Peer Review File

Manuscript Title: Time-resolved structural analysis of an RNA-cleaving DNA catalyst

Editorial Notes:

Redactions – unpublished data
Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data.

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referee #1:

The manuscript from the Etzkorn lab reports on the highly impressive and astonishingly comprehensive analysis of structures and their functional dynamics to understand the catalytic function of a DNA-based enzyme cleaving an RNA substrate strand. The overall enzyme architecture involves a single stranded DNA that upon binding of RNA forms a two-armed DNA-RNA hetero-duplex and a large exposed loop that acts as the catalytic pocket for the RNA cleavage reaction. The manuscript reports the concerted effort representing a multiple technique-based characterization that leaves out no aspects of the structural characterization of five relevant states along the catalytic cycle represented in Figure 3a. The collection of a massive amount of NMR, EPR and FRET experimental restraints guides molecular dynamics simulations to provide an atomistic description of all relevant states. Mg^{2+} binding is quantified in the absence and presence of Na^{+} ions, and mechanistic findings are tested experimentally by an impressively large amount of mutational data. As highlight, time-resolved liquid-state NMR spectroscopy is utilized to characterize the changes in the transition from the Mg^{2+} apo state to the catalytic active state.
Due to its architecture, DNAzyme can target a large variety of RNA substrates, and such universal applicability of DNAzymes makes the presented comprehensive characterization of the states populated along the catalytic cycle worthy of publication in Nature. The work my Etzkorn and colleagues provides yet another hallmark in promoting this extremely important tool in cell biology but also for translation applications, paralleling previous outstanding work particularly by Slivermann and Hoebartner in the field of DNAzymes.

Not surprisingly, given the huge amount of presented data including eleven extended data figures, each of which contains more than 10 subpanels, there are points that need to be revised prior to acceptance of the manuscript. I am indicating points for revision below. From the apparent large number of points that I raise, I do not wish to imply weakness in the current submission.

1.) While I understand the current interest in SARS-CoV research, I find reference 9 not very convincing. This publication has been cited seven times since 2007, for me indication that the technique is not really useful. Thus: delete this reference.

2.) The exact nature of the RNA substrate to be cleaved by the DNAzyme is irrelevant. Thus, three references to Creutzfeld-Jakob and Alzheimer are misplaced, in particular reference 2003 that certainly does not deal with the application of a DNAzyme to combat Alzheimer. Delete references 15-17.

3.) Reference 18 and its title is misfortunate, in particular in generating hopes that the failure of in vivo function of DNAzymes could now be overcome as result of the studies. Such expectation is too high, thus I recommend to delete this reference.

4.) Suggestion: Figure 1, b: the resolution of the pdf-file is not extremely high. This limited resolution makes reading of the annotations extremely difficult. This aspect has to be improved almost through all figures and extended data figures.

5,) The authors use 2'F substituted RNA substrate as catalytically inactive form for the substrate, thus, as substrate analogon. The effect of 2'F substitution on pseudorotation phase has been documented before and should be tested by measurement of ^{3}J couplings for the residues around the cleavage site.

6.) Figure 2, b: The author explain the use of eNOEs to fit multiple distances from a single NOE cross peak by detailed recording of auto- and cross-correlated relaxation rates from mixing time-dependent quantification of cross peaks and diagonal peaks. As far as I read the results, such fitting to multiple distances has not been applied. Thus, I recommend to delete the insert to explain eNOE effects.

7.) Figure 2,b: The insert explain ^{19}F-STD is too small.

8.) Figure 2c: the explanatory insert for RDCs should be omitted.

9.) Figure 2h and throughout the text: in Figure 2f, the authors correctly provide the molar ratio of $[Mg^{2+}]$:[RNA], while in figure 2h and at many other places in the manuscript, only $[Mg^{2+}]$ is given. Please always provide: $[Mg^{2+}]$:[RNA], because this is absolutely essential.

10.) Figure 2d, 2e: I do not find the ribbon representation of the RNA structure as given sufficiently insightful and suggest to also add 2D representations of RNA to make it easier to identify metal binding sites. Figure 2e is not "understandable" currently.

11.) Figure 3d: see point #10.

12.) Figure 3e: provide better x-axis ticks (at each hour).

13.) Figure 3g: delete Figure 3g, as it does not add substantial new information.

14.) Reference 26, 27, 28: reduce to a single reference for EPR studies to detect Mn^{2+} binding stoichiometry.

15.) p. 16 line 443: I think the CD data are not very insightful and can be deleted from the manuscript.

16.) p. 16, line 457: D_z:RNA -> D_z·RNA throughout the manuscript.

17.) p. 16, EPR measurements: Details and fitting model for fitting the affinities of Mn^{2+} to the complex are not provided. Differentiate between microscopic and macroscopic binding constants as detailed e.g. in Nucl. Acids Res. (2011) 39, 8258-8270.

18.) p. 18, line 548: Provide details on ^{1}H decoupling in ^{19}F detected experiments.

19.) p. 19, line 598: Explicitly state the details of the labelling pattern for the isotope labelled DNA. Is this uniformly labelled? This is important to judge the ^{13}C T_2-relaxation rate determination. The

systematic error introduced in relaxation rate determination in uniformly 13C labelled samples has been discussed, e.g. in Thakur et al. J. Biomol. NMR (2012) 52, 103-114 and references cited therein.

20.) p. 20, line 608: Change temperature-gradient to temperature-dependent measurements throughout the manuscript. Temperature gradient experiments in the context of NMR implies a gradient within a single sample, e.g. induced by rf decoupling.

21.) Throughout the manuscript: data are always plural.

22.) Extended Data Figure 2: Omit the middle zoom into the H3'-H8/H6 spectral region of the NOESY spectra. Enlarge the sequential assignment walk in the H1'-H8/H6 spectral region. Use arrows to ensure that readers can follow your assignment. Provide unambiguous annotation for all cross peaks. The chemical structure of the DNA oligonucleotide has been enlarged without keep the size ratio constant so that the chemical structure appears distorted. Delete the entire HSQC and retain only the assigned C1',H1' spectral region. 1'-CH groups -> C1',H1 sites. Given the chemical shift resolution apparent in the C1',H1' spectral region, HCCH-TOCSY would allow complete chemical shift assignments of all sugar carbon chemical shifts.

