Burstein et al., 2017). Comparative sequence and protein architecture analysis showed that CasY (now known as Cas12d) proteins are most closely related to the CRISPR-C2c3 family of enzymes (renamed Cas12c); for simplicity, we refer to this CRISPR-Cas subclass as Cas12c/d (Figures 1A and 1B). These proteins belong to the CRISPR-Cas type V superfamily, enzymes that contain a single RuvC nuclease domain that, in other type V family enzymes, is responsible for RNA-guided DNA cleavage.

We identified, based on comparative sequence analysis, 23 distinct variants of Cas12c/d proteins from microbial organisms populating diverse environments, including hot springs, Antarctic sea ice, and insect microbiomes (Figure S1A). Notably, Cas12d genes and their CRISPR-Cas genomic loci occur primarily in the compact genomes of CPR bacteria, a microbial super-phylum characterized by small cell and genome sizes (Figures 1B and 1C). Consistent with this phylogenetic distribution, Cas12c/d systems are streamlined relative to other type V CRISPR-Cas enzymes, frequently occurring in CRISPR-Cas operons lacking any other *cas* genes, except for *cas1*, which encodes the CRISPR integrase (Yosef et al., 2012; Nuñez et al., 2015; Wright et al., 2019) (Figures 1A, S1A, and S1B).

Although initial results demonstrated indirectly that Cas12c and Cas12d are capable of RNA-guided DNA interference (Burstein et al., 2017; Yan et al., 2019), no direct RNA-programmed DNA targeting activity has been detected for Cas12c/d proteins. We hypothesized that these proteins require a short sequence in DNA known as the protospacer-adjacent motif (PAM) for the recognition of RNA-guided dsDNA. To test this possibility, we transformed *Escherichia coli* expressing a minimal Cas12d locus with a dsDNA plasmid containing a randomized PAM region next to a sequence matching the target-encoding sequence (spacer) in the Cas12d CRISPR array. Depletion analysis of plasmids in resulting *E. coli* transformants revealed that Cas12d requires a T-enriched PAM sequence for DNA cleavage, similar to the PAM preference detected for other type V family CRISPR-Cas enzymes (Figures 1D and 1E). The Cas12d PAM is a minimal TR (R = A/G) sequence (Burstein et al., 2017; Chen et al., 2019), in contrast to the 4-nt PAM required for most Cas12a orthologs (Zetsche et al., 2015). This TR PAM allows for a 10-fold increase, relative to Cas12a proteins, in the number of targetable sites in recently published CPR bacteriophage genomes (Figure 1F).

Cas12d Requires scoutRNA, a Noncoding Transcript Necessary for DNA Interference

Efforts to detect RNA-guided Cas12c/d-catalyzed DNA cleavage directly, or to reconstitute this activity biochemically, has proved elusive, raising the possibility of a missing component that is necessary for enzymatic activity. Inspection of multiple *cas12d*-containing genomic loci revealed the presence of a noncoding region between the CRISPR array and *cas12d* (Figure 2A). To test the requirement for this noncoding sequence for Cas12d function, we conducted plasmid transformation experiments in *E. coli* in which the CRISPR-Cas12d locus was expressed with a plasmid-complementary crRNA and with or without the noncoding sequence in the locus (Figure 2A). The results showed that plasmid transformation could only be prevented by crRNA-guided Cas12d targeting when the full-length noncoding sequence and a repeat sequence upstream of the spacer was present in the CRISPR-Cas12d locus (Figure 2A).

Examination of Cas12c- and Cas12d-containing CRISPR-Cas genomic loci identified potential homologs of this noncoding sequence that in many cases includes a short conserved

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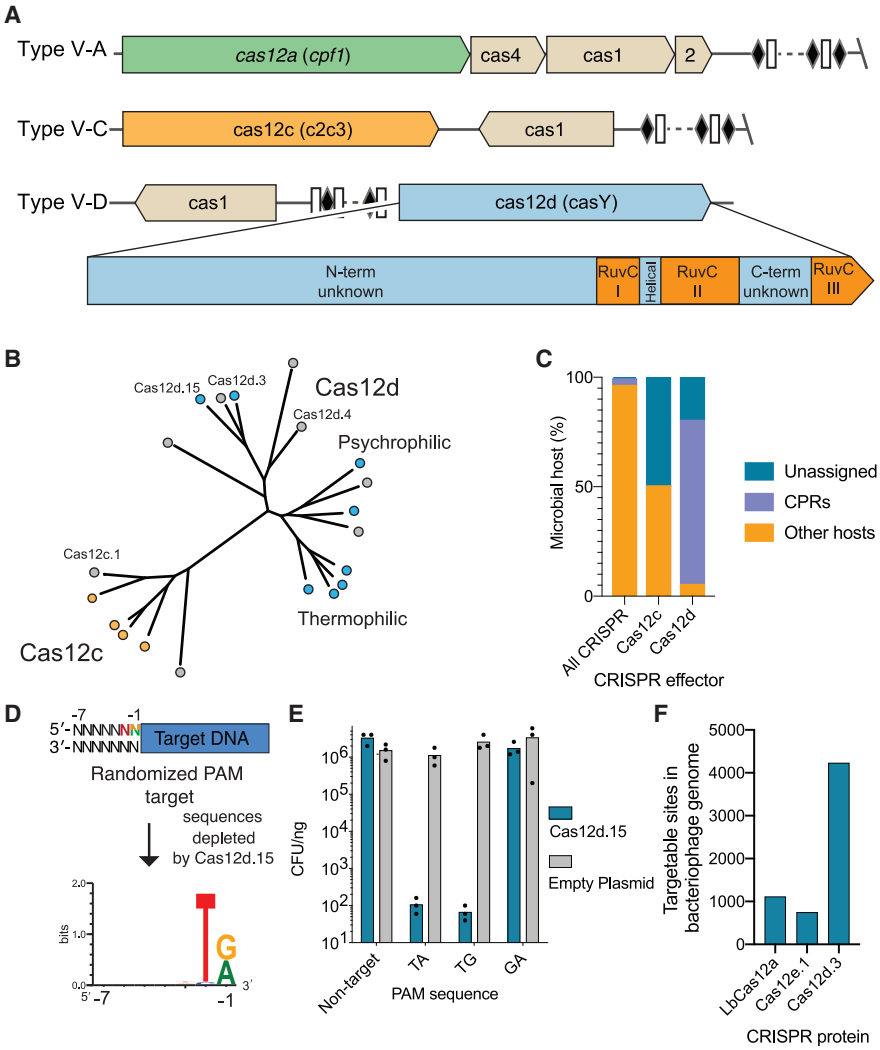


Figure 1. Cas12c/d Are Part of Compact CRISPR Systems Found in Tiny Genomes

(A) Diagram of type V-C and type V-D CRISPR-Cas loci. Cas12c (C2c3) and Cas12d (CasY) share minimal sequence similarity with Cas12a (Cpf1), except for the RuvC catalytic domain.

(B) Unrooted phylogentic tree showing Cas12c and Cas12d representatives. Newly identified orthologs are highlighted with colored circles (orange, Cas12c; blue, Cas12d), and grayed-out circles mark previously described orthologs. Orthologs used for experiments in this study are identified by name.

(C) Host assignment for all CRISPR systems, Cas12c, and Cas12d, illustrating that Cas12d is highly enriched in candidate phyla radiation (CPR) bacteria.

