Identification of protein structural elements responsible for the diversity of sequence preferences among Mini-III RNases

Dawid Głów, Małgorzata Kurkowska, Justyna Czarnecka, Krzysztof Szczepaniak, Dariusz Pianka, Verena Kappert, Janusz M. Bujnicki & Krzysztof J. Skowronek

Many known endoribonucleases select their substrates based on the presence of one or a few specific nucleotides at or near the cleavage site. In some cases, selectivity is also determined by the structural features of the substrate. We recently described the sequence-specific cleavage of double-stranded RNA by Mini-III RNase from *Bacillus subtilis* in vitro. Here, we characterized the sequence specificity of eight other members of the Mini-III RNase family from different bacterial species. High-throughput analysis of the cleavage products of *Φ6* bacteriophage dsRNA indicated subtle differences in sequence preference between these RNases, which were confirmed and characterized by systematic analysis of the cleavage kinetics of a set of short dsRNA substrates. We also showed that the sequence specificities of Mini-III RNases are not reflected by different binding affinities for cognate and non-cognate sequences, suggesting that target selection occurs predominantly at the cleavage step. We were able to identify two structural elements, the α4 helix and α5b–α6 loop that were involved in target selection.

Characterization of the sequence specificity of the eight Mini-III RNases may provide a basis for better understanding RNA substrate recognition by Mini-III RNases and adopting these enzymes and their engineered derivatives as tools for RNA research.

Studies on enzymes that are involved in RNA metabolism are highly important for the field of RNA biology. In addition to uncovering the molecular mechanisms of RNA processing, they can provide novel tools for RNA research. Advances in RNA studies are hampered by the lack of key enzymatic tools, the counterparts of which are available for DNA and protein research. The availability of sequence-specific endoribonucleases (endoRNases) would enable easy, reproducible and controlled fragmentation of RNA molecules both *in vivo* and *in vitro*.

Many endoRNases that act on single-stranded RNA exhibit some sequence preference, but their target sites are usually limited to one or a few specific nucleotides. Additionally, cleavage frequently depends on the structure of the substrate. Examples include the following prokaryotic single-strand specific endoRNases: T1, which cleaves 3′ to guanosine residues with high specificity1, RegB, which cleaves its mRNA substrates in the middle of the GGAG sequence2 if the last G is located in a short stem between two loops3, and α–sarcin RNase, which cleaves 28S rRNA in the GAGA sequence within a loop4.

Recently, successful attempts were described to engineer sequence specific single-stranded RNases, leading to the development of novel enzymes with narrow, well-defined sequence specificities, e.g., fusion of RNase T1 and the TAT peptide5 and fusion of a PIN nuclease with a PUF domain6. Other recent developments involve hammerhead ribozymes7, catalytically active DNA molecules (DNAzymes)8, peptide nucleic acid-based artificial nucleases (PNAzymes)9 and Cas9 nuclease10, which have been engineered to enable the site-specific cleavage of RNA molecules.

Until recently, no endoRNase with sequence specificity that is comparable to restriction enzymes has been described for double-stranded RNA (dsRNA). The best studied endoRNase that cuts dsRNA is bacterial RNase III. A conserved catalytic domain of RNase III is present in a large group of enzymes that comprise the RNase III superfamily. The classification of RNase III enzymes into subfamilies is based on the presence of various
is not required for dsRNA binding. Here, we characterize the sequence preferences of eight other members of the Mini-III family. We confirmed the involvement of the loop that connects the helix 5b and short loop that connects the α5b and α6 helices.

We previously reported that BsMiniIII was able to cleave long dsRNA substrates, as a standalone enzyme, in addition to its previously known ability to cleave, in complex with the L3 protein, the irregular structure in pre-rRNA. We also defined the cleavage site sequence (ACC^U, where ^ = any residue, and ^ = a scissile phosphodiester bond) of members of class 4 do not possess dsRBD; thus, the mechanism of the substrate recognition process must differ from canonical RNase III and remains unclear. Another difference is the substitution of a long loop of the Mini-III family. We confirmed the involvement of the loop α5b-α6 in canonical RNase III with a helix (α5b) and short loop that connects the α5b and α6 helices.

We previously reported that BsMiniIII was able to cleave long dsRNA substrates, as a standalone enzyme, in addition to its previously known ability to cleave, in complex with the L3 protein, the irregular structure in pre-rRNA. We also defined the cleavage site sequence (ACC^U, where ^ = any residue, and ^ = a scissile phosphodiester bond) of members of class 4 do not possess dsRBD; thus, the mechanism of the substrate recognition process must differ from canonical RNase III and remains unclear. Another difference is the substitution of a long loop of the Mini-III family. We confirmed the involvement of the loop α5b-α6 in canonical RNase III with a helix (α5b) and short loop that connects the α5b and α6 helices.

**Table 1. Mini-III RNases that were used in the present study.** Each enzyme was tested in all buffers listed in Supplementary Table S1 to select conditions leading to well pronounced cleavage pattern.

| Enzyme     | Microorganism             | Strain | Gene identifier (GI) | DSMZ no. | Reaction temperature (°C) | Cleavage buffer |
|------------|---------------------------|--------|----------------------|----------|---------------------------|----------------|
| BsMiniIII  | Bacillus subtilis         | 168    | 2632362              | 402      | 37                        | Bs             |
| CkMiniIII  | Caldicellulosiruptor kristjansonii | 177R1B | 311792827            | 12137    | 65                        | G1             |
| CrMiniIII  | Clostridium ramousum      | 113-I  | 167756029            | 1402     | 37                        | Bs             |
| CzMiniIII  | Clostridium thermocellum  |        | 125974551            | 1237     | 55                        | B1             |
| FnMiniIII  | Fusobacterium nucleatum   | 1612-A | 19704899             | 15643    | 37                        | BG             |
| FpMiniIII  | Faecalibacterium prausnitzii | A2-165 | 166943938            | 17677    | 37                        | Bs             |
| SeMiniIII  | Staphylococcus epidermidis| PCI 1200 | 27467211              | 1798     | 37                        | R              |
| TmMiniIII  | Thermotoga maritima       | MSB8   | 15644486             | 3109     | 65                        | R              |
| TtMiniIII  | Thermoanaerobacter tengcongensis (Caldanaerobacter subterraneus subsp. tengcongensis) | MB4 | 20808680            | 15242    | 65                        | B1             |

**Results**

**Characterization of the limited cleavage of the Φ6 RNA by Mini-III RNases.** In our previous study, we reported evidence of the sequence-dependent cleavage of long dsRNA molecules and characterized the sequence preferences of BsMiniIII. Simple sequence database searches identified more than 600 members of class 4 RNase III enzymes. We selected eight proteins with diverse amino acid sequences in the loop α5b-α6 and we tested their cleavage specificity using the Φ6 bacteriophage dsRNA as a substrate (Table 1, Supplementary Table S1). The band patterns that were generated by different enzymes were clearly discernible and apparently different from the pattern generated by the previously characterized BsMiniIII enzyme (Fig. 1). In vitro cleavage of the 23S pre-rRNA, a natural substrate of BsMiniIII, is substantially increased in the presence of the ribosomal protein L3. In contrast, BsMiniIII cleavage of the Φ6 dsRNA in the presence of the B. subtilis L3 protein is slightly decreased and no change in the band pattern is observed, which suggests that L3 has no influence on the cleavage site selection in long dsRNA (Supplementary Fig. S2).