23.) Extended Data Figure 3: a) Annotate RNA and DNA in the chemical structure. Why do you show only the chemical shifts of a single amino-^{1}H? Delete extended data figure 3b as the data are incomplete. Delete extended data figure 3c, as it is better visible in extended data figures 3d-f. Delete extended data figure 3g, this is unnecessary.

24.) Extended Data Figure 4: It is unclear to me what information could be extract from the 19F STD data shown in extended data figure 4i. Information derived from HOESY type experiments mostly yields trivial restraints and can be deleted from the manuscript extended data figure 4e,f.

25.) Extended Data Figure 8: Extended Data Figure 8a: the annotations are two small. The presentation of RNA structures in Extended Data Figure 8a, 8f,j should be supported also by 2D representations; these figure drive too much attention to the oligonucleotide phosphodiester backbone. Extended Data Figure 8g: the double bond has gotten lost. Information on the fitting of the EPR detected binding can also be provided here (see also point 17).

26.) Extended Data Figure 9d: this figure is too small.

27.) Extended Data Figure 10a, 10c: provide ticks on the time axes, annotate the time axes. Extended Data Figure 10l can be deleted as the data do not provide additional strong insight.

Referee #2:

The authors use NMR spectroscopy to identify the high-resolution structure of the 10-23 DNAzyme, known since 1997 (ref. 2) and often used in studies and applications of RNA cleavage. Despite the fact that DNAzymes have been known since 1994, there are currently only two high-resolution structures available, both via X-ray crystallography, as reported recently in ref. 7 (2016) for the RNA-ligating 9DB1 DNAzyme and in ref. 8 (2017) for the RNA-cleaving 8-17 DNAzyme. The latter was originally reported in the same 1997 study (ref. 2) as the 10-23 DNAzyme as well as found independently in several other reports.

The lack of an X-ray structure for 10-23 is surely not for lack of trying by many labs, and here the

authors have obtained the structure instead using NMR spectroscopy. The time-resolved NMR spectroscopy data lead to a multi-step model (Fig. 3) in which several types of structural transition are important for catalysis. The model suggests a specific atomic modification (6-thio at nucleotide G14), which was found experimentally to improve the catalysis via 6-fold increase in the RNA cleavage rate constant.

Overall this manuscript is an important achievement for the field of nucleic acid enzymes. As only the third high-resolution structure of a DNAzyme and the first such structure to be obtained using NMR spectroscopy, it will be considered an important and likely landmark achievement in the field.

I have several comments for the authors to consider. Addressing the first of these comments could involve additional experiments, although such experiments are arguably not critical to establishing the conclusions of the manuscript.

1. The 6-thio-dG14 experiment, described on p. 12, is especially interesting. The finding of a 6-fold increase in RNA cleavage rate constant upon 6-thio substitution at G14 appears to match well with the expectation derived from the authors' model. Of course, thio substitutions and metal ion specificity switches (Mg/Mn) have been used for many years to study ribozymes and DNAzymes. From such literature, it can be valuable to evaluate not just a single strategic substitution but other substitutions at several nearby positions, to evaluate whether the observed effect at the key position arises for the claimed reason. In other words, negative control experiments are valuable to confirm that the basis for the observed effect is as assigned rather than merely coincidental. In the present case, the only other Gs in the catalytic loop are at G1, G2, and G6. Thio substitutions can be made readily on T (the 2-thio and 4-thio monomer are readily available), and the catalytic loop has T4 and T8. More broadly, from the authors' ref. 25, several of the nearby catalytic loop nucleotides can be mutated to G while retaining high activity (e.g., each of T8, A9, C10, and A11), in which case 6-thio-G at those mutated G positions could be examined in comparison to G. On this basis I suggest that the authors can consider making additional thio substitutions at positions other than G14. If the thio effect observed at G14 is not found at the other positions, then that would strengthen the authors' conclusion by providing evidence that the catalytic improvement effect is specific to G14 rather than more generically observed.

2. In the summary paragraph, sentence 2, the authors claim that "the high expectations [for therapeutic and biotechnological potential] have not been met yet, a fact that can be traced back to the lack of high-resolution and time-resolved information about its mode of action". However, in my view this claim oversells the value of such information for this potential, both in vitro and in vivo. Most such applications do not depend on knowing mode of action, i.e., mechanism, as the applications would be enabled by nearly any RNA-cleaving entity regardless of how it cleaves the RNA. High-resolution information (including time-resolved information that enables model formulation) can indeed aid in fine-tuning catalytic activity, e.g., by single-atom replacement as the authors have done here at G14, and increases in catalytic activity (e.g., faster rate) can improve applications in vitro and in vivo. But the need for improved catalytic activity is hardly the sole reason that therapeutic and biotechnological potential of RNA-cleaving DNAzymes has not been fully realized, and in many cases especially in vitro, the existing catalytic activity is not limiting for applications. As the authors cite (e.g., ref. 18 from these authors), in vivo DNAzyme activity is limited by the free Mg2+ concentration, and having a faster DNAzyme would help to counteract the low free Mg2+ concentration in vivo. But other serious concerns (delivery, turnover, stability...) are just as important and are not rendered unimportant by improving catalytic activity. Also there has been considerable success in identifying RNA-cleaving DNAzymes with substantial catalytic activity yet little or no free Mg2+ requirement by including one or more kinds of modified nucleobases, in numerous publications primarily by D. Perrin but not cited here by the authors. If the main obstacle to in vivo activity of RNA-cleaving DNAzymes were simply the rate constant in a low-Mg2+ environment, then Perrin's approach has likely already solved the problem without the benefit of high-resolution structural information.

3. On p. 3, lines 51-54 include a list of advantages of DNAzymes relative to protein or RNA. But many of these advantages are relevant only relative to protein or are not really advantages at all. In the first category are "straightforward design" and "independence of protein cofactors", as both ribozymes and DNAzymes can be targeted to nucleic acid substrates in straightforward fashion, and both can readily be independent of protein cofactors. In the second category is "absence of permanent effects on the genome", as none of the three biopolymers necessarily causes permanent effects on the genome, and all three biopolymers are used therapeutically.