(D) A plasmid depletion screen for PAM-dependent inhibition of plasmid transformation showing that only target sequences adjacent to a TR sequence were efficiently depleted.

(E) Plasmid interference against individual PAM targets showing clearance of plasmids containing a TA or TG adjacent to the targeted sequence.

(F) Predicted number of sites in a CPR-associated bacteriophage genome that are targetable by Cas12a, Cas12e, and Cas12d.

pyrimidine-rich sequence with base-pairing complementarity to a short purine-rich sequence in the corresponding CRISPR array repeat (Figures S2A–S2C). Northern blotting of RNA extracted from a Cas12c protein expressed in *E. coli* from its cloned native locus demonstrated the presence of the corresponding transcript of similar size to the *in vitro*-transcribed transcript (Figure 2B). Notably, this RNA was not detected when the corresponding genomic region was deleted from the expression plasmid or when an oligonucleotide probe with a sequence complementary to the opposite genomic strand was used. These results suggest conservation of this noncoding RNA between the Cas12c and Cas12d subtypes.

To examine the *in vivo* expression of the CRISPR-Cas12d locus, we sequenced the RNA isolated from affinity-purified Cas12d protein expressed in *E. coli* harboring a CRISPR-Cas12d locus-containing plasmid. In addition to transcripts corresponding to the CRISPR array, as expected, we found an abundant small RNA species produced from the noncoding sequence between the CRISPR array and the *cas12d* gene (Figure 2C). This 50- to 100-nt RNA is transcribed in the same direction as the

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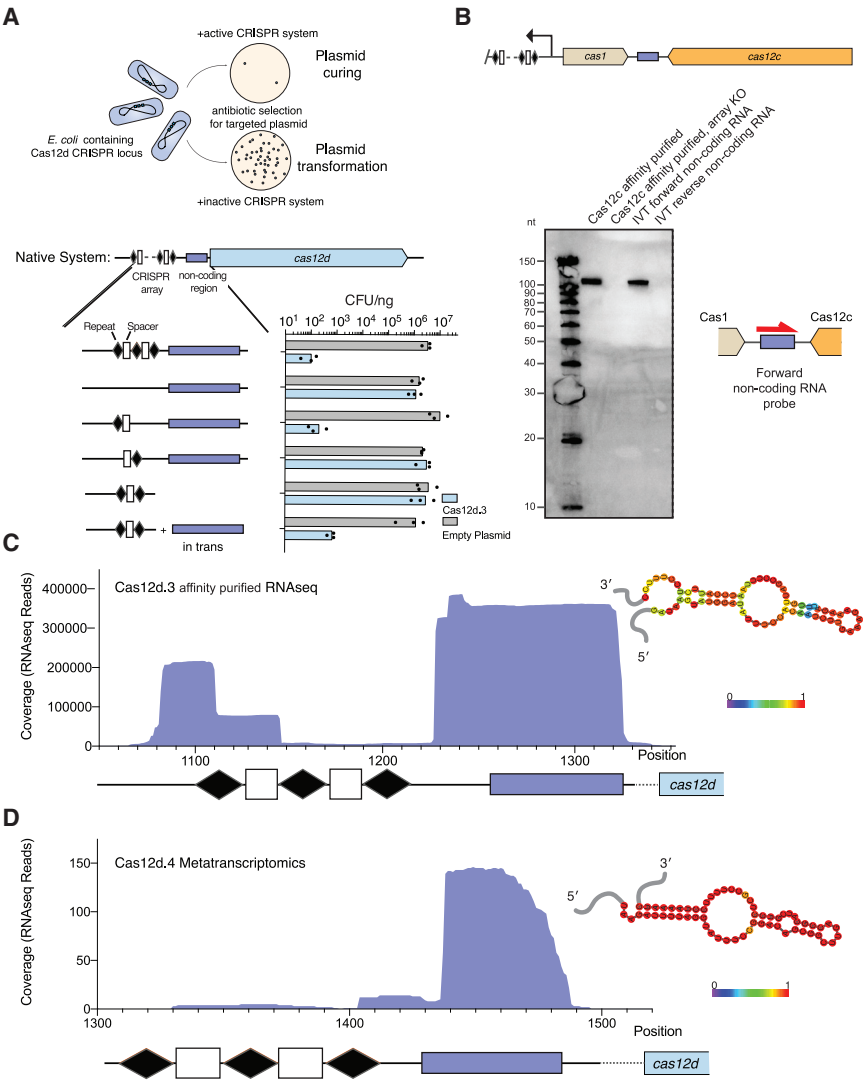


Figure 2. Cas 12c/d Requires a New Kind of tracrRNA for DNA Interference

(A) Plasmid transformation assay testing RNA-guided DNA targeting by CRISPR-Cas systems expressed in *E. coli*. Deletions were made of noncoding regions of the CRISPR locus, and resulting plasmid transformation efficiencies are shown.

(B) Diagram of CRISPR-Cas12c genomic loci indicating a noncoding sequence between the *cas1* and *cas12c* genes; northern blot using a radiolabeled DNA oligonucleotide probe (represented by red arrow) and affinity-purified samples of Cas12c when co-expressed with noncoding regions of the CRISPR locus, (IVT, *in vitro* transcribed; KO, knockout).

(C and D) RNA-sequencing data corresponding to the CRISPR-Cas noncoding locus, from samples that were affinity purified from *E. coli* expression (C) or obtained from metatranscriptomic analysis (D). The black diamonds in the CRISPR loci illustrations represent repeats and the white rectangles represent spacers. Purple rectangles correspond to the noncoding region, and the predicted secondary structure of this region is shown at right. Color scale represents base-pair probabilities.

CRISPR array. Unlike tracrRNA, originally identified in type II CRISPR-Cas systems and required for pre-crRNA maturation (Deltcheva et al., 2011) and CRISPR-Cas9 cleavage activity (Jinek et al., 2012), this transcript bears only very short sequence complementary to the repeat region of Cas12d crRNAs (Figure S2C). Furthermore, its predicted secondary structure differs from tracrRNA and contains a short unpaired RNA segment that exposes the limited region of crRNA complementarity (Figure 2C).

We next examined environmental metatranscriptomic data (Brown et al., 2015) to determine whether this RNA is also produced in native uncultured hosts of Cas12d. We found limited RNA reads mapping to the CRISPR array, likely due to array diversity not represented in the reference genome. However, a transcript analogous to the scoutRNA identified in *E. coli,* with similar secondary structure and limited complementarity to the CRISPR array repeat sequence, was observed (Figure 2D). We noted that scoutRNA transcript boundaries detected in metatranscriptomic data were variable, perhaps reflecting differential RNA processing at transcript ends and in cells within a large population. As observed for tracrRNAs, variability between *in silico* prediction and mature, processed transcripts could affect the ability to predict scoutRNA sequences in other systems.