**Characterization of the sequence preference based on high-throughput sequencing.** To determine the quantitative characteristics of the cleavage preference of the Mini-III family members, we applied high-throughput sequencing of the ends that were generated in the time-limited cleavage reactions of Φ6 dsRNA. For each enzyme we analyzed between 175,000 and 376,000 reads, that passed quality filter and were mapped to the Φ6 genome. In the control experiment, in which Φ6 dsRNA was incubated in the Bs reaction buffer for...
5 min at 37 °C in the absence of any enzyme and then processed similarly to the other samples, 93% of the reads started from the physical ends of the genomic segments. This confirmed that the internal reads that were obtained for dsRNA after cleavage were almost certainly genuine cleavage products and not artifacts of the preparation method. 200 14-base-pair sequences, each representing one of 200 of the most frequently observed cleavage sites, were used to build sequence profiles for each Mini-III enzyme, from which sequence logos and consensus sequences were inferred (Fig. 2). For the majority of the enzymes, the consensus sequences could be summarized as WSSW (where S = G or C and W = A or U). Nevertheless, in agreement with the gel analysis of the band patterns after limited cleavage of the same substrate (Φ6 dsRNA), clear differences could be seen between consensus sequences of the preferred cleavage sites for the enzymes analyzed herein. BsMiniIII appeared to be the most specific, with a clear preference for the ACCU sequence. CtMiniIII and SeMiniIII appeared to be less specific for position 7 of the sequence logo, which could be occupied by any nucleotide. For FnMiniIII, the consensus was less stringent for the central dinucleotide, and positions 7 and 8 in the cleavage site (numbering according to the 14-nt sequence logo) could be occupied by either C or G. For FpMiniIII and TmMiniIII, also position 6 appeared to be less stringently discriminated as it could be occupied by either A or U. CkMiniIII, CrMiniIII, and TtMiniIII appeared to be the least specific among the enzymes analyzed herein, and their consensus sequences suggested that any variation of a WSSW motif could be efficiently cleaved.

High-throughput sequencing results also indicated that some of the Mini-III enzymes discriminated between substrates depending on sequence positions outside the tetranucleotide core. The most apparent was the preference of CrMiniIII and TmMiniIII for C in position 4 and for G in position 11, respectively. Moreover, for CkMiniIII and TtMiniIII, the preference for C in positions 12 and 13 was detected.

To relate observed differences in sequence preferences among Mini-III enzymes to differences in their natural substrates, we aligned 23 S pre-rRNA sequences from bacterial hosts studied in this work and analyzed sequence conservation and predicted structure in the region homologous to the known cleavage site of BsMiniIII (Supplementary Fig. S3). For all microorganisms we were able to find sequences corresponding to the sequence profile of the preferred cleavage sites in the double stranded stems of the 23 S pre-rRNA. In five out of eight cases the location of the predicted cleavage site is similar to that in B. subtilis, i.e. at the end of the stem. In T. maritima 23 S pre-rRNA the predicted helical region is extended by three additional base pairs in such a way that the cleavage site may be shifted in relation to B. subtilis 23 S pre-rRNA. In E. nucleatum 23 S pre-rRNA the potential cleavage site (AGGU/AUCU) contains a wobble pair U•G and is present within the dsRNA region. Finally, in C. ramosum 23 S pre-rRNA the region under consideration with the AGGU/AUCU site has a very weak propensity to form a helical structure, and the 3’ segment exhibits much stronger tendency to pair with another region further downstream. The predicted cleavage site is located within short dsRNA region.

To validate the results of the high-throughput analysis, we prepared five short fragments of the Φ6 genome that encompassed single cleavage sites. According to the sequencing results, these substrates were cleaved with high frequency, and we used them as substrates for in vitro cleavage assays (Supplementary Table S2, Supplementary Fig. S4). The sizes of the major reaction products agreed with cleavage within sites found in the high-throughput data. Additionally, the relative cleavage efficiencies of the selected substrates agreed with the relative cleavage frequencies that were observed in the sequencing results. For example, the 949 S substrate was...
cleaved efficiently only by FpMiniIII, for which it was highly scored in the high-throughput data, whereas other enzymes, for which this position was not found among the most frequent sequencing reads, did not cleave it at all or cleaved it barely (FnMiniIII). Secondary cleavage sites (i.e., sites responsible for generation of additional bands) were found in several cases; see BsMiniIII cleavage of 4486 L substrate; CrMiniIII cleavage of 3292 L substrate or FnMiniIII cleavage of 2021 L substrate in Supplementary Fig. S4. They may be explained by the additional cleavage events that were observed in the high-throughput data, however with frequency much lower than the primary cleavage sites.

Consistent with the differences that were visible in the Φ6 cleavage patterns and in consensus sequences of the preferred cleavage sites derived from high-throughput sequencing, these results indicated differences in the sequence preferences of some of the enzymes.

Effect of base-pair substitutions on the substrate cleavage efficiency. Substitutions in the central tetranucleotide. To further investigate the differences between the Mini-III enzymes in a systematic way we analyzed the effects of substitutions in the ACCU sequence of the 910S substrate on the cleavage activity of four enzymes: BsMiniIII, CtMiniIII, FpMiniIII, and SeMiniIII as they represent well the diversity of the cleavage consensus sequences (Fig. 2). Nineteen variants of the substrate that were obtained by site-directed mutagenesis were used to study the single-turnover kinetics of selected Mini-III enzymes (Fig. 3, Supplementary Fig. S5). The only derivative of the 910S substrate that was cleaved by BsMiniIII, CtMiniIII, and SeMiniIII was one reversely complementary to the ACCU sequence. SeMiniIII was unable to cleave any other substrate variant with efficiency greater than 50% of the efficiency that was measured for the 910S-ACCU substrate. BsMiniIII cleaved only the ACCA variant with efficiency that was greater than 50% of the efficiency that was measured for the wild type substrate. For CtMiniIII, ACCA, ACUU, AUCU, and AGUU were also cleaved with efficiency that was greater than 50% of the efficiency that was measured for the wild type substrate. For

---

Figure 2. Sequence profiles of the preferred cleavage sites of Mini-III enzymes based on high-throughput sequencing. Blue arrows indicate cleavage sites in the sequences shown; orange arrows indicate cleavage sites in the complementary strands.

Figure 3. Effects of substitutions introduced into the cleavage site of substrate 910S-ACCU on cleavage rates. For each enzyme, cleavage rates were normalized to the rate measured for the original 910S-ACCU substrate. *The sequence of the complementary strand is shown.
FpMiniIII, the best cleaved substrate contained the UGCA sequence and was cleaved threefold faster than the original sequence. Furthermore, FpMiniIII cleaved substrates that contained UCCU, ACCA, ACCU, and UCGU sequences with efficiencies similar to or greater than the cleavage of its original substrate. Results of this experiment showed that all deviations from the ACCU consensus sequence in the cleavage site resulted in noticeable decrease of the cleavage rate, with the exception of the least specific FpMiniIII.