4. The authors refer in their manuscript title to "DNA-mediated catalysis". Because several kinds of catalyst whose actions are mediated by DNA are not DNAzymes, such as the use of double-stranded DNA as a chiral ligand as well as DNA-templated synthesis, I suggest that it would be clearer and more accurate to use the more specific term "DNAzyme catalysis" in the title.

5. The summary paragraph never states that NMR spectroscopy is the method used here for structure determination. Given the importance of using NMR for the achievement, the method should be stated clearly in this paragraph. Perhaps NMR should be included in the manuscript title as well.

6. In Fig. 1, are the data in panel d the same as in the upper part of panel e (i.e., Dz-5C:RNA-2'F, 1H-1H correlation data)? If so, then it would be reasonable to omit the upper part of panel e and just show the lower part for Dz-5A. If not, then the difference between panel d and the upper part of panel e needs to be made clear.

7. For metal ions I, II, and III (Fig. 2e), in the associated text (pp. 8-9) the authors identify the first two of these three ions with steps that they designate as scaffolding and conformational activation, respectively. Referring to "steps" strongly implies a consecutive series of actions or motions, and the setup of describing metal ions I and II in this fashion reinforces the impression. Subsequently in Fig. 3, the authors present their model that involves multiple rate-limiting transient intermediate states. The relationship between this model and the scaffolding and conformational activation states and steps is not made clear, at least in my reading of the submitted manuscript. The sole place that scaffolding and conformational activation steps are mentioned in association with Fig. 3 is briefly on p. 11, line 284.

8. On p. 8, line 188, the Co(NH3)6 ion should have a 3+ charge rather than no charge. Same in Methods p. 15 and Ext. Data Fig. 7 (several parts of caption as well as panels j and k).

9. In Ext. Data Fig. 8b caption, what does "(Absent)" mean? In 8c caption, "(PAGE-data)" can presumably be deleted. Phrases accidentally remaining from manuscript editing?

10. Ref. 18 should be year 2018 (not 2017) and pp. 333-343 (not 1-11). I did not comprehensively check all of the references regarding such details.

Referee #3:

This manuscript reports detailed studies of the structure and dynamics of the RNA-cleaving deoxyribozyme, 10-23, using a broad range of NMR spectroscopic methods combined with molecular dynamics simulations. The study was motivated by the goal to rationally improve the catalytic efficiency of DNAzymes for therapeutic applications in vivo.

The 10-23 deoxyribozyme was among the first DNAzymes discovered by in vitro selection in the 1990s and has since been studied in detail, but high-resolution structural insights and a detailed understanding of the structural dynamics have been missing. The work is highly significant, and the methods employed provide much needed insights for comprehending fundamental aspects of DNA catalysis.

The authors report an intricate fold of the precatalytic RNA-DNA complex, and studied the metal ion-mediated dynamics of the system. The results are interpreted to support a stepwise kinetic reaction scheme. Using the metal ion binding data, the authors suggest a rational nucleobase modification to modulate the metal ion affinity and thereby accelerate the cleavage reaction.

The experimental approaches are original and the insights generated in this work are novel, but some important aspects have not been addressed. The authors should rationalize their results by a more comprehensive comparison with previously reported data on mutations, functional group exchanges, and modifications in the catalytic core of 10-23 (see below).

Missing aspects need to be addressed:

a) The 10-23 deoxyribozyme strongly prefers purine|pyrimidine (R|Y) junctions in the RNA substrates, while other combinations are poor substrates or are not cleaved at all. Do the insights reported in this work give us any clue about the nucleotide preference at the cleavage site? Even if the answer is "no", this aspect should be included in the discussion. Along these lines, the abstract should be revised, as the opening statement on 10-23 and "its ability to cleave virtually any RNA with high selectivity" is not correct, or at least misleading.

b) It is well established that the DNA-catalyzed cleavage involves in-line attack of the 2'-OH of R on the scissile phosphate, with 5'-OH of Y as leaving group and generation of a cyclic phosphate on R (here G0). Any relevant structure needs to allow for such an in-line orientation, but this aspect is not considered in the manuscript. The authors should search for relevant conformational states in their MD ensembles and include an appropriate discussion. Along these lines, the authors should more clearly address the long-standing question if 10-23 catalyzes RNA cleavage by a general acid-base mechanism and/or an metal ion-assisted mechanism (with M2+ facilitating 2'-OH deprotonation and coordinating to the the 5'-leaving group). There are some hints in the section that discusses the involvement of C13/G14 "strongly suggesting a direct involvement of C13 and/or G14 in catalysis" (but how?), and the MD data that "corroborate interactions of most functional groups of G14 with hexa-hydrated Mg2+". But how these two statements come together remains unclear from Fig. 3f, where the caption says that Mg2+ also interact with the cleavage site.

Also, the authors should compare and contrast their new insights on the structure and mechanism of 10-23 with the previously available structure of the 8-17 deoxyribozyme and its mechanistic implications.

c) How can the results help to rationalize the faster cleavage rate of the Dz5A vs Dz5C variant? All studies were performed with Dz5C in order to avoid a potentially problematic palindromic sequence motif in the catalytic loop. However, the sequence containing the palindrome cleaves RNA faster. Please also include rate constants to allow for a more quantitative description.

d) It is surprising that the authors did not comment on or use any information from 31P-NMR. Previous phosphorothioate substitution and rescue experiments with thiophilic Mn2+ ions have identified functionally important phosphate groups in the 10-23 DNAzyme (e.g. Nawrot et al., FEBS J., 2007, 274:1062). The same work also used 6-thioguanosine (s6G) and its metal ion interactions in the 10-23 DNAzyme, and this earlier work should be cited and discussed, especially because the authors also performed studies using Mn2+ and s6G.

Further considerations for a revision:

1. The "unexpected but effective fold" needs a better structural description. Fig. 2d and the description in the text that says "a condensed core region involving an additional turn of the Dz's loop around the RNA", is not sufficient to understand and appreciate the complexity of the 3D fold.