Reconstitution of a Cas12d-scoutRNA-crRNA DNA-Targeting Complex

We next tested whether purified Cas12d is capable of crRNA-guided DNA cleavage in the presence of scoutRNA. We incubated purified Cas12d-crRNA complexes with radiolabeled target oligonucleotides (single-stranded DNA [ssDNA], dsDNA, and ssRNA) bearing 18-nt sequence complementary to the crRNA guide sequence in the absence or presence of scoutRNA and analyzed these substrates for Cas12d-mediated cleavage. Cleavage products for a crRNA-complementary dsDNA were observed only in the presence of scoutRNA (Figures 3A and S3B). However, no cleavage was observed for the Cas12c ortholog tested in this study under the current reaction conditions (Figure S3C). Cleavage site mapping showed that like other type V family CRISPR-Cas enzymes, Cas12d generates a staggered dsDNA cut with an ~9-nt overhang (Figure S3D). These results establish the scoutRNA as a required component of Cas12d-catalyzed RNA-guided dsDNA cleavage.

Type V CRISPR-Cas systems have been shown to target ssDNA, dsDNA, and ssRNA (Zetsche et al., 2015; Chen et al., 2018; Yan et al., 2019). Using the functionally reconstituted Cas12d, we investigated the substrate preferences

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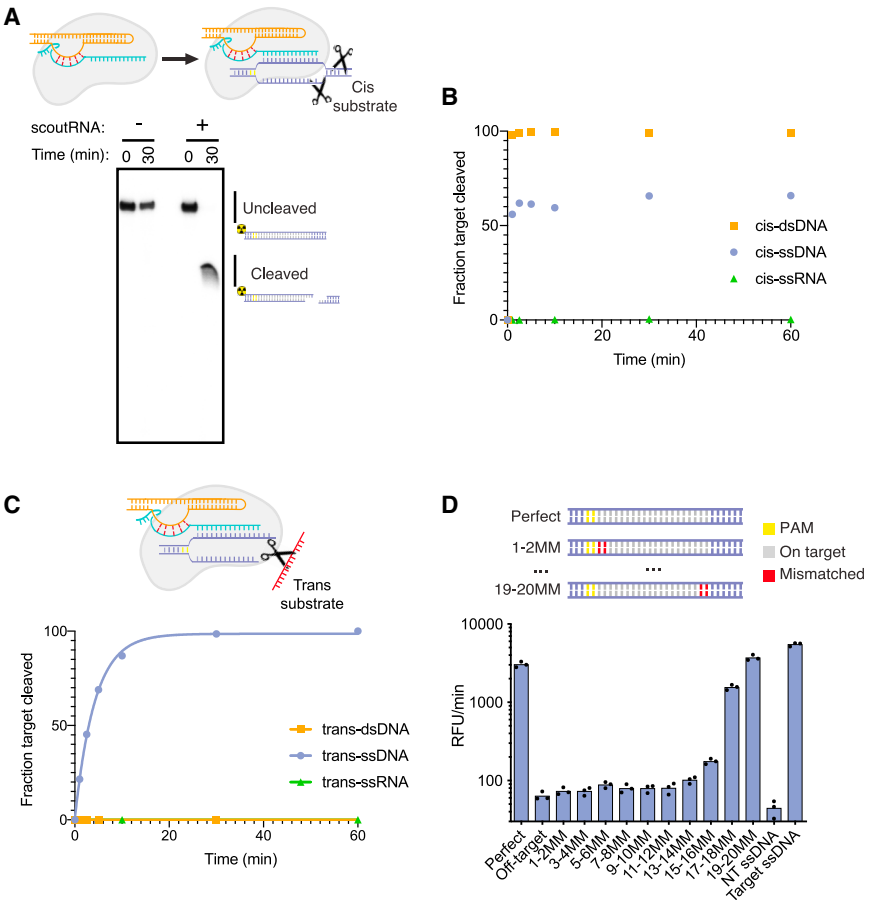


Figure 3. *cis*- and *trans*-Cleavage Activities of Cas12d-Catalyzed and crRNA-Targeted DNA Cleavage

(A) ScoutRNA is essential for Cas12d-mediated dsDNA cleavage. In this assay, the nontarget strand is 5′ end-labeled, and the reactions were conducted in the absence (–) or presence (+) of scoutRNA.

(B) Time course plots of *cis*-cleavage activity of Cas12d.

(C) Time course plots of *trans*-cleavage activity of Cas12d. The substrates of dsDNA, ssDNA, and ssRNA used in this assay are nonspecific to Cas12d crRNA.

(D) Cas12d cleavage activities on mutated dsDNA targets. In this assay, pairs of mismatched base pairs were tiled across the crRNA-target DNA strand duplex, and the resulting extent of crRNA-guided Cas12d-catalyzed dsDNA cleavage is shown.

of this complex (Figures 3B and S3E). We observed rapid and precise cleavage of both ssDNA and dsDNA substrates with base-pairing complementarity to the Cas12d guide RNA sequence. In contrast, no detectable cleavage was observed for RNA. Following the recognition of on-target substrates, many type V proteins are activated as nonspecific ssDNA endonucleases (Chen et al., 2018). We tested whether this activity was also a property of Cas12d by providing a dsDNA activator molecule matching the guide RNA sequence (Figures 3C and S3F). Incubating this activated complex with nonspecific ssDNA, dsDNA, and ssRNA revealed that Cas12d displays robust *trans* ssDNA cutting activity; no such nonspecific activity was detected for dsDNA or ssRNA substrates. We used this *trans* cleavage activity to further investigate the fidelity of Cas12d for its on-target dsDNA substrate, using *trans* cleavage as a proxy for on-target DNA binding and cleavage. By tiling mismatches across the target DNA, we observed a PAM-proximal seed region similar to other class 2 CRISPR effectors (Figure 3D). Notably, the protein was sensitive to mismatches across the majority of the guide sequence, in contrast to Cas12a, which shows a more focused seed region. Further analysis will be needed to compare more directly the fidelity of these systems. These results establish Cas12d as a dual-guided, programmable DNA-targeting nuclease.

Mechanism of crRNA Recognition

Inspection of multiple different scoutRNA sequences identified a 5-nt sequence (5′-GCCUU-3′) that is conserved in Cas12d-associated scoutRNAs (Figure S2B) and predicted to occur within a secondary structural unpaired region (Figures 4A and S2A). This sequence is complementary to a 5-nt sequence found in the CRISPR array repeat sequence, and thus present in every crRNA transcript generated from the Cas12d arrays, suggesting a possible base-pairing interaction between scoutRNA and crRNA. Nitrocellulose filter-binding experiments showed that the co-existence of crRNA and scoutRNA bound with higher affinity to purified Cas12d protein than either RNA alone (Figures 4B and S4B). We next tested mutated versions of scoutRNA bearing altered sequences in the conserved segment, and tested these in Cas12d-catalyzed dsDNA cleavage assays (Figures 4C, 4D, and S4A). We also tested crRNA bearing compensatory mutations designed to restore base pairing with the altered scoutRNAs (Figure 4C). DNA cleavage results showed that scoutRNA mutations disrupted Cas12d-catalyzed DNA cleavage, and this disruption was not restored by creating compensatory mutations in the crRNA. These findings differ from those observed in analogous experiments with *Streptococcus pyogenes* Cas9, in which tracrRNA sequence mutations had no effect on DNA cleavage efficiency when the compensatory mutation was made in the repeat (Figure 4C).