Substitutions outside the central tetranucleotide. We next tested the importance of the positions 3, 4, 11 and 12 (i.e., outside the central tetranucleotide) by studying the effects of substitutions at these positions on the cleavage reactivity (Supplementary Fig. S6). These effects did not always agree with the predictions based on consensus sequences obtained from high-throughput sequencing experiments. For instance, TtMiniIII was able to cut all substrates tested, including those with C to A substitution at position 12, which violated the consensus sequence for this enzyme (Fig. 2) calculated from high-throughput cleavage experiments.

CrMiniIII (consensus sequence with C being the dominant residue at position 4; Fig. 2) was able to cleave the 910S-ACCU substrate with A at position 4. Surprisingly, the A4C substitution in this substrate that made it more “consensus-like” decreased the cleavage efficiency of CrMiniIII. A similar decrease was seen also for the A4U substitution in this substrate. The only residue that was well tolerated by CrMiniIII at position 4 was G (seen also in the consensus as the second most common residue at position 4). Hence, tests of 910S-ACCU substrate variants indicated the preference of CrMiniIII for A or G at position 4, rather than C and G observed in high-throughput experiments. The other results were more consistent with the high-throughput sequencing results. Consistent with these data, CkMiniII was mostly impaired by the C12A substitution in the 910S-ACCU substrate, however it was also affected by a A4C substitution, which could not be predicted from high-throughput data. TmMiniIII activity on 910S-ACCU substrate variants agreed with the predictions from high throughput data concerning the preference for G at position 11, and additionally indicated a relative preference for G over U at position 3, C over A in position 4, and C over A at position 12.

The general conclusion from above-mentioned experiments is that effects of substitutions at positions 3, 4, 11 and 12 in the 910S-ACCU substrate, similarly to the effects of substitutions in the central tetranucleotide (positions 6-9) generally agreed with predictions of sequence preferences of individual enzymes inferred from the high-throughput sequencing results. These results also confirmed that there are differences in sequence preferences of the Mini-III enzymes.

Effects of swapping the α4 helix and the α5b-α6 loop on activity and specificity of Mini-III enzymes. The results of our previous study suggested that the α5b-α6 loop of BsMiniIII is involved in the recognition and selection of the target sequence in dsRNA. According to the structural model of the Mini-III-dsRNA complex, in addition to this loop, the α4 helix is also in close proximity to the sequence cleaved (Supplementary Fig. S7). In the absence of a high-resolution crystal structure of the protein-RNA complex, which could verify the putative involvement of these structural elements in RNA sequence recognition, we used biochemical experiments that consisted of swapping amino acid sequences in one or the other of these two regions between related Mini-III enzymes, followed by assaying cleavage activities of such chimeric proteins. To guide the planning of such swaps and construction of chimeric proteins, we used sequence alignment of Mini-III enzymes (Supplementary Fig. S7). We selected conserved amino acid residues L45 and V53 as the boundaries for α4 helix swaps and residues N84 and Y99 as the boundaries for α5b-α6 loop swaps (the numbering refers to the native BsMiniIII sequence). We assayed the endoribonucleolytic activity of chimeric proteins that consisted of an acceptor Mini-III protein scaffold and one or two structural elements that were derived from another protein. As a substrate for activity assays, we used 910S RNA (ACCU target sequence) and its three selected substitution variants, with the target changed to AGGU, UGCA, or AUCU (Fig. 4). The nomenclature of the chimeric proteins that were obtained indicates (in parentheses) the source and identity of the swapped element. For example, CtMiniIII with part of the α4 helix from FpMiniII was named Ct(FpH)MiniIII (Fig. 4A). Initially we constructed four chimeric proteins: Fp(CtH)MiniIII, Fp(CtL)MiniIII, Ct(FpH)MiniIII and Ct(FpL)MiniIII. Both constructs based on the FpMiniIII scaffold and with CtMiniIII “grafts” were inactive, whereas the reverse chimeras (CtMiniIII scaffold with “grafts” from FpMiniIII) were active. We also constructed chimeric proteins where the same fragments of FpMiniIII were grafted into scaffolds of BsMiniIII and SeMiniIII. One of these constructs, Se(FpL)MiniIII, was inactive. To test if the chimeric proteins are folded properly we measured FT-IR spectra of native Mini-III enzymes and their swapped variants. No apparent differences in shapes of amide I peak that would indicate significant differences in secondary structures content were visible suggesting that none of the chimeric proteins was misfolded (Supplementary Fig. S8, Supplementary Table S3). The Bs(FpL)MiniIII variant retained 34% of the activity of the wild type BsMiniIII enzyme, and the Se(FpH)MiniIII variant had approximately the same activity as the wild type SeMiniIII enzyme.

In contrast, three variants in which FpMiniIII fragments were introduced into the CtMiniIII scaffold Ct(FpH)MiniIII, Ct(FpL)MiniIII and Ct(FpHL)MiniIII, as well as the Bs(FpH)MiniIII variant, all had much greater activity than the original enzymes. For instance, the activity of the Ct(FpH)MiniIII chimeric enzyme increased more than 30-fold compared to CtMiniIII or FpMiniIII (Fig. 4D).

In the case of chimeras with the CtMiniIII scaffold and FpMiniIII “grafts”, changes in cleavage preferences were observed. These changes were not as striking as in the case of the enzymatic activity, but were clearly visible. The 910S-UGCA RNA was a very poor substrate for CtMiniIII cleavage. Ct(FpH)MiniIII cleaved this substrate at a rate that was similar to that of the 910S-ACCU substrate. The Ct(FpHL)MiniIII variant cleaved 910S-UGCA threefold faster than the original 910S-ACCU substrate, similarly to the donor enzyme FpMiniIII. No changes in specificity were seen for any variant with the BsMiniIII or SeMiniIII scaffold (Fig. 4C).

The results of swapping experiments showed that a swap of the α4 helix and/or the α5b-α6 loop between different Mini-III proteins led in several cases to a substantial increase of enzymatic activity and alteration of

---

**SCIENTIFIC REPORTS | 6:38612 | DOI: 10.1038/srep38612**
sequence preferences. In the double chimera Ct(FpHL)MiniIII, where both the $\alpha_{5b}$-$\alpha_{6}$ loop and the $\alpha_{4}$ helix were swapped, the sequence preference was changed from that of the “acceptor” to that of the “donor” protein.

Multiple turnover assay of the dsRNA cleavage. To test sequence preference of Mini-III enzymes on relatively large dsRNA substrates, which present many potential cleavage sites, including sites that deviate from the consensus and represent poorer targets, we used single turnover cleavage assays described above. Such assays provide important insight into cleavage site selection at the endonucleolytic step of the enzymatic reaction, while single turnover experiments neglect the product release step, which for many endonucleases is a rate-limiting in the enzymatic cycle. Multiple turnover cleavage measurements were also performed on two 28 bp dsRNAs (Supplementary Table S6), one that contained the preferred cleavage site from the 910S substrate (POS-ACCU) and another one with the same nucleotide composition, but derived from a different fragment of 910S sequence, in which no cleavage could be detected (NEG). The NEG substrate is therefore “scrambled”, uncleavable derivative of POS-ACCU. The POS-ACCU substrate was cleaved by all enzymes, and they all generated unique products (Supplementary Fig. S9). Only TmMiniIII and CkMiniIII cleaved the NEG substrate, although with significantly lower efficiency than the POS-ACCU substrate, and with no retention of discrete products. These results lead us to conclude that Mini-III RNases maintain sequence preference also under multiple turnover conditions. There
is also qualitative correspondence between relative activities of the Mini-III enzymes in multiple turnover and in single turnover assays (Fig. 1).