Also, the authors need to explain more precisely how the DNA architecture with the RNA threaded through a structured DNA loop is compatible with the multi-state scheme in Fig. 3a, which suggests that both binding arms are hybridized before the core loop folds in the activated state. However, the "additional turn" mentioned above is absent in Fig. 3a, and the supplementary discussion describes a pre-structured DNA loop even in the absence of the RNA.

2. On what basis were the six 2'F positions chosen for the Dz6xF variant used for 19F-STD experiments? The effect of 2'-F substitution in the catalytic core should be compared with previously available data on 2'-OMe derivatization (Schubert et al., NAR, 2003, 31:5982), which was found to be tolerated at six positions in the catalytic core.

3. Some figure panels and labels are too tiny for good readability at 100% (on screen and on printout). The figure captions need to more precisely describe what is shown. For example, Fig. 2a and ED Fig. 1d mention "nucleotide-specific resonances", but it remains unclear which resonances for each nucleotide were in fact analyzed (or sum or average?).

4. Fig. 3f: Please label the yellow ball; is it the P atom of the scissile phosphate? Please also include the 2'-OH nucleophile, and indicate from which MD snapshot this image was taken.

5. Fig. 3g: The kinetics trace for 6-oxo-G14 is a reference, and should correspond to data shown for Dz(5C) in Fig. 1b, but shows significantly slower cleavage. How are the conditions different? Please include the rate constants.

6. Nomenclature: Syn and anti orientation of the nucleobase describe conformations, and not configurations as stated in the manuscript. (syn and anti conformers can be accessed via rotation around the glycosidic bond; no covalent bonds needs to be broken and reconnected, as it would be the case for syn/anti configurations).

Author Rebuttal to Initial Comments:

Point-by-point reply

We are delighted by the extraordinarily high quality of the review process and the very positive feedback by all reviewers. It is apparent that each reviewer invested a substantial amount of time and thoroughly went through our manuscript. We would therefore like to express our sincere gratitude for this commitment and the very helpful and constructive feedback, which helped us to further improve our manuscript as reported in detail in the following point-by-point reply.

General points:

Overall, the major changes to the manuscript include:

I. An increased focus on the key aspects, i.e., a fundamental understanding of the system, by also reducing the focus on the therapeutic potential. Large parts of the introduction concerning the latter have been deleted or rewritten.

II. A number of additional experimental results and data analysis, including new NMR spectra of existing Dz variants and of a new (inactive) Dz variant, new activity data on an additional thio-modified Dz, and a comprehensive analysis of mechanistic features visible in the MD data.

III. A rearrangement of the subfigures shown in Fig. 1 and 2 to incorporate the additional data and to increase readability.

Editorial requests:

- In line with the reviewer's and editorial suggestions, we have strongly reduced the focus on the therapeutic potential and instead increased the focus on the catalytic mechanism.
- As requested, we have (i) separated the legends from the Extended Data Figures and included them sequentially at the end of the main text, and (ii) provide individual files for each Extended Data Figure without caption.
- We now refer to each Figure, Extended Data Figure and Supplementary Figure in the main text.

Reviewer 1:

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1) *While I understand the current interest in SARS-CoV research, I find reference 9 not very convincing. This publication has been cited seven times since 2007, for me indication that the technique is not really useful. Thus: delete this reference.*

We agree that this reference is not essential and, together with the restructuring of the introduction, deleted it from the manuscript.

2) *The exact nature of the RNA substrate to be cleaved by the DNAzyme is irrelevant. Thus, three references to Creutzfeld-Jakob and Alzheimer are misplaced, in particular reference 2003 that certainly does not deal with the application of a DNAzyme to combat Alzheimer. Delete references 15-17.*

These references were also deleted in the new version of the introduction.

3) *Reference 18 and its title is misfortunate, in particular in generating hopes that the failure of in vivo function of DNAzymes could now be overcome as result of the studies. Such expectation is too high, thus I recommend to delete this reference.*

The mentioned reference (which was carried out by several co-authors of the current manuscript) motivated our current work to understand the metal-ion interactions better. While we here provide the desired insights, we fully agree that this does not imply that the in vivo limitations are already resolved.
However, please note that the above-mentioned reference is also the first introduction of the used DNAzyme sequence, including the binding arm region. In this respect, the reference describes, how the binding-arm sequence was selected and, e.g., demonstrates its activity to target full-length and folded RNA transcripts. It also already proposes a cooperative binding behaviour of the metal ions, which is also cited later in the current manuscript.
Accordingly, we changed the text in the introduction to lower the expectations regarding overcoming in vivo limitations and would like to keep the respective citation when referring to the DNAzyme construct, in vivo limitations, and the metal-ion cooperativity.

4) *Suggestion: Figure 1, b: the resolution of the pdf-file is not extremely high. This limited resolution makes reading of the annotations extremely difficult. This aspect has to be improved almost through all figures and extended data figures.*

To increase the readability of the figures, we deleted several unnecessary and potentially distracting annotations. In addition, we increased the size of several subfigures. We further rearranged Fig. 1 and Fig. 2 to (i) make better use of the available space and (ii) better structure the different key aspects of our work. Note that this rearrangement does not affect the content with respect to the previous version but, stimulated by the reviewer's comments, should improve readability of the manuscript.
To improve the limited resolution, we (will) also include vector graphics of all figures in the manuscript (this did not work for the pdf file automatically created by the submission platform). Here, we provide the respective figures also as separate pdf files and are confident that the typesetting team will be able to include the vector graphics format in a potentially published version.

5) *The authors use 2'F substituted RNA substrate* as *catalytically inactive form for the substrate, thus,* as *substrate analogon. The effect of 2'F substitution on pseudorotation phase has been documented before and should be tested by measurement of* ^{3}J *couplings for the residues around the cleavage site.*

We thank the reviewer for this suggestion and fully agree that it would indeed be interesting to assess the pseudorotation phase effects in the 2'FRNA substrate.