These results suggest that unlike tracrRNA, which forms an extensive base-pairing interaction with crRNA in type II CRISPR-Cas systems (Deltcheva et al., 2011; Chylinski et al., 2013), scoutRNA assembly with Cas12d and crRNA may involve only short sequence-specific recognition of the conserved 5-nt scoutRNA sequence. Our data neither confirm nor refute the hypothesis that scoutRNA forms a base-paired interaction with crRNA, since compensatory mutations that maintain this

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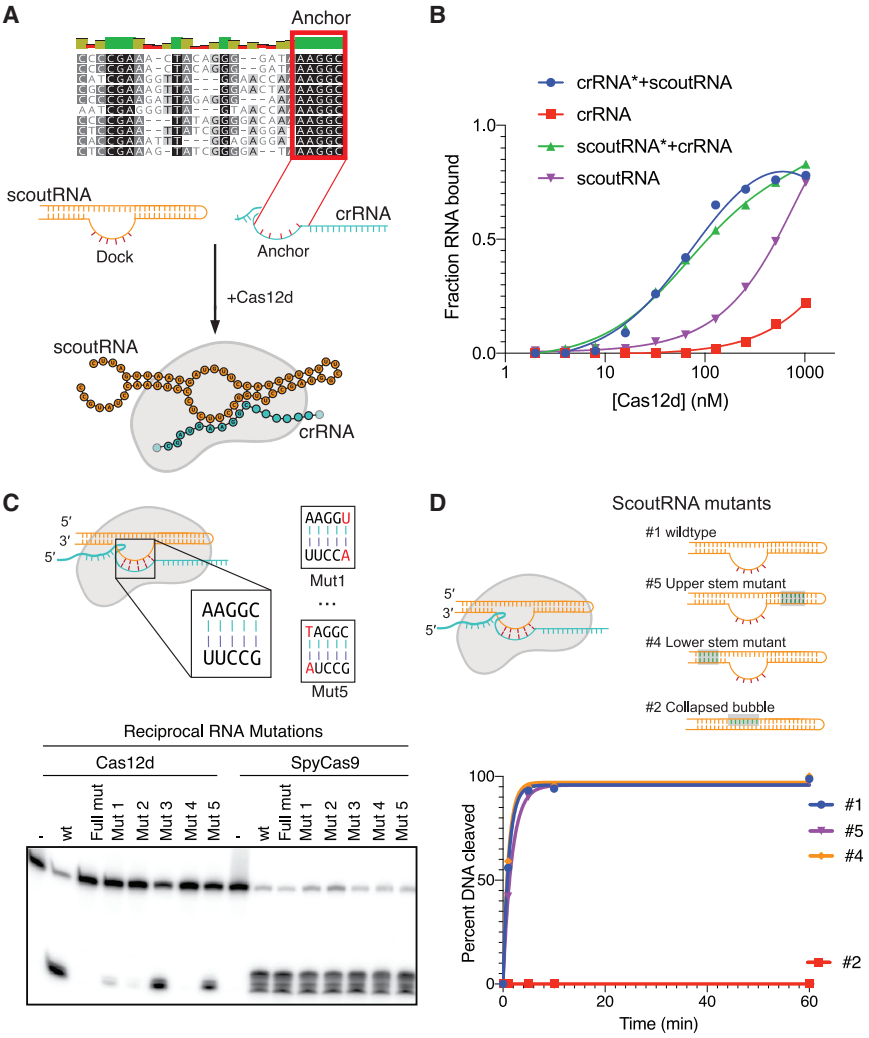


Figure 4. A Short Conserved Sequence in scoutRNA Is Required for dsDNA Targeting

(A) Cas12d-associated crRNA repeat sequence alignment. Conserved sequences are shown in black; predicted scoutRNA secondary structure and possible short base-paired interaction between scoutRNA and crRNA repeat are also shown.

(B) Cas12d strongly binds to the complex from scoutRNA and crRNA. Data are from nitrocellulose filter binding assays with radiolabeled crRNA and/or scoutRNA as a function of Cas12d protein concentration; asterisk indicates radiolabeled species when two RNAs were present in the binding reaction.

(C) The effect of reciprocal changes in the guide RNA stem on Cas12d-mediated dsDNA cleavage. mut, mutation; wt, wild type.

(D) Importance of five conserved nucleotides in Cas12d scoutRNA. Mutants #4 and #5 contains sequence changes that maintained base-pairing complementarity in the regions shown; mutant #2 contains nucleotide changes to create a complementary sequence on the strand opposite the conserved 5-nt sequence.

base-pairing potential but alter the RNA sequence were defective or inactive for RNA-guided Cas12d activity (Figure 4C). To test this further, we created a mutant scoutRNA that collapsed the predicted unpaired region containing the conserved 5-nt sequence without altering the conserved sequence itself. No Cas12d-catalyzed RNA-guided dsDNA cleavage was detected in the presence of this modified scoutRNA (Figures 4D and S4A). In contrast, mutations that maintain base pairing in the flanking regions of scoutRNA had no impact on cleavage rate (Figure 4D). These results support an essential role for the conserved 5-nt sequence in scoutRNA and suggest, but do not confirm, its formation of a base-pairing interaction with a short complementary region of the crRNA.

A Dual-RNA-Guided Pre-crRNA Autoprocessing Mechanism

In bacteria, CRISPR transcripts are often generated as precursors that must be cleaved to produce the mature crRNAs that guide DNA recognition. Type II CRISPR systems comprising Cas9 use tracrRNA to create an extensive double-stranded structure with pre-crRNA for recognition and processing by ribonuclease III (Chylinski et al., 2013; Deltcheva et al., 2011). In contrast, the Cas12a subfamily of type V CRISPR systems possesses internal ribonucleolytic activity for auto-cleavage of crRNA precursors (Fonfara et al., 2016). We wondered how crRNAs are produced in Cas12c/d systems, given that limited base-pairing complementarity between scoutRNA and crRNA may preclude association in the absence of a Cas12c/d protein. In addition, the genomes from which these systems are derived do not always harbor genes encoding ribonuclease III, implying that another mechanism for crRNA production may be involved.

To test the possibility that Cas12c itself catalyzes pre-crRNA maturation, we generated a set of substrates designed to detect Cas12c-mediated pre-crRNA processing. Initial experiments in which cleavage was expected at a position in the repeat upstream of the spacer, analogous to the processing site in pre-crRNAs of Cas12a-type systems, resulted in no detectable cleavage product. However, we were surprised to observe robust scoutRNA-dependent processing of a pre-crRNA substrate that enabled the detection of cutting at a position on the opposite end of the pre-crRNA (Figure 5A), suggesting a cleavage mechanism that is distinct from that observed for other CRISPR-Cas enzymes known to process their own pre-crRNAs, including Cas12a or Cas13a (East-Seletsky et al., 2016; Fonfara et al., 2016).