Selected swapped variants of CtMiniIII were assayed in multiple turnover cleavage reaction on two substrates POS-ACCU and POS-UGCA (Fig. 5) that contained the same target sequence as 910-ACCU and 910-UGCA molecules, respectively, which were used as substrates in single turnover assays. In contrast to the single turnover assay on longer dsRNA (Fig. 4), no increase in enzymatic activity was observed for Ct(FpH)MiniIII and Ct(FpHL)MiniIII. On the contrary, Ct(FpHL)MiniIII activity was decreased at least threefold in comparison to FpMiniIII (Fig. 5C). A threefold preference for POS-UGCA over POS-UCCA was observed for the Ct(FpHL)MiniIII variant (Fig. 5D), which is in agreement with the single turnover assay results. On the other hand no preference for POS-UCCA substrate was observed for FpMiniIII.

Cleavage sequence selection is not strictly dependent upon preferential binding. In a previous report we showed that deletion of the α5b-α6 loop in BsMiniIII did not alter the binding of this enzyme to dsRNA with a preferred cleavage sequence. We hypothesized that the α5b-α6 loop is involved in selecting.

**Figure 5. Sequence preference of swap variants in multiple turnover assay.** Cleavage assays were performed with 2.5 μM substrates in enzyme:substrate ratio 1:10 for CtMiniIII and Ct(FpHL)MiniIII and 1:20 for FpMiniIII and Ct(FpH)MiniIII. (A) Comparison of cleavage activity on POS-ACCU and POS-UGCA substrates. Aliquots were collected after 0.5, 1, 1.5, 2 and 2.5 hours of reaction. (B) Cleavage of NEG substrate without preferred sequence. Aliquots were collected after 1, 2 and 3 hours of cleavage. Some unspecific cleavage of NEG substrate can be observed but only in CtMiniIII reaction transient accumulation of cleavage products is visible. (C) Comparison of the POS-ACCU cleavage rates. (D) Sequence preference of the swap variants and native enzymes. Cleavage rate of POS-UGCA substrate for each enzyme is normalized to the POS-ACCU cleavage rate of the same enzyme.
the cleavage site and further posited that this selection occurred at the step of cleavage itself and did not involve selective binding. In the current study we measured the dissociation constants of several Mini-III RNases and CtMiniIII swap variants for two 28 bp dsRNAs, one that contained the preferred cleavage site from the 910 S substrate (POS-ACCU) and the other with the same nucleotide composition but derived from the other fragment of 910 S sequence, in which no cleavage could be detected (NEG). No clear preference for binding the cleavage site was found (Table 2, Supplementary Fig. S10). The only exception was BsMiniIII, but even in this case, the difference in affinity was only twofold and the affinity for the preferred cleavage site was lower than for the uncleaved dsRNA. All of the measured $K_D$ values were within the range of 70 nM to 2.25 $\mu$M, with the exception of FpMiniIII, in which no binding saturation was observed at the range of concentrations tested, indicating that the $K_D$ of this enzyme was substantially higher. These results suggest that Mini-III enzymes do not select their cleavage sequence at the substrate binding step, but most probably they bind dsRNA irrespective of its sequence. In other words, as there is no difference in binding cleavable vs not-cleavable substrates, the binding preference is unlikely to be important for the cleavage site selection.

**Discussion**

The presented results led to four important findings:

- Preference for cleaving distinct sequences in dsRNA is a common feature of Mini-III RNases
- There is a clear diversity of target sequences preferred by MiniIII enzymes studied in this work although they can all be summarized by the WSSW consensus sequence
- Helix α4 and loop α5b-α6 are involved in sequence selection
- Sequence preference for dsRNA cleavage is not a result of preferential substrate binding

In our previous study we showed that Mini-III RNase from *B. subtilis* is able to cleave long dsRNA in a sequence-dependent manner\(^1\). In the present study, we report that this is a common feature of the Mini-III subfamily of RNase III enzymes (class 4). We also found that swapping two structural elements, the α4 helix and α5b-α6 loop, between different MiniIII enzymes, led to significant changes in the catalytic activity and in some cases to alterations in sequence preference of the chimeric enzymes. These results suggest the involvement of both structural elements in catalytic activity and sequence selection.

Sequence specificity of the canonical RNase III from *E. coli*, has been only partially characterized; for instance there are no data concerning limited cleavage of long dsRNA molecules. In our own experiments we were unable to obtain discernible bands in limited cleavage of Φ6 dsRNA with RNase III (Supplementary Fig. S1). Most information has been obtained from *in vitro* studies with the μR1.1 RNA and its derivatives, composed of a relatively short RNA chain that forms a double stranded stem of 13–25 base pairs, including wobble G-U base pairs\(^8,19\). In these RNAs the cleavage site is located in a double-stranded region at the very end of the stem. These cleavage events are determined by recognition of two regions of the stem: (i) the distal box (UU/AG) at positions 10–11 from the center of the cleavage, and (ii) the proximal box (consensus sequence CWUU/WAWG) at positions 2–5 from the center of the cleavage. No sequence preference was observed for the central dinucleotide at the RNase III cleavage site.

The residues in the consensus of the preferred cleavage sequence of Mini-III enzymes, which were determined in this work based on limited cleavage of Φ6 dsRNA, and residues in the consensus sequence of *E. coli* RNase III have different localization in relation to the cleavage site. For Mini-III enzymes no conservation is observed for positions corresponding to the proximal box and the distal box. The most conserved region of four base pairs directly encompasses the cleavage site. Some additional preferences are exhibited by several of the enzymes at the distance of 4–5 residues from the cleavage site. The differences in target selection between class 1 (canonical) and class 4 (Mini-III) RNase III enzymes may be explained based on different structures of the two classes of the enzymes and differences in the enzyme-substrate contacts.