Unfortunately, we can only reliably determine the $^{3}_{1HF}$ and also the $^{2}_{1HF}$ couplings of the modified nucleotide. In this respect it should be noted that our samples only included ^{13}C enrichments of the DNAzyme and not the RNA substrate, restricting the determination of ^{13}C-1H cross correlated relaxation rates as well as ^{13}C-seperated $^{3}_{1HH}$ that would be the desired readout to assess the pseudorotation phase of the stabilized RNA substrate (Richter et al., 1999).

It would arguably be best to directly compare e.g., the respective $^{3}_{1HH}$ values of the modified RNA with the non-modified RNA target. Unfortunately, without ^{13}C-encoding none of the relevant splittings is resolved due to peak overlap. We now also tried to resolve this with our newest spectrometer (at 1.2 GHz) but still could not reliably determine the respective couplings.

Based on previous studies, it can be assumed that the included 2'F substitution has only moderate effects on the pseudorotation phase compared to the non-modified RNA. For instance, Anasova and co-workers (Anosova et al., 2016) report a comparative study in which out of the three analysed fully 2'F modified RNAs, two show a C3'-endo to C4'-exo shift with a pseudorotation phase shift of about (only) 36°, whereas one 2'F modified RNA does not show any shift in comparison to regular RNA

[REDACTED]

To still be able to provide better experimental insights into this issue, we have now also used the opportunity to record new data at 1.2 GHz to obtain the best possible insights into the effects of the modification on the surrounding atoms via chemical shift perturbations.

We have recorded high-resolution 2D NOESY spectra of Dz^{5C}·RNA and Dz^{5C}·$RNA^{2'F}$ under identical conditions and could use this new data to obtain the complete resonance assignment of the non-stabilized substrate in addition. We included a full chemical shift comparison of all resolved ^{1}H resonances as an additional Figure in Extended Data Fig. 1d. The data show astonishingly restricted CSPs induced only in the direct surrounding of the modified position. While we cannot exclude minor local distortions of the pseudorotation phase angle, our data strongly suggest that this effect is not spread/amplified throughout the rest of the complex.

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6) *Figure 2, b: The author explain the use of eNOEs to fit multiple distances from a single NOE cross peak by detailed recording of auto- and cross-correlated relaxation rates from mixing time- dependent quantification of cross peaks and diagonal peaks. As far as I read the results, such fitting to multiple distances has not been applied. Thus, I recommend to delete the insert to explain eNOE effects.*

We thank the reviewer for bringing this possible source of misunderstanding to our attention. We deleted the respective insert.
In addition, to enable a stronger focus on the obtained structural and mechanistic results, to remove redundancies with the extended data, and to resolve readability concerns due to too small illustration sizes, we removed (or shifted to the Extended Data) all other methodology schematics from Fig. 2.

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7) *Figure 2,b: The insert explain ^{19}F-STD is too small.*

This part of the Figure was deleted to increase the readability of the main Figures (see point 6).

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8) *Figure 2c: the explanatory insert for RDCs should be omitted.*

The insert has been removed.

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9) *Figure 2h and throughout the text: in Figure 2f, the authors correctly provide the molar ratio of $[Mg^{2+}]$:[RNA], while in figure 2h and at many other places in the manuscript, only $[Mg^{2+}]$ is given. Please always provide: $[Mg^{2+}]$:[RNA], because this is absolutely essential.*

We have included the molar ratio at all relevant positions in the main figures and throughout the main text.

10) *Figure 2d, 2e: I do not find the ribbon representation of the RNA structure as given sufficiently insightful and suggest to also add 2D representations of RNA to make it easier to identify metal binding sites. Figure 2e is not "understandable" currently.*

We thank the reviewer for the suggestions and included a larger representation of the 3D structure (formerly Fig. 2d), including a view from the previously not shown side to enable a better comprehension of the structure (now Fig. 1g). In addition, we included a schematic '2D-like' model that further highlights the fold of the catalytic loop (Fig. 1h).
We also increased the size of the previous Fig. 2e (now Fig. 2a) and included a simplified representation of the metal binding sites using the 2D model (Fig. 2b).

11) *Figure 3d: see point #10.*

Fig. 3d displays the 3D view of the orange highlighted nucleotides in the simplified model in Fig. 3a state C_0 (magenta area in 3d) and state C_1 (purple area in 3d). We added a respective comment in the caption to make this clearer.

12) *Figure 3e: provide better x-axis ticks (at each hour).*

We thank the reviewer for bringing this to our attention. We noticed that a few other tick marks 'got lost' in the Figure, likely due to moving the subfigures around during Figure preparation. We have now prepared a new version that contains the correct tick marks, including the hour marks in Fig. 3e.

13) *Figure 3g: delete Figure 3g, as it does not add substantial new information.*

This aspect has been explicitly highlighted by reviewer 2, point 1, as 'especially interesting' and we have included additional data and discussion following reviewer 2's suggestions (see reply to the respective point below). We would therefore like to keep the data as it is now presented in the manuscript.

14) *Reference 26, 27, 28: reduce to a single reference for EPR studies to detect* Mn^{2+} *binding stoichiometry.*

We have selected one reference (previous ref. 26) and deleted the additional references.

15) *p. 16 line 443: I think the CD data are not very insightful and can be deleted from the manuscript.*

We have removed the CD data from the manuscript.

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16) *p. 16, line 457: D_Z:RNA -> D_Z·RNA throughout the manuscript.*

We appreciate the suggestion and changed the nomenclature of the complex accordingly throughout the manuscript.

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17) *p. 16, EPR measurements: Details and fitting model for fitting the affinities of Mn^{2+} to the complex are not provided. Differentiate between microscopic and macroscopic binding constants as detailed e.g. in Nucl. Acids Res. (2011) 39, 8258-8270.*

The details and fitting models are now given in the Methods section and are referred to in Extended Data Fig. 8. For the lowest K_D, we now also differentiate between macroscopic and microscopic binding sites referring to the above-mentioned reference. However, we cannot provide a more detailed analysis because the EPR approach uses only the Mn(II)-EPR signal intensity and does not, in contrast to the excellent NMR study of Rinnethal et al. 2011, provide differentiable spectral signatures for the different sites.

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18) *p. 18, line 548: Provide details on ^1H decoupling in ^{19}F detected experiments.*

The missing information regarding the ^1H decoupling was included in the Methods section.