Next, we mutated the regions upstream or downstream of the processed crRNA spacer sequence to determine the mechanism of substrate recognition. Mutation of the upstream repeat sequence resulted in complete ablation of the RNA processing

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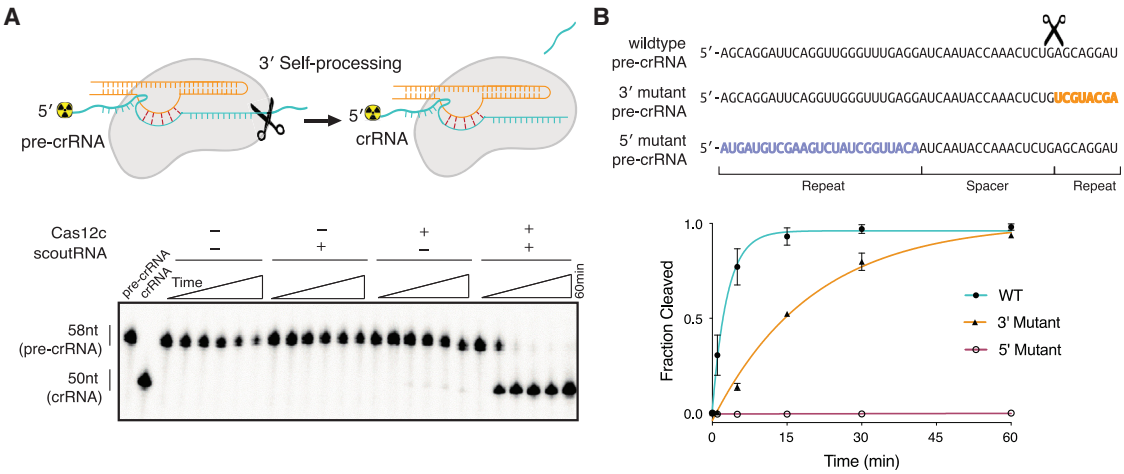


Figure 5. An RNase III-Independent Dual-RNA-Guided Pre-crRNA-Processing Mechanism

(A) Time courses of pre-crRNA cleavage in the presence or absence of purified Cas12c and scoutRNA, using a 5′ end-radiolabeled 58-nt pre-crRNA.

(B) Kinetics of scoutRNA-dependent Cas12c-catalyzed pre-crRNA cleavage using the pre-crRNA substrates shown.

activity on the downstream spacer, likely due to the lack of binding to the scoutRNA. By comparison, mutation of the predicted cleavage site still supported pre-crRNA processing (Figure 5B). These results suggest that the spacer is measured by a ruler mechanism whereby Cas12c recognizes the sequence of the upstream repeat and cleaves downstream of the recognition site 18 nt away. This mechanism is distinct from Cas12a and Cas13a enzymes, which catalyze pre-crRNA cleavage at the recognized CRISPR repeat sequence. Mutations of the scoutRNA to alter the predicted secondary structure at or near the short conserved sequence had variable effects on the rate of pre-crRNA processing (Figure S5A), and we did not observe conclusive pre-crRNA processing by Cas12d in the same reaction conditions (Figure S5B). The disproportionate impact of scoutRNA mutations on Cas12d-mediated DNA cleavage compared to Cas12c-mediated pre-crRNA processing could reflect differences in enzyme catalytic activities, CRISPR-Cas system functionality, or both.

These results reveal a new mechanism of crRNA maturation that requires both the scoutRNA and Cas12c, but not an external ribonuclease. Based on scoutRNA conservation, it is likely that this mechanism extends to the Cas12c/d family of enzymes and that scoutRNA-dependent pre-crRNA processing is an inherent activity of these proteins that may enable their propagation in organisms lacking ribonuclease III and related activities.

DISCUSSION

CRISPR-Cas systems have evolved in diverse microbial populations to provide adaptive protection from bacteriophage infection and plasmid transformation. These systems have been shown to use two kinds of noncoding RNA molecules, crRNA and tracrRNA. Whereas crRNA is used universally to identify foreign nucleic acids by base pairing, tracrRNA has been found only in type II and the Cas12b (C2c1) and Cas12e (CasX) type V CRISPR systems, where it functions both during pre-crRNA maturation and Cas9/Cas12b/CasX targeting complex assembly. We show in this study that Cas12c/d type V CRISPR-Cas systems encode and use a distinct type of noncoding RNA, scoutRNA, which is required for pre-crRNA maturation, as shown for Cas12c, and for DNA targeting, as shown for Cas12d. For the CRISPR-Cas12c/d genomic loci examined in this study, none were found to encode a tracrRNA and all encoded a scoutRNA, according to the criteria described here. Unlike tracrRNAs, scoutRNA sequences have minimal base-pairing complementarity to the corresponding crRNA repeat sequence, and our data do not confirm the existence of base pairing between scoutRNA and crRNA. The definition of the scoutRNA as distinct from tracrRNA also sets the stage for defining and naming CRISPR-Cas components according to their function rather than according to their order of discovery or proposed phylogenetic relationships.

In addition to a predicted secondary structure that precludes an extensive pre-crRNA base-pairing interaction, the scoutRNA supports a mechanism of pre-crRNA processing that is distinct from those of either tracrRNA-dependent or -independent processing systems. Instead of substrate recognition and cleavage occurring together in the tracrRNA-pre-crRNA duplex or pre-crRNA alone, scoutRNA supports Cas12c-catalyzed maturation by a mechanism in which substrate recognition and cleavage occur on separate segments of the pre-crRNA. This is notably inconsistent with ribonuclease III-catalyzed RNA processing, which involves dsRNA recognition and cutting that generates 2-nt 3′ overhangs in the cleavage product (Court et al., 2013; Nicholson, 2014). This difference in pre-crRNA processing mechanisms supports the conclusion that scoutRNA is functionally distinct from tracrRNAs as originally defined (Deltcheva et al., 2011; Chylinski et al., 2013).

Until now, CRISPR-Cas systems have been categorized according to their protein components, and phylogenetic relationships are derived from protein homologies. The existence of scoutRNA suggests a new possibility for categorization based

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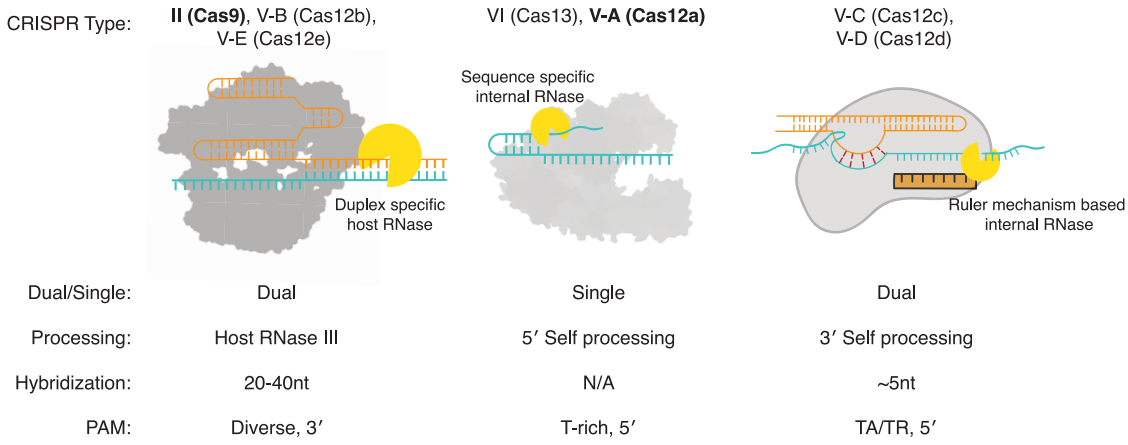


Figure 6. Three Different Types of RNA-Guided CRISPR-Cas Families Defined by RNA Components

Noncoding RNAs enable the functional classification of CRISPR-Cas enzymes into 3 distinct categories. All use crRNA, whereas a subset uses either a canonical tracrRNA and another subset uses a scoutRNA.

on noncoding RNA composition. Three RNA-based classes of CRISPR-Cas systems include those using crRNA and tracrRNA, those using crRNA alone, and those using crRNA and scoutRNA (Figure 6). The role of a conserved 5-nt crRNA-complementary segment in some scoutRNAs suggests a possible direct base-pairing interaction with crRNA that would presumably occur only within the context of the Cas12c/d protein. The possible short segment of scoutRNA-crRNA base pairing is reminiscent of the short RNA-RNA base pairing that occurs between small nuclear RNAs (snRNAs), forming the interactions required for association with proteins to form small nuclear ribonucleoprotein particles (snRNPs). It remains to be determined how scoutRNA creates a stable interaction with crRNA and whether, like tracrRNA, it creates a structural scaffold for Cas protein assembly and conformational dynamics.