The first prominent difference in substrate recognition between the two classes of enzymes is the fact that RNase III recognizes the distal box, whereas the high throughput data presented in this work indicate that Mini-III RNases do not show preferences toward any sequence located at a distance longer than 5 bp from the cleavage site. For *E. coli* RNase III the distal box forms contacts with RBM4, which is located in a long loop

Table 2. Dissociation constants for the preferred cleavage substrate and uncleaved dsRNA. The equilibrium dissociation constants ($K_D$) were obtained from global fitting of the results of triplicate measurement in nitrocellulose filter binding assay (see Materials and Methods) at enzyme concentration ranges listed in the right column to the one site specific binding model (with the Prism 6.0 software)\(^1\). Brackets indicate the standard error of fitting.

| Protein   | $K_D$(POS-ACCU) | $K_D$(NEG) | Protein concentration |
|-----------|----------------|------------|-----------------------|
| BsMiniIII | 0.14 $\mu$M ($+/−/0.02)\(^1\) | 0.07 $\mu$M ($+/−/0.01)\(^1\) | 0.03125–1$\mu$M |
| CtMiniIII | 1.14 $\mu$M ($+/−/0.11)\(^1\) | 1.34 $\mu$M ($+/−/0.28)\(^1\) | 0.3125–10$\mu$M |
| FpMiniIII | > 100 $\mu$M | 19.9 $\mu$M ($+/−/3.20)\(^1\) | 0.3125–10$\mu$M |
| SeMiniIII | 0.16 $\mu$M ($+/−/0.02)\(^1\) | 0.19 $\mu$M ($+/−/0.02)\(^1\) | 0.0375–1.2$\mu$M |
| Ct(FpH)MiniIII | 2.62 $\mu$M ($+/−/0.38)\(^1\) | 1.94 $\mu$M ($+/−/0.23)\(^1\) | 0.14–4.6 $\mu$M |
| Ct(FpL)MiniIII | 1.33 $\mu$M ($+/−/0.33)\(^1\) | 1.8 $\mu$M ($+/−/0.28)\(^1\) | 0.156–5$\mu$M |
| Ct(FpHL)MiniIII | 2.17 $\mu$M ($+/−/0.59)\(^1\) | 2.25 $\mu$M ($+/−/0.86)\(^1\) | 0.109–3.5$\mu$M |
between helices α5 and α612. This region in Mini-III RNases is replaced by a short helix α5b followed by a loop α5b-α6, which have been shown to be indispensable for the cleavage of the preferred sequence in dsRNA substrate13. We speculate that due to the structural differences between class 1 and class 4 enzymes, this region in Mini-III RNase is involved in interactions with the part of the substrate encompassing the cleavage site and not with the distal box.

The second prominent difference between the two classes of RNases III is the recognition by canonical RNase III of the so-called proximal box, formed by nucleotides 2–5 from the center of the cleavage. Nucleotides 5–6 form contacts with RBM1, an element located in dsRBD, which is absent from all Mini-III enzymes11,12,19. A common element between class 1 and 4 RNases III is helix α4, which in canonical RNase III enzymes forms the RBM3 element that interacts with the second and third nucleotide from the center of the cleavage site. We have shown that swapping of helices α4 between Mini-III enzymes resulted in an alteration of sequence preferences and cleavage activities. Therefore, we hypothesize that this helix plays a similar role in both class 1 and class 4 RNase III enzymes, and interacts with nucleotides in close proximity of the cleavage site. Since the sequence preference observed for Mini-III enzymes is mostly limited to residues 1 and 2 from the center of the cleavage site, it is possible that the interaction of helix α4 in this family is shifted one nucleotide towards the center of the target site relative to the canonical RNase III. It is worth noting that amino acid sequences in two Mini-III regions implicated in sequence preference, helix α4 and loop α5b-α6, are rather divergent, which might explain the observed differences in sequence preferences of the studied Mini-III RNases.

All the above analyses of the structural basis of sequence recognition by Mini-III enzymes would benefit greatly from the availability of a high-resolution structure for the nuclease-RNA complex. So far, our numerous attempts to crystalize Mini-III enzymes in a complex with dsRNA have unfortunately been unsuccessful. An important element that influences the substrate preference of RNases III is their domain composition. The majority of known RNA-binding proteins have modular structures and include one or more specialized RNA-binding domains20. Alone, these domains often bind short RNA stretches with weak affinity, however by existing in multiple copies, they endow a protein with the ability to bind RNA with high specificity and affinity. Thereby, RNA-binding modules of the same or of different structural types combined with enzymatic domains define the targets of many enzymes acting on RNA. Most members of the RNase III family (including canonical RNase III enzymes, Dicer and Drosha) contain dsRBD and this domain is responsible for dsRNA substrate bind by these enzymes. For example, for E. coli RNase III it was demonstrated that dsRBD deletion severely impairs this enzyme for RNA cleavage under standard in vitro conditions21, suggesting that interactions of dsRBD with RNA are crucial to form a cleavage-competent complex of RNase III with its substrate22. Among RNases III, the unique property of Mini-III enzymes is that they are single-domain proteins containing only the RNase III catalytic domain. The role of additional domains can be played by interacting proteins. For example studies carried out to date by the Condon group indicate that efficient cleavage of pre-rRNA can be achieved by Mini-III only in the presence of the L3 protein, which establishes high affinity binding of the enzyme to the substrate RNA and thereby might serve as a functional analog of an RNA binding domain, e.g. dsRBD in E. coli RNase III15. We were unable to see any effect of L3 protein on target selection in cleavage of a long dsRNA of Φ6 bacteriophage (Supplementary Fig. S2).

Majority of cleavage assays used in this study to investigate Mini-III sequence preferences were performed in single turnover conditions. We have repeated selected assays in multiple turnover settings on smaller dsRNA substrates. The results of these assays on swapped variants of Mini-III do not show as many differences between these enzymes as were apparent in single turnover conditions, such as substantial increase of the catalytic activity in variant C(tFPFH) or differences in cleavage of 910-ACCU and 910-UGCA substrates by CtMiniIII and FpMiniIII. There are several factors that can explain these differences. For practical reasons, we used substrate concentration of 2.5 μM, which is not substantially higher than the measured dissociation constants (Table 2). In such conditions most probably both product release and rebinding of a new substrate steps had big impact on measured cleavage rates, which may obscure differences at the cleavage step that in turn are emphasized in a single turnover assay. Such effect is most noticeable for FpMiniIII, for which K_D is significantly higher than for other MiniIII enzymes. This can explain the lack of preference of this enzyme for the UGCA sequence in the multiple turnover assay, which was observed under single turnover conditions. Based on the binding affinity measurements we postulate that substrate selection takes place at the cleavage step, as no preferential binding of the “cleavable” substrate over “uncleavable” one was observed (Table 2). Therefore, in this case the single turnover assay is more informative to study the sequence selection phenomenon. It is worth emphasizing that for a similar reason, the single turnover assay is commonly used in investigations concerning sequence specificity and its alteration, for different enzymes acting on a double-stranded DNA23-25. Besides, the endonucleolytic activity of Mini-III enzymes is rather low. Therefore, the measurement of multiple turnover cleavage requires long incubation times, at which enzyme and substrate stability becomes an issue.

In our experiments we observed small differences between high-throughput sequencing data and the results of in vitro cleavage assays of isolated dsRNA substrates. This indicates that possibly factors other than just sequence consensus recognition are involved in the selection of cleavage sites by Mini-III enzymes. One such factor could be altered geometry of RNA in the cleaved sequence or in its close proximity, perhaps influenced by the surrounding sequence. We analyzed target sequences for evident and recurrent sequence-dependent distortions in base-step geometries using values for shift, slide, rise, tilt, roll, and twist from the RNA STEPS database26, but we did not find such anomalies (data not shown). This makes the involvement of altered geometry in sequence preference mechanism unlikely.