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19) *p. 19, line 598: Explicitly state the details of the labelling pattern for the isotope labelled DNA. Is this uniformly labelled? This is important to judge the ^{13}C T_2-relaxation rate determination. The systematic error introduced in relaxation rate determination in uniformly 13C labelled samples has been discussed, e.g. in Thakur et al. J. Biomol. NMR (2012) 52, 103-114 and references cited therein.*

We used uniform ^{13}C-enriched DNA, a piece of information that should indeed be included in the manuscript. We thank the reviewer for bringing this oversight to our attention and have included the missing information. We also agree that, although no quantitative interpretation is attempted here, and, as suggested in point 23), we removed the T2-relaxation rates from Extended Data Fig. 3, it is helpful to explicitly mention the effects of this labelling pattern. Therefore, we have included a brief discussion and the above-mentioned reference in the respective Methods section.

20) *p. 20, line 608: Change temperature-gradient to temperature-dependent measurements throughout the manuscript. Temperature gradient experiments in the context of NMR implies a gradient within a single sample, e.g. induced by rf decoupling.*

We agree that this is a potential source of misunderstanding and changed the respective phrase throughout the manuscript.

21) *Throughout the manuscript: data are always plural.*

The respective sentences have been adjusted accordingly.

22) *Extended Data Figure 2: Omit the middle zoom into the H3'-H8/H6 spectral region of the NOESY spectra. Enlarge the sequential assignment walk in the H1'-H8/H6 spectral region. Use arrows to ensure that readers can follow your assignment. Provide unambiguous annotation for all cross peaks.*

The chemical structure of the DNA oligonucleotide has been enlarged without keep the size ratio constant so that the chemical structure appears distorted. Delete the entire HSQC and retain only the assigned C1',H1' spectral region. 1'-CH groups -> C1',H1 sites. Given the chemical shift resolution apparent in the C1',H1' spectral region, HCCH-TOCSY would allow complete chemical shift assignments of all sugar carbon chemical shifts.

We appreciate the reviewer's useful suggestions to improve the readability of Extended Data Fig. 2 and have modified the Figure accordingly.

Regarding the chemical shift assignments, we had initially recorded a 3D-HCCH-TOCSY experiment, which, although partly useful, only contained limited information due to the rather low signal-to-noise ratio due to comparatively low sample concentrations. Nevertheless, we could obtain good data from a 2D version of a HSQC-TOCSY, which allowed us to assign most (but not all) 2' and 3' ribose ^{13}C as well as several additional carbons. The information of all assigned carbons is included in our BMRB deposition with ID 34654.

23) *Extended Data Figure 3: a) Annotate RNA and DNA in the chemical structure. Why do you show only the chemical shifts of a single amino-^{1}H? Delete extended data figure 3b as the data are incomplete. Delete extended data figure 3c, as it is better visible in extended data figures 3d-f. Delete extended data figure 3g, this is unnecessary.*

We agree that several panels can be removed from Extended Data Fig. 3 and modified the Figure according to the reviewer's suggestions.

Regarding the detection of amino peaks, the respective spectral extract (Extended Data Fig. 3a) was selected since it represents a well-resolved region that contains exclusively correlations involving the amino group and H5 of cytosine. Indeed, we detected the respective correlations for all cytosines present in the binding arm regions (all peaks are labelled). On the contrary, no signal was detected for any of the 5 cytosines present in the loop region, indicative of the absence of stable hydrogen bonding of the respective amino groups. We rewrote the caption to make this point clearer.

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24) *Extended Data Figure 4: It is unclear to me what information could be extract from the 19F STD data shown in extended data figure 4i. Information derived from HOESY type experiments mostly yields trivial restraints and can be deleted from the manuscript extended data figure 4e,f.*

We agree that most peaks in the HOESY spectrum are trivial (and/or not resolved). However, the spectra also contain several useful pieces of information, including sequential correlations used for the resonance assignment as well as some long-range restraints. In a new version of the Figure, we now zoomed into the respective spectral regions, labelled the highlighted peaks, and modified the figure caption to make this clearer.

While homonuclear [^{19}F,^{19}F]-NOESY spectra did not yield any visible correlations for our system (not shown), we found that ^{19}F-STD NMR provides an interesting addition to detect long-range contacts. Extended Data Figure 4i shows ^{1}H-detected STD spectra with saturation on the ^{19}F frequencies shown in Extended Data Fig. 4d, lower panel (using matching colour codes). Several STD peaks appearing in the individual spectra can be assigned to sufficiently isolated proton resonances and translated into distance restraints between the assigned proton and the respective ^{19}F position. To compensate for the imprecise distance dependency of the STD transfer, the respective restraints were set to 5 Å with rather loose boundaries of

[REDACTED]

25) *Extended Data Figure 8: Extended Data Figure 8a: the annotations are two small. The presentation of RNA structures in Extended Data Figure 8a, 8f,j should be supported also by 2D representations; these figure drive too much attention to the oligonucleotide phosphodiester backbone. Extended Data Figure 8g: the double bond has gotten lost. Information on the fitting of the EPR detected binding can also be provided here (see also point 17).*

We thank the reviewer for the useful suggestions and have deleted unnecessary annotations and increased the size of the remaining annotations in the respective figure panels. In addition, we have prepared a schematic '2D' model that highlights the central aspects seen in the 3D representations. We have also corrected the missing double bounds. In the caption of Extended Data Fig. 8, we now refer to the Methods section, in which we have included the details on the fitting of the EPR data.

26) *Extended Data Figure 9d: this figure is too small.*

We have increased the size of the respective Figure.

27) *Extended Data Figure 10a, 10c: provide ticks on the time axes, annotate the time axes. Extended Data Figure 10l can be deleted as the data do not provide additional strong insight.*

We agree that it is helpful to include the time information in Extended Data Fig. 10a and 10c. Due to minor deviations of the reaction time (which arguably is the more interesting information) and the experimental time, which are related to a slightly delayed start and the incremental nature of the experimental time axis, we added a dedicated reaction time axis to the respective sub figures.

In line with point 13) and the comments of reviewer 2 - point 1), we have added additional data concerning the thio-dG modifications and would therefore like to keep the previous Extended Data Fig. 10l (now Extended Data Fig. 10m).