The unique properties of scoutRNA, including variable length and sequence diversity, offer possibilities for engineering that include the creation of shorter forms that retain function and possibly fusions with crRNA to form an sgRNA-type construct. These possibilities, combined with the minimal PAM required for DNA target recognition, could enhance Cas12c/d functionality for genome editing by providing ways to induce cellular delivery or append RNA-encoded capabilities. The continued exploration of scoutRNA diversity should reveal whether its detection can signal the presence of new CRISPR-Cas systems or protein variants that have yet to be identified.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.molcel.2020.06.022.

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AUTHOR CONTRIBUTIONS

L.B.H., J.S.C., I.P.W., and E.M. developed the project idea, conducted experiments, and prepared the manuscript and figures. D.B., D.P.-E., D.G., J.F.B., and N.C.K. conducted and designed the computational biology work and

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reviewed the manuscript. J.A.D. provided financial support, assisted in experimental design, and wrote the manuscript.

DECLARATION OF INTERESTS

J.A.D. is a cofounder of Caribou Biosciences, Editas Medicine, Scribe Therapeutics, and Mammoth Biosciences. J.A.D. is a scientific advisory board member of Caribou Biosciences, Intellia Therapeutics, eFFECTOR Therapeutics, Scribe Therapeutics, Mammoth Biosciences, Synthego, Algen Biotechnologies, Felix Biosciences, and Inari. J.A.D. is a Director at Johnson & Johnson and has research projects sponsored by Biogen, Pfizer, AppleTree Partners, and Roche. The Regents of the University of California have filed patents related to this work on which D.B., J.F.B., L.B.H., D.P.-E., J.S.C., and J.A.D. are inventors. L.B.H., J.S.C., and J.A.D. are co-founders of Mammoth Biosciences. I.P.W. served as a consultant to Mammoth Biosciences. J.F.B. is a co-founder of Metagenomi.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and Virus Strains		
E. coli BL21(DE3)	Novagen	70235
Chemicals, Peptides, and Recombinant Proteins		
Gamma-^{32}P ATP	PerkinElmer	Blu002Z001MC
ATP	Sigma-Aldrich	A1852
CTP	Sigma-Aldrich	C1506
GTP	Sigma-Aldrich	G8877
TTP	Sigma-Aldrich	T0251
Cas12c Protein	This paper	N/A
Cas12d (CasY)Protein	This paper	N/A
SpyCas9 Protein	This paper	N/A
TEV Protease	This paper	N/A
Protease inhibitor cocktail	MilliporeSigma	4693159001
TCEP	Sigma-Aldrich	75259
Critical Commercial Assays		
T4 Polynucleotide kinase	Thermo Scientific	#EK0032
NEBnext small RNA sequencing Illumina library kit	New England BioLabs	E7330S
Oligonucleotides		
Target (T) DNA Oligo used in cleavage assay GACTGGAAAGTTTAC GCCGCCAGAGTTTGGTATTGATTAGTCTGCGGGCAGGC	This Paper	N/A
Non-target (NT) DNA Oligo used in cleavage assay GCCTGCCCGC AGACTAATCAATACCAAACTCTGGCGGCGTAAACTTTCCAGTC	This paper	N/A
DNA Oligo used in trans-cleavage assays GACGACAAAACTTTAGA TCGTTACGCTAACTATGAGGGCTGTCTGTGGAATGCTA	This paper	N/A
cRNA-RNA_382 ACCCGUAAAGCAGAGCGAUGAAGGCAUCAAUA CCAAACUCUGG	This paper	N/A
cRNA-RNA_386 GCGAUGAAGGCAUCAAUACCAAACUCUGG	This paper	N/A
cRNA-RNA_387 GCGAUGAAGGCAUCAAUACCAAACUCUG	This paper	N/A
cRNA-RNA_391 CGAUGGGCGUAUCAAUACCAAACUCUGG	This paper	N/A
scoutRNA-RNA_396 CUUAGUUAAGGAUGUUCCAGGUUCUUUCG GGAGCCUUGGCCUUCUCCCUUAACCUAUGCCACUAAUGAUU	This paper	N/A
RNA used for Cas12c (C2c3) RNA processing_C2C3_1 Scout (143.1) GGAUACCACCCGUGCAUUUCUGGAUCAAUGAUCCGUACCUCAA UGUCCGGGCGCGCAGCUAGAGCGACCUGAAAUCU	This paper	N/A
RNA used for Cas12c (C2c3) RNA processing_C2c3 RSRS (147) GGAGCAGGAUUCAGGUUGGGUUUGAGGAUCAAUACCAAACU CUGAGCAGGAUUCAGGUUGGGUUUGAGGGAGACCACGCAG GUCUC	This paper	N/A
Recombinant DNA		
Modified pET vector (2CT10)	UC Berkeley MacroLab	His10-MBP-N10-tev-yORF
Software and Algorithms		
Prism 7	Graphpad Software, Inc.	https://www.graphpad.com
PAM depletion and plasmid interference assays	Burstein et al., 2017	https://www.ncbi.nlm.nih.gov
MAFFT_a multiple sequence alignment program	This paper	https://mafft.cbrc.jp/alignment/software/

(*Continued on next page*)

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Raxml_ a phylogenetic tree construction program	This paper	http://evomics.org/learning/phylogenetics/raxml/
FigTree- a program to graphically view phylogenetic trees	This paper	http://evomics.org/resources/software/molecular-evolution-software/figtree/
Bowtie2	Langmead and Salzberg, 2012	http://bowtie-bio.sourceforge.net/bowtie2/index.shtml
Cutadapt	Martin, 2018	https://cutadapt.readthedocs.io/en/stable/
Other		
Sequence data, analyses, and resources related to the deep sequencing of small RNA libraries.	This paper	https://genomics.qb3.berkeley.edu/

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jennifer A. Doudna (doudna@berkeley.edu).

Materials Availability

Materials generated in this study are available from Addgene.org or upon request from doudna@berkeley.edu.

Data and Code Availability

No data or code was generated in this study for deposition in public databanks.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Bacterial strain used in this study to expression the CRISPR Proteins is *E. coli* BL21(DE3). The CRISPR-protein expression plasmids were first transformed into the *E. coli* BL21(DE3). The bacteria were then overnight cultured on agar plates containing Ampicillin and Chloramphenicol at 37°C . To make starters for protein expression, single colony was picked up and incubated in 50 mL of Terrific Broth (TB) containing the antibiotics overnight. For expression the CRISPR proteins, 10 mL of starter bacteria were transferred into 1 l TB-containing flask and incubated at 37°C until the OD reaches 0.7. The bacteria were then induced with 0.6 mM of IPTG and incubated at 16°C for 14 hours before harvesting for protein purification.