The molecular basis of the target sequence selection by Mini-III enzymes is not known but RNA binding assays indicate that preferential binding is not involved in this process. An alternative possibility is the contribution of a mechanism described for E. coli RNase III, based on a combination of biochemical and structural data on the enzyme-RNA complex of its close homolog. In this mechanism antideterminants and positive determinants
within the RNA sequence mask or expose particular phosphodiester bonds for the endoRNase cleavage by local alteration of the dsRNA structure12,18. A similar mechanism may explain the sequence preference of Mini-III enzymes. Another possible mechanism are conformational changes of the dsRNA substrate upon Mini-III binding required to generate a cleavage-proficient complex. Such a conformational change may be more likely to occur for some of the target sequences than for others. Such a behavior was observed for many restriction endonucleases and included various distortions of the substrate27,28. Although restriction enzymes do bind dsDNA in a sequence-independent manner, this binding is typically much weaker compared to the binding of the cognate sequence29–31. There are, however, restriction endonucleases, such as the Clf9I, which bind cognate and non-cognate sequences with similar affinities32. One possibility is that a similar lack of selectivity at the substrate binding step is a reason why the sequence preference of Mini-III is rather weak. Importantly, the preference of BsMiniIII to cleave pre-RNA \( \text{in vivo} \) is brought about by the presence of ribosomal protein L3 in close proximity to the cleavage site, as well as the complicated secondary and tertiary structure of the whole substrate molecule15.

We demonstrate that in addition to targeting pre-rRNA \( \text{in vivo} \), Mini-III RNases exhibit preferences for sequences in dsRNA, dictated by structural elements within the catalytic domain. We found that this sequence specificity is exerted not at the level of substrate binding, but at the level of cleavage. This is a unique feature of Mini-III enzymes, since all other RNase III superfamily members use the dsRBD domain(s), rather than the catalytic domain, to bind dsRNA substrates with high affinity12,33–35 see also ref. 36.

Identification of new structural elements in the RNase III catalytic domain that participate in target selection, together with the discovery of a diversity of solutions used by RNase III enzymes to achieve this goal, provides a platform for engineering substrate specificity in Mini-III enzymes to make them useful tools for RNA research.

**Methods**

**Cloning, protein expression, and purification.** Bacterial genomic DNA was isolated from reconstituted cultures that were obtained from the DSMZ strain collection (Germany; Table 1). DNA was purified by phenol extraction and ethanol precipitation. The Mini-III coding sequences and \( B. \ subtilis \) L3 coding sequence were amplified from genomic DNA by polymerase chain reaction (PCR) with Pfu DNA polymerase and the primers that are listed in Supplementary Table S4. The reaction products were digested with NdeI and XhoI and inserted into a PET28a vector that was cleaved with the same enzymes. The resulting plasmids encoded endoRNases with an N-terminal His\(_6\)-tag. The L3 coding sequence was prepared in the same way but cloned into pET30a vector resulting in the plasmid encoding L3 protein with a C-terminal His\(_6\)-tag. The E106Q substitution in BsMiniIII catalytic center, which leads to complete inactivation of BsMiniIII endonucleolytic activity, was introduced by the inside-out PCR amplification of wt BsMiniIII construct with E106Qf and E106Qrev primers, phosphorylation of PCR product and recircularization with T4 DNA ligase. All the constructs were verified by DNA sequencing.

To construct plasmids that encoded chimeric versions of Mini-IIIIs with parts of the \( \alpha 4 \) helix and \( \alpha 5b-\alpha 6 \) loop that were swapped, original plasmids that expressed one Mini-III (acceptor) were subjected to inside-out PCR with Pfu DNA polymerase that amplified the entire template, with the exception of the sequence that encoded the structural element that was to be swapped (Supplementary Table S5). The PCR products were treated with T4 polynucleotide kinase and ligated with short double-stranded oligonucleotides that contained a corresponding sequence from the other Mini-III (donor). Inserts that replaced the sequence encoding the \( \alpha 5b-\alpha 6 \) loop were created by filling in 5′-protruding sticky ends formed by annealing partially complementary oligonucleotides with the Klenow fragment of DNA polymerase I. Constructs with a confirmed correct orientation of the insert were selected after DNA sequencing.

The nomenclature of the chimeras that were obtained indicates (in parentheses) the Klenow fragment of DNA polymerase I. Constructs with a confirmed correct orientation of the insert were verified by DNA sequencing.

To obtain recombinant proteins, plasmids were transformed into the \( E. \ coli \) BL21 (DE3) strain. Enzymes were produced by 24-h autoinduction at \( 37 ^\circ C \). Cells were harvested by centrifugation, resuspended in buffer L0 (50 mM Tris HCl [pH 7.5] and 300 mM NaCl) and lysed by a single passage through a Constant Systems cell disruptor at 20 kpsi. Lysates were clarified by centrifugation at 50,000 \( \times \) g for 20 min at 4 °C. The proteins were purified by Ni-NTA affinity chromatography on a His-Select Nickel Affinity Gel (Sigma). After 1-h incubation at 4 °C. The proteins were purified by Ni-NTA affinity chromatography on a His-Select Nickel Affinity Gel (Sigma). After 1-h incubation at 4 °C. The proteins were purified by Ni-NTA affinity chromatography on a His-Select Nickel Affinity Gel (Sigma). After 1-h incubation at 4 °C.

**RNA substrate preparation.** \( \Phi 6 \) phage was produced and purified as described previously36 with minor modifications. LB broth was used as a liquid medium. Phage particles were concentrated by PEG precipitation and purified by equilibrium CsCl gradient ultracentrifugation at 150,000 \( \times \) g for 24 h at 4 °C. Purified virions were diluted five-fold in 10 mM potassium phosphate (pH 7.1), 1 mM MgSO\(_4\), pelleted by centrifugation at 70,000 \( \times \) g for 75 min at 4 °C, and resuspended in TE buffer (10 mM Tris HCl [pH 7.5], 1 mM ethylenediaminetetraacetic acid...
dsRNA was isolated by phenol extraction and ethanol precipitation. Purified dsRNA was dissolved in 1 mM sodium citrate (pH 6.4) at 1 mg/ml, aliquoted, and stored at −20 °C.

Isolated fragments of the Φ6 genome were prepared by enzymatic in vitro synthesis. The product of the reverse transcription of Φ6 dsRNA with random hexamer primers and Maxima Reverse Transcriptase (Thermo Scientific) was used as the PCR template to produce dsDNA that was equivalent to the desired phage genome flanked by the T7 RNA polymerase promoter (TAATACGACTCCTATAGGG) on one end and the Φ6 RNA polymerase promoter (GGAAAAAAA) on the other end (Supplementary Table S6). These PCR products were used to produce defined dsRNA substrates with a replicator RNAi Kit (Thermo Scientific).

To generate a template for dsRNA synthesis of the 910S RNA molecule and its variants that had various substitutions, the dsDNA template used to produce dsRNA was cloned into the SmaI site of the pUC19 plasmid, thus providing the pUC910S plasmid. Substitutions of each position in the cleavage site were obtained by inside-out PCR of amplification of the pUC910S plasmid with three sets of the primers that contained a degenerate sequence in the cleavage site (Supplementary Table S7). The 5′ ends of the PCR products were phosphorylated with T4 polynucleotide kinase and circularized with T4 DNA ligase. The resulting plasmids were subjected to DNA sequencing to characterize substitutions in each clone.