Reviewer 2:

I have several comments for the authors to consider. Addressing the first of these comments could involve additional experiments, although such experiments are arguably not critical to establishing the conclusions of the manuscript.

1. The 6-thio-dG14 experiment, described on p. 12, is especially interesting. The finding of a 6-fold increase in RNA cleavage rate constant upon 6-thio substitution at G14 appears to match well with the expectation derived from the authors' model. Of course, thio substitutions and metal ion specificity switches (Mg/Mn) have been used for many years to study ribozymes and DNAzymes. From such literature, it can be valuable to evaluate not just a single strategic substitution but other substitutions at several nearby positions, to evaluate whether the observed effect at the key position arises for the claimed reason. In other words, negative control experiments are valuable to confirm that the basis for the observed effect is as assigned rather than merely coincidental. In the present case, the only other Gs in the catalytic loop are at G1, G2, and G6. Thio substitutions can be made readily on T (the 2-thio and 4-thio monomer are readily available), and the catalytic loop has T4 and T8. More broadly, from the authors' ref. 25, several of the nearby catalytic loop nucleotides can be mutated to G while retaining high activity (e.g., each of T8, A9, C10, and A11), in which case 6-thio-G at those mutated G positions could be examined in comparison to G. On this basis I suggest that the authors can consider making additional thio substitutions at positions other than G14. If the thio effect observed at G14 is not found at the other positions, then that would strengthen the authors' conclusion by providing evidence that the catalytic improvement effect is specific to G14 rather than more generically observed.

We fully agree with the reviewer that it is essential to have suitable control experiments, in particular if ‘unusual’ effects are observed. We, therefore, had already measured an additional 6-thio-dG modification in parallel to the shown 6-thio-G14. Since this data ‘just’ show what is expected, we had not included it in the last version. However, we understand the reviewer’s concern that the control would increase confidence in the more interesting modification and therefore gladly included the data together with a brief discussion into the new version.

In more detail, our control was 6-thio-G6, which the reviewer correctly acknowledges as the sequentially closest guanine of the loop to G14. This was also one reason for this selection. The other reason was that a 6-thio modification of G6 has already been studied for the Dz^{5A} variant (Nawrot et al., 2007). In strong contrast to our data of the G14 modification, the 6-thio-G6 shows a strong decrease of cleavage activity in the presence of Mg^{2+} while still maintaining moderate activity in the presence of Mn^{2+} (see new Extended Data Fig. 10m). While an accurate quantitative analysis is not attempted here due to the very slow Mg^{2+} induced rates that do not allow proper curve-fitting, this data nicely reproduce the observation reported earlier for the Dz^{5A} variant (Nawrot et al., 2007), including the thio effect and the Mn^{2+} rescue effect, and therefore serve as a suitable control.

We, in general, also agree that additional modifications could be interesting. In respect to the suggested positions, we think that the two remaining guanines, i.e., G1 and G2, may not be ideal, due to their central role in the metal-ion interaction at the metal-ion binding site II, which will likely be disturbed by the thio modification. While also interesting, this is unlikely to add reliable information to the effects seen at position G14. The same applies to T4.
Concerning position T8, we agree that this may be an interesting position, and we particularly like the suggestion of using the additional A15G mutation, although it may be more difficult to separate the effects of the thio modification from the effects of the mutation in this variant. We contacted our supplier regarding the preparation of these samples. Unfortunately, the company (Sigma) has ran out of the thio-modified nucleotides and needs to order them first. Since we also do not want to change suppliers in a comparative analysis, including these new constructs would therefore lead to a delay of about 6–8 weeks. Since the reviewer also acknowledges that this will '*be not critical to establishing the conclusion of the manuscript*', and we can readily include the arguably most suitable 6-thio-G6 control experiment, we believe that the additional data, albeit potentially interesting, do not justify the required delay. Nevertheless, we thank the reviewer for this suggestion, which we will gladly include in a follow-up study.

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2. In the summary paragraph, sentence 2, the authors claim that "the high expectations [for therapeutic and biotechnological potential] have not been met yet, a fact that can be traced back to the lack of high-resolution and time-resolved information about its mode of action". However, in my view this claim oversells the value of such information for this potential, both in vitro and in vivo. Most such applications do not depend on knowing mode of action, i.e., mechanism, as the applications would be enabled by nearly any RNA-cleaving entity regardless of how it cleaves the RNA. High-resolution information (including time-resolved information that enables model formulation) can indeed aid in fine-tuning catalytic activity, e.g., by single-atom replacement as the authors have done here at G14, and increases in catalytic activity (e.g., faster rate) can improve applications in vitro and in vivo. But the need for improved catalytic activity is hardly the sole reason that therapeutic and biotechnological potential of RNA-cleaving DNAzymes has not been fully realized, and in many cases especially in vitro, the existing catalytic activity is not limiting for applications. As the authors cite (e.g., ref. 18 from these authors), in vivo DNAzyme activity is limited by the free Mg2+ concentration, and having a faster DNAzyme would help to counteract the low free Mg2+ concentration in vivo. But other serious concerns (delivery, turnover, stability...) are just as important and are not rendered unimportant by improving catalytic activity. Also there has been considerable success in identifying RNA-cleaving DNAzymes with substantial catalytic activity yet little or no free Mg2+ requirement by including one or more kinds of modified nucleobases, in numerous publications primarily by D. Perrin but not cited here by the authors. If the main obstacle to in vivo activity of RNA-cleaving DNAzymes were simply the rate constant in a low-Mg2+ environment, then Perrin's approach has likely already solved the problem without the benefit of high-resolution structural information.

We are grateful for the reviewer's discussion of this point and have modified the respective statements in the summary paragraph and the introduction, which now also acknowledges the important contributions by the Perrin lab.

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3. On p. 3, lines 51-54 include a list of advantages of DNAzymes relative to protein or RNA. But many of these advantages are relevant only relative to protein or are not really advantages at all. In the first category are "straightforward design" and "independence of protein cofactors", as both ribozymes and DNAzymes can be targeted to nucleic acid substrates in straightforward fashion, and both can readily be independent of protein cofactors. In the second category is "absence of permanent effects on the genome", as none of the three biopolymers necessarily causes permanent effects on the genome, and all three biopolymers are used therapeutically.