METHOD DETAILS

Phylogenetic Analysis

Amino acid sequences of proteins previously identified and new orthologs described in this manuscript were aligned using MAFFT and phylogenetic trees were constructed using RAxML. Trees were visualized using FigTree 1.4.4.

PAM depletion and plasmid interference

PAM depletion and plasmid interference assays were conducted as previously described (Burstein et al., 2017). Expression plasmids containing the native contig and non-coding sections (https://benchling.com/s/seq-c4cx5V2kzCCOLGsLplyY/edit) were transformed into BL21(DE3) *E. coli.* After selection, these cells were grown to OD600 = 0.4 before pelleting and washing 3 times with ice cold 10% glycerol. The resulting cells were transformed 200ng of a plasmid library containing a randomized 7-nt section upstream of the region matching the spacer. After transformation the resulting cells were plated on selective medium containing carbenicillin and chloramphenicol for ~36hrs at room temperature. For plasmid interference assays the same procedure was followed but clonal plasmids were used in place of the randomized libraries. Serial dilutions of the electroporated cells were serially diluted and CFUs were counted.

Northern blotting

Both RNAs extracted from an affinity-purified Cas12c protein expressed in *E. coli* from its cloned native locus and transcribed *in vitro* were separated on 10% UREA-PAGE at 1 W in 0.5X TBE after denatured in denature buffer of 95% of formamide, 0.001% bromophenol blue and 0.001% of xylene cyanol. The separated RNAs were blotted onto nylon membrane via semi-dry electroblotting in 0.5X TBE at 20 V for 2 hours. The RNA blot was cross-linked in UV-cross linker and then pre-incubated for 3 hours at 45°C in

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hybridization buffer (40% formamide, 5X SSC, 3X Denhardt's, 200 ug/ml of salmon sperm DNA, and 0.1% SDS). The pre-incubated RNA blot was further incubated at 45°C overnight with 5′ end labeled DNA oligo in hybridization buffer. The blot was then washed once with 4X SCC, followed by 3 times with 0.1X SSC. The hybridization signals were detected and analyzed with Amersham Typhoon and ImageQuant (GE Healthcare).

Small RNA sequencing

RNaseq was conducted as previously described with modification (Minnier et al., 2018). Cells transformed with the native expression plasmid were grown in SOB to saturation overnight at 30°C. The resulting bacterial cell pellet was lysed by treatment with Lysozyme, SDS and hot phenol extraction. To prepare the RNA for sequencing it was treated with trubo DNase, rSAP and T4 PNK before inputting into the NEBnext small RNA sequencing illumine library kit. Resulting reads were trimmed with Cutadapt (Martin, 2018) and mapped using Bowtie 2 (Langmead and Salzberg, 2012).

Protein expression and purification

Cas12d (CasY) and Cas12c proteins were expressed in a modified pET vector containing an N-terminal 10 × His-tag, maltose-binding protein (MBP) and TEV protease cleavage site. Proteins were purified as described elsewhere **(**Chen et al., 2018**)**, with the following modifications: *E. coli* BL21(DE3) containing Cas12d expression plasmids were grown in Terrific Broth at 16°C for 14 hr. Cells were harvested and re-suspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 5% (v/v) glycerol, 1 mM TCEP, 1 tablet of protease inhibitor/50 mL (Sigma-Aldrich)), disrupted by sonication, and purified using Ni-NTA resin. After overnight TEV cleavage at 4°C, proteins were purified over an OrthoTrap HP column, the elutes were further purified through a HiTrap Heparin HP column for cation exchange chromatography. The final gel filtration step (Superdex 200) was carried out in elution buffer containing 20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5% (v/v) glycerol and 1 mM TCEP. Purified Cas12d is shown in Figure S3A.

Nucleic acid preparation

DNA oligos were synthesized commercially (IDT, Integrated DNA Technologies, Inc., San Diego, CA USA), and PAGE-purified in-house before being radiolabeled for cleavage assays.

For generation of scout RNAs, the commercially synthesized T7-promoter-tagged DNA oligos served as templates for *in vitro* transcription reactions, which were performed as described elsewhere (Chen et al., 2018). crRNAs were commercially synthesized by IDT and PAGE-purified in-house. All DNA and RNA substrates are listed below.

DNA cleavage assays

Generally, Cas12d-mediated cleavage assays were carried out in cleavage buffer consisting of 20 mM Tris (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, 1% glycerol and 0.5 mM DTT. For radiolabeled cleavage assays, the substrates of either target strand or non-target strand were 5′ end-labeled with T4 PNK (NEB, New England Biolabs) in the presence of gamma ^{32}P-ATP in 30 μl reactions. To form dsDNA substrates, the labeled substrate was annealed with excess cold target or non-target strand according to the labeled strand. In a typical Cas12d cleavage reaction, the concentrations of Cas12d, guide RNA and ^{32}P-labeled substrates were 100 nM, 120 nM and 2-4 nM, respectively. To carry out the assay, Cas12d was first incubated with its guide RNA(s) at room temperature for 15 min before addition of the labeled substrates at 37°C. Reactions were incubated for certain periods (min) of time as indicated and quenched with formamide-containing loading buffer (final concentration 45% formamide and 15 mM EDTA, with trace amount of xylene cyanol and bromophenol blue) for 3 min at 90°C. The reaction products were resolved by 12% urea-denaturing PAGE gel and quantified with Amersham Typhoon (GE Healthcare). The fraction of DNA cleaved at each time point was plotted as a function of time, and these data were fit with a single exponential decay curve using Prism 6 (GraphPad Software, Inc.), according to the equation: Fraction cleaved = $A \times (1 - \exp(-k \times t))$, where A is the amplitude of the curve, k is the first-order rate constant and t is time. All experiments were carried out at least in triplicate, with representative replicates shown in the figure panels.

For *trans*-cleavage assays, the Cas12d was first incubated with guide RNA(s) at room temperature for 15 min, then further incubated for another 15 min with activator at room temperature before addition of labeled substrates that are unrelated to guide RNA(s). The cleaved products were separated and quantified similarly as stated above.

Filter Binding Assays

Filter binding reaction was carried out in 30 ul reaction in filter-binding buffer (20 mM Tris [pH 7.5], 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 5% glycerol, 0.01% Igepal CA-630, 10 μg/ml yeast tRNA, and 10 μg/ml BSA). 1.2 × concentration of Cas12d protein to unlabeled RNA was incubated with radiolabeled RNA (< 0.05nM) for 1 hr at room temperature. Tufryn, Protran, and Hybond-N+ were assembled onto a dot-blot apparatus in the order of Tufryn, Protran, and Hybond-N+ (from top to bottom). The membranes were washed twice with 50 μL equilibration buffer (20 mM Tris [pH 7.5], 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 5% glycerol) before the sample was applied to the membranes. Membranes were again washed twice with 50 μL equilibration buffer, air-dried, and visualized by phosphorimaging. Data were quantified with ImageQuant TL Software (GE Healthcare) and fit to a binding isotherm using Prism (GraphPad Software). Dissociation constants (K_D) is reported in the figure legends.