The 28 bp dsRNAs that were used for the filter binding assays and for the multiple turnover cleavage assays were generated by annealing complementary RNA oligonucleotides (Supplementary Table S6). For binding assays dsRNAs were labeled with [32P]ATP using a T4 polynucleotide kinase. Labeled dsRNA molecules were purified using mini Quick Spin Oligo Columns (Roche).

dsRNA cleavage assays. The cleavage buffers and reaction temperatures that were used for each enzyme are listed in Table 1. The enzyme and substrate concentrations and reaction times in each experiment are described in the Results section. The reactions were terminated by mixing the reaction aliquot with the gel loading buffer that contained EDTA (10 mM final concentration) and a 1/10 volume of a 1:1 phenol:chloroform mixture. The products were separated by non-denaturing gel electrophoresis in 1.5% agarose (Φ6 bacteriophage genome) and in 8% or 15% polyacrylamide for the single turnover and multiple turnover cleavage assays respectively, stained with ethidium bromide, and visualized under ultraviolet light using the LAS 4010 imaging system (GE Healthcare). Cleavage kinetics were measured based on a densitometric analysis of each reaction time point on the electropherograms using ImageQuantTL software (GE Healthcare). Cleavage rates were calculated from the initial linear part of the reaction progress curves. Measurements were made in triplicate.

RNA sequencing analyses. A 5 μg sample of the Φ6 genome was used as a substrate in a time-limited cleavage reaction, to achieve a 10% decrease in the intensity of the substrate bands. The cleavage reaction of the substrate was performed for 10 min for the majority of the enzymes, with the exception of SeMiniIII and TmMiniIII, in which the reaction times were 40 and 5 min, respectively. The preparation of the sequencing libraries was performed as described previously27. High-throughput sequencing was performed using a MiSeq (Illumina) platform at Genomed (Warsaw, Poland). The processing of sequencing data was performed as described previously27. Two hundred of the most frequent cleavage sites in each experiment were selected for further analysis. Fourteen-nucleotide-long sequences of these sites were used to build consensus sequences of the preferred cleavage sites for each enzyme using MEME software39 and default parameters, with the exception of setting the minimum width of the motifs to 14.

Filter binding assay. Nitrocellulose filters (GE Healthcare) were soaked for 30 min at room temperature in binding buffers that were identical to the cleavage buffers, with the exception that MgCl2 was replaced with 1 mM CaCl2. The binding reactions were performed for 1 h at room temperature in a total volume of 50 μl with 0.0015 μM M 32P end-labeled 28 bp dsRNA and 0.03–10 μM of enzyme. The binding reactions were filtered through nitrocellulose equilibrated with binding buffer, followed by three 150-μl washes with the binding buffer. The filters were dried, and radioactivity was recorded autoradiographically using the Storage Phosphor Screen (GE Healthcare) and scanned with Typhoon Trio+ Imager (GE Healthcare). The quantitative analysis of the autoradiograms was performed using ImageQuant TL software (GE Healthcare). Dissociation constants were calculated by GraphPad Prism 6 (GraphPad Software, Inc.) with the single-site-specific binding model.

Bioinformatics analysis. Sequences of 23S rRNA genes were obtained from NCBI database along with 100-nucleotide long flanking regions and aligned using T-Coffee web server40 with standard server parameters. 14–16 nucleotide long sequences flanking in the alignment the position of the known 5′ and 3′ BsmIII cleavage sites in B. subtilis 23S pre-rRNA were extracted and used in secondary structure prediction with CentroidFold web server41 using default parameters. Secondary structure prediction was carried out using PSSpred using default parameters42.

References
1. Czaia, R. et al. RNase T1 variant RV cleaves single-stranded RNA after purines due to specific recognition by the Asn46 side chain amide. Biochemistry 43, 2854–2862 (2004).
2. Saias, F., Uzan, M. & Bontems, F. The phage T4 restriction endonuclease RegB: a cyclizing enzyme that requires two histidines to be fully active. Nucleic Acids Res. 31, 2751–2758 (2003).
3. Lebars, I., Hu, R. M., Lallemant, J. Y., Uzan, M. & Bontems, F. Role of the substrate conformation and of the S1 protein in the cleavage efficiency of the T4 endonuclease RegB. J. Biol. Chem. 276, 13264–13272 (2001).
4. Endo, Y., Gluck, A., Chan, Y. L., Tsurugi, K. & Wool, I. G. RNA-protein interaction. An analysis with RNA oligonucleotides of the recognition by alpha-sarcin of a ribosomal domain critical for function. J. Biol. Chem. 265, 2216–2222 (1990).
5. Dow-Tien, C., Yuan-Jhui, T. & Alan, L. Creating a ribonuclease T-tat that preferentially recognizes and hydrolyzes HIV-1 TAR RNA in vitro and in vivo. *Nucleic Acids Res* **36**, 963–969 (2008).

6. Choudhury, R., Tsai, Y. S., Dominguez, D., Wang, Y. & Wang, Z. Engineering RNA endonucleases with customized sequence specificities. *Nat. Commun.* **3**, 1147 (2012).

7. Usman, N., Beigelman, L. & McSwiggen, J. A. Hammerhead ribozyme engineering. *Curr. Op. Struct. Biol.* **6**, 527–533 (1996).

8. Feldman, A. R. & Sen, D. A new and efficient DNA enzyme for the sequence-specific cleavage of RNA. *J. Mol. Biol.* **313**, 283–294 (2001).

9. Murtola, M., Wenska, M. & Stromberg, R. PNAzymes That Are Artificial RNA Restriction Enzymes. *J. Am. Chem. Soc.* **132**, 8984–8990 (2010).

10. Price, A. A., Sampson, T. R., Ratner, H. K., Grakoui, A. & Weiss, D. S. Cas9-mediated targeting of viral RNA in eukaryotic cells. *Proc. Natl. Acad. Sci. USA* **112**, 6164–6169 (2015).

11. Redko, Y., Bechhofer, D. H. & Condon, C. Mini-III, an unusual member of the RNase III family of enzymes, catalyses 23S ribosomal RNA maturation in *B. subtilis*. *Mol. Microbiol.* **68**, 1096–1106 (2008).

12. Gan, J. H. et al. Structural insight into the mechanism of double-stranded RNA processing by ribonuclease III. *Cell* **124**, 355–366 (2006).

13. Kim, K., Sim, S. H., Jeon, C. O., Lee, Y. & Lee, K. Base substitutions at scissile bond sites are sufficient to alter RNA-binding and cleavage activity of RNase III. *FEMS Microbiol. Lett.* **315**, 30–37 (2011).

14. Krinke, L. & Wulf, D. L. The cleavage specificity of RNase III. *Nucleic Acids Res.* **18**, 4809–4815 (1990).

15. Redko, Y. & Condon, C. Ribosomal RNA L3 bound to 23S RNA stimulates its maturation by Mini-III ribonuclease. *Mol. Microbiol.* **71**, 1145–1154 (2009).

16. Hotto, A. M. et al. Arabidopsis Chloroplast Mini-Ribonuclease III Participates in RNA Maturation and Intron Recycling. *Plant Cell* **27**, 724–740 (2015).