We appreciate the reviewer's feedback on the mentioned statement, which initially contained an explicit pairwise comparison between Dz to protein, siRNA, antisense oligonucleotides, ribozymes, and CRISP/Cas9, but was than reduced due to space limitations (the advantages of DNAzymes over ribozymes would be the smaller size and more cost-effective production). We agree that this version was misleading and largely removed the comparative aspect.

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4. The authors refer in their manuscript title to "DNA-mediated catalysis". Because several kinds of catalyst whose actions are mediated by DNA are not DNAzymes, such as the use of double-stranded DNA as a chiral ligand as well as DNA-templated synthesis, I suggest that it would be clearer and more accurate to use the more specific term "DNAzyme catalysis" in the title.

We thank the reviewer for this suggestion and changed the title accordingly.

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5. The summary paragraph never states that NMR spectroscopy is the method used here for structure determination. Given the importance of using NMR for the achievement, the method should be stated clearly in this paragraph. Perhaps NMR should be included in the manuscript title as well.

We agree that this information would be helpful and included the 'NMR information' into the summary section. Since we would like to keep the title simple and overall used an integrative approach covering a wide range of techniques, we would prefer not to include NMR in the title.

6. In Fig. 1, are the data in panel d the same as in the upper part of panel e (i.e., Dz-5C:RNA-2'F, 1H-1H correlation data)? If so, then it would be reasonable to omit the upper part of panel e and just show the lower part for Dz-5A. If not, then the difference between panel d and the upper part of panel e needs to be made clear.

The difference in the shown spectra is the Mg^{2+} concentration. While Fig. 1d is recorded in the absence of Mg^{2+}, the spectra in Fig. 1e are recorded in the presence of 3 mM Mg^{2+} leading to the disappearance of peaks for the loop region in Dz^{5A} but not Dz^{5C}. We added the Mg^{2+} addition to panel e) and modified the caption to make this point clear.

7. For metal ions I, II, and III (Fig. 2e), in the associated text (pp. 8-9) the authors identify the first two of these three ions with steps that they designate as scaffolding and conformational activation, respectively. Referring to "steps" strongly implies a consecutive series of actions or motions, and the setup of describing metal ions I and II in this fashion reinforces the impression. Subsequently in Fig. 3, the authors present their model that involves multiple rate-limiting transient intermediate states. The relationship between this model and the scaffolding and conformational activation states and steps is not made clear, at least in my reading of the submitted manuscript. The sole place that scaffolding and conformational activation steps are mentioned in association with Fig. 3 is briefly on p. 11, line 284.

We thank the reviewer for this helpful comment and agree that the phrase 'steps' inherently (albeit unintentionally) implies a consecutive action. We have omitted this in the new version by just referring to the two aspects as 'scaffolding' and 'conformational activation'.
Concerning Fig. 3a, it should be noted that our experimental approach enabled us to monitor the subsequent/individual addition of monovalent and divalent ions that give rise to the respective states in (Fig. 3a). Under standard buffer or cellular conditions, the individual interactions will form a dynamic equilibrium. We tried to highlight this via the indicated arrows between the states that point in the direction of this equilibrium. To make this point clearer, we now added a brief discussion in the Figure caption.
In general, scaffolding and conformational activation are both effects that we observe and discuss in the manuscript as a consequence of M^{2+} interaction. As correctly pointed out by the reviewer, we cannot deduce a step-wise character of the two effects from our data. Now, when additionally bringing the role of monovalent ions into the system, it gets a bit more complicated. In this case an additional species occurs in the absence of any metal ions (state B_0, see Extended Data Fig. 9a). As shown in Extended Data Fig. 9e-k, the monovalent ions can even mimic some characteristics of the scaffolding and activation effects without being able to promote Dz-mediated substrate cleavage. (The latter is already demonstrated in (Rosenbach, Borggräfe, et al., 2020)). Therefore, we do not explicitly include the scaffolding and activation 'step' in Fig. 3a, but summarize the 'monovalent ion interacting state' as inactive (state B1) and the M^{2+} interacting state as activated (state B2). We included a short discussion when first mentioning Fig. 3a to make this point clearer.

8. On p. 8, line 188, the Co(NH3)6 ion should have a 3+ charge rather than no charge. Same in Methods p. 15 and Ext. Data Fig. 7 (several parts of caption as well as panels j and k).

We have included the 3+ charge to $[Co(NH_3)_6]^{3+}$ throughout the manuscript.

9. In Ext. Data Fig. 8b caption, what does "(Absent)" mean? In 8c caption, "(PAGE-data)" can presumably be deleted. Phrases accidentally remaining from manuscript editing?

In the respective Figure panel, the characteristic peaks are absent, which in this case is a strong indication of an out-of-register flip. We have rephrased the respective sentence in the caption to make this clearer.
The phrase 'PAGE-data' refers to 'Polyacrylamide gel electrophoresis' that was used, in contrast to the detection via FRET. Since this is, however, clearly visible in the Figure itself, we removed the phrase to avoid future confusion.

10. Ref. 18 should be year 2018 (not 2017) and pp. 333-343 (not 1-11). I did not comprehensively check all of the references regarding such details.

We thank the reviewer for bringing this mistake to our attention and have changed the respective reference and carefully checked all other references.

Reviewer 3:

a) The 10-23 deoxyribozyme strongly prefers purine|pyrimidine (R|Y) junctions in the RNA substrates, while other combinations are poor substrates or are not cleaved at all. Do the insights reported in this work give us any clue about the nucleotide preference at the cleavage site? Even if the answer is "no", this aspect should be included in the discussion. Along these lines, the abstract should be revised, as the opening statement on 10-23 and "its ability to cleave virtually any RNA with high selectivity" is not correct, or at least misleading.

We appreciate the reviewer's interest in the implications of our results regarding the known nucleotide preference of the cleavage site. In general, while the observed conformational plasticity of the nucleotides involved in the cleavage reaction offers new insights into the intrinsic molecular