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Cas12c pre-crRNA autoprocessing experiments

Processing reactions (total volume of 100 uL) contained 100 nM Cas12c, 120 nM scoutRNA, 3 nM 5′ radiolabeled pre-crRNA (wild-type, 3′ mutant, or 5′ mutant), and 1X Cleavage Buffer (20 mM Tris-HCl pH 7.5, 150 mM Kcl, 5 mM MgCl2, 1 mM TCEP). Prior to the addition of Cas12c to the reaction, scoutRNA and pre-crRNA were annealed in 1X Cleavage Buffer by incubating at 70°C for 5 min followed by −2°C/min to 25°C. To test which components were essential for autoprocessing, Cas12c and scoutRNA were omitted from the reactions as indicated in Figure 5A. Reactions were incubated at 37°C, and 15 uL of each reaction were quenched with 2x Quench Buffer (90% formamide, 25 mM EDTA, and trace bromophenol blue) at 0, 1, 5, 15, 30, and 60 min. Quenched reactions were heated to 95°C for 2 min and run on a 15% denaturing polyacrylamide gel (7M Urea, 0.5xTBE). Products were visualized by phosphorimaging and band intensities were quantified using ImageQuant software.

DNA and RNA sequences

DNA substrates for cleavage assays:

Non-target (NT) GCCTGCCCGCAGACTAatcaataccaaactctggCGGCGTAAACTTTCCAGTC
Target (T) GACTGGAAAGTTTACGCCGccagagtttggtattgatTAGTCTGCGGGCAGGC
Used in *trans*-cleavage assays:
GACGACAAAACTTTAGATCGTTACGCTAACTATGAGGGCTGTCTGTGGAATGCTA
crRNAs used in this study:
RNA_382 ACCCGUAAAGCAGAGCGAUGAAGGCaUcaaUaccaaacUcUgg
RNA_386 GCGAUGAAGGCaUcaaUaccaaacUcUgg
RNA_387 GCGAUGAAGGCaUcaaUaccaaacUcUg
RNA_391 GCGAUGGGCGUaUcaaUaccaaacUcUgg
sccoutRNA:
RNA_396 CUUAGUUAAGGAUGUUCCAGGUUCUUUCGGGAGCCUUGGCCUUCUCCC
UUAACCUAUGCCACUAAUGAUU
scoutRNAs of wild-type and mutations used in reciprocal mutation studies:
396-w.t. CUUAGUUAAGGAUGUUCCAGGUUCUUUCGGGAGCCUUG**GCCUU**CUCCC UUAACCUAUGCCACUAAUGAUU
396-full mut CUUAGUUAAGGAUGUUCCAGGUUCUUUCGGGAGCCUUG**ACGCC**CUCCC UUAACCUAUGCCACUAAUGAUU
396-mut1 CUUAGUUAAGGAUGUUCCAGGUUCUUUCGGGAGCCUUG**A**CCUUCUCCC
UUAACCUAUGCCACUAAUGAUU
396-mut2 CUUAGUUAAGGAUGUUCCAGGUUCUUUCGGGAGCCUUGG**G**CUUCUCCC
UUAACCUAUGCCACUAAUGAUU
396-mut3 CUUAGUUAAGGAUGUUCCAGGUUCUUUCGGGAGCCUUGGC**G**UUCUCCC
UUAACCUAUGCCACUAAUGAUU
396-mut4 CUUAGUUAAGGAUGUUCCAGGUUCUUUCGGGAGCCUUGGCC**A**UCUCCC
UUAACCUAUGCCACUAAUGAUU
396-mut5 CUUAGUUAAGGAUGUUCCAGGUUCUUUCGGGAGCCUUGGCCU**A**CUCCC
UUAACCUAUGCCACUAAUGAUU
crRNAs of wild-type and mutations used in reciprocal mutation studies:
386-w.t. GCGAUG**AAGGC**aUcaaUaccaaacUcUgg
386-full mut GCGAUG**GGCGU**aUcaaUaccaaacUcUgg
386-mut1 GCGAUGAAGG**U**aUcaaUaccaaacUcUgg
386-mut2 GCGAUGAAG**C**CaUcaaUaccaaacUcUgg
386-mut3 GCGAUGAA**C**GCaUcaaUaccaaacUcUgg
386-mut4 GCGAUGA**U**GGCaUcaaUaccaaacUcUgg
386-mut5 GCGAUG**U**AGGCaUcaaUaccaaacUcUgg
RNA used for Casd12c (C2c3) RNA processing:
C2C3_1 Scout (143.1) ggaUaccacccgUgcaUUUcUggaUcaaUgaUccgUaccUcaaUgUccgggcgcgcagcUagagcgaccUgaaaUcU
C2c3 RSRS (147)
ggagcaggaUUcaggUUgggUUUgaggAUCAAUACCAAACUCUGagcaggaUUcaggUUgggUUUgaggGAGACCacgcaGGUCUC
Casd12d scoutRNAs of wild-type (#1) and mutations:
#1 CUUAGUUAAGGAUGUUCCAGGUUCUUUCGGGAGCCUUGGCCUUCUCCCUUAACCUAUGCC
#2 CUUAGUUAAGGAGAAGGCCAGGUUCUUUCGGGAGCCUUGGCCUUCUCCCUUAACCUAUGCC
#3 CUUAGUUAAGGAUGUUUCCAGGUUCUUUCGGGAGCCUUGGCCUUCUCCCUUAACCUAUGCC
#4 CUUAGUGCUGGAUGUUCCAGGUUCUUUCGGGAGCCUUGGCCUUCUCCCAGCACCUAUGCC
#5 CUUAGUUAAGGAUGUUCCAGGCGAUUUCGGUCGCCUUGGCCUUCUCCCUUAACCUAUGCC
#6 CUUAGUUAAGGAUGUUCCAGGUUCUUUCGGGAGCCUUGGCUUUCUCCCUUAACCUAUGCC

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Casd12c scoutRNAs of wild-type (#1) and mutations:

#1 GGAUACCACCCGUGCAUUUCUGGAUCAAUGAUCCGUACCUCAAUGUCCGGGCGCGCAGCUAGAGCGACCUG
#2 GGAUACCACCCGUGCAUUGAGGUAUGGAUCAAUGAUCCGUACCUCAAUGUCCGGGCGCGCAGCUAGAGCGACCUG
#3 GGAUACCACCCGUGCAUUUUUUUCUGGAUCAAUGAUCCGUACCUCAAUGUCCGGGCGCGCAGCUAGAGCGACCUG
#4 GGAUACCACCCGUGCAUUUCUGGAUCAAUGAUCCGUUCUUCAAUGUCCGGGCGCGCAGCUAGAGCGACCUG
#5 GGAUACCACCCGUGCAUUUCUGACUCAAUGAGUCGUACCUCAAUGUCCGGGCGCGCAGCUAGAGCGACCUG
#6 GGAUACCACCCGUGGGAUUCUGGAUCAAUGAUCCGUACCUCAUCCUCCGGGCGCGCAGCUAGAGCGACCUG
#7 GGAUACCACCCGUGCAUUAAUGGAUCAAUGAUCCGUACCUCAAUGUCCGGG**CGCGCAGCUAGAGCGACCUG**

QUANTIFICATION AND STATISTICAL ANALYSIS

Amino acid sequences of proteins previously identified and new orthologs described in this manuscript were aligned using MAFFT and phylogenetic trees were constructed using RAxML. Trees were visualized using FigTree 1.4.4. Products/images from both DNA/RNA cleavage assays and filter binding assays were visualized by phosphorimaging and band/dots intensities were quantified using ImageQuant software. Graphs from these data were generated from GraphPad Prism.