17. Glow, D. et al. Sequence–specific cleavage of dsRNA by Mini-III RNase. *Nucleic Acids Res.* **43**, 2864–2873 (2015).

18. Zhang, K. & Nicholson, A. W. Regulation of ribonuclease III processing by double-helical sequence antitumoridants. *Proc. Natl. Acad. Sci. USA* **94**, 13437–13441 (1997).

19. Pertev, A. V. & Nicholson, A. W. Characterization of RNA sequence determinants and antitumoridants of processing reactivity for a minimal substrate of Escherichia coli ribonuclease III. *Nucleic Acids Res.* **34**, 3708–3721 (2006).

20. Lunde, B. M., Moore, C. & Varani, G. RNA-binding proteins: modular design for efficient function. *Nat. Rev. Mol. Cell Biol.* **8**, 479–490 (2007).

21. Sun, W., Jun, E. & Nicholson, A. W. Intrinsic double-stranded-RNA processing activity of Escherichia coli ribonuclease III lacking the dsRNA-binding domain. *Biochemistry* **40**, 14976–14984 (2001).

22. Nicholson, A. W. Ribonuclease III mechanisms of double-stranded RNA cleavage. *Wiley Interdiscip. Rev. RNA* **5**, 31–48 (2014).

23. Wenz, C., Hahn, M. & Pingoud, A. Engineering of variants of the restriction endonuclease EcoRV that depend in their cleavage activity on the flexibility of sequences flanking the recognition site. *Biochemistry* **37** (1998).

24. Parry, D., Moon, S. A., Lisa, H. H., Hoslop, P. & Connolly, B. A. DNA Recognition by the EcoRV Restriction Endonuclease Probed using Base Analogues. *J. Mol. Biol.* **331**, 1005–1016 (2003).

25. Chahar, S., Elsawy, H., Ragozin, S. & Jeltsch, A. Changing the DNA recognition specificity of the EcoDam DNA-(adenine-N6)-methyltransferase by directed evolution. *J. Mol. Biol.* **395**, 79–88 (2010).

26. Xie, Y. & Olson, W. K. BPS: a database of RNA base-pair structures. *Nucleic Acids Res.* **37**, D83–88 (2009).

27. Winkler, F. K. et al. The crystal structure of EcoRV endonuclease and of its complexes with cognate and non-cognate DNA fragments. *EMBO J.* **12**, 1781–1795 (1993).

28. Vladiu, H. & Aggarwal, A. K. Structure of BamHI bound to nonspecific DNA: a model for DNA sliding. *Mol. Cell* **5**, 889–895 (2000).

29. Langowski, J., Pingoud, A., Goppelt, M. & Maass, G. Inhibition of EcoRI action by polynucleotides - a characterization of the nonspecific-binding of the enzyme to DNA. *Nucleic Acids Res.* **8**, 4727–4736 (1980).

30. Alves, J., Selen, U. & Wolfe, H. Accuracy of the EcoRV restriction endonuclease - binding and cleavage studies with oligodeoxynucleotide substrates containing degenerate recognition sequences. *Biochemistry* **34**, 11191–11197 (1995).

31. Vipond, I. R. & Halford, S. E. Specific DNA recognition by EcoRV restriction endonuclease induced by calcium-ions. *Biochemistry* **34**, 1113–1119 (1995).

32. Sikins, V. & Pleckaityte, M. Catalytic and binding properties of restriction endonuclease Ccr1. *Eur. J. Biochem.* **217**, 411–419 (1993).

33. Gan, J. et al. Intermediate states of ribonuclease III in complex with double-stranded RNA. *Structure* **13**, 1435–1442 (2005).

34. Wu, H., Henras, A., Chanfreau, G. & Feigion, J. Structural basis for recognition of the AGNNN tetraloop RNA fold by the double-stranded RNA-binding domain of Rnt1p RNase III. *Proc. Natl. Acad. Sci. USA* **101**, 8307–8312 (2004).

35. Wang, Z., Hartman, E., Roy, K., Chanfreau, G. & Feigion, J. Structure of a yeast RNase III dsRBD complex with a noncanonical RNA substrate provides new insights into binding specificity of dsRBDs. *Structure* **19**, 999–1010 (2011).

36. Masliha, G., Barraud, F. & Allain, F. H. RNA recognition by double-stranded RNA binding domains: a matter of shape and sequence. *Cell Mol. Life Sci.* **70**, 1875–1895 (2013).

37. Studier, F. W. Protein production by auto-induction in high-density shaking cultures. *Protein Expres. Purif.* **41**, 207–234 (2005).

38. Vidaver, A. K., Koski, R. K. & Van Etten, J. I. Bacteriophage phi6: a Lipid-Containing Virus of Pseudomonas phaseolicola. *J. Virol.* **11**, 799–805 (1973).

39. Bailey, T. L. et al. MEME SUITE: tools for motif discovery and searching. *Nucleic Acids Res.* **37**, W202–W208 (2009).

40. De Tierrano, I. I. et al. T-Coffee: a web server for the multiple sequence alignment of protein and RNA sequences using structural information and homology extension. *Nucleic Acids Res.* **39**, W13–17 (2011).

41. Sato, K., Hamada, M., Asai, K. & Mituyama, T. CENTROIDFOLD: a web server for RNA secondary structure prediction. *Nucleic Acids Res.* **37**, W277–280 (2009).

42. McGuffin, L. J., Bryson, K. & Jones, D. T. The PSIPRED protein structure prediction server. *Bioinformatics* **16**, 404–405 (2000).

Acknowledgements

Polish National Science Center [NCN, grant 2011/01/B/NZ1/00209 to K.J.S.]; Polish National Center for Research and Development [NCBR, grant 245550 to J.M.B.]; European Research Council [ERC, StG grant RNA + P = 123D grant to J.M.B. PoC grant eRNases to J.M.B.]; D.G. and D.P. were additionally supported by scholarships from the European Social Fund, Human Capital Operational Programme [UDA-POKL.08.02.01-14-041/09]. J.M.B. was also supported by the “Ideas for Poland” fellowship from the Foundation for Polish Science. The authors want to thank Marcin Nowotny, Elżbieta Puria and Monika Radlinska for critical reading of the manuscript.

Author Contributions

D.G. and K.J.S. performed the experiments, analyzed data, wrote the main manuscript text and prepared all figures, J.M.B. participated in writing the manuscript, K.J.S. designed research, K.J.S. and J.M.B. oversaw the
project, J.M.B. and K.Sz. performed computational analyses of 23 S pre-rRNA sequences, M.K. performed experiments presented on Supplementary Figures S1 and S2, J.C. performed experiments presented on Supplementary Figure S9, D.P. and V.K. cloned coding sequences and performed initial optimization of the cleavage assay conditions.

**Additional Information**

**Supplementary information** accompanies this paper at http://www.nature.com/srep

**Competing financial interests:** The authors declare no competing financial interests.

**How to cite this article:** Głów, D. *et al.* Identification of protein structural elements responsible for the diversity of sequence preferences among Mini-III RNases. Sci. Rep. 6, 38612; doi: 10.1038/srep38612 (2016).

**Publisher's note:** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/

© The Author(s) 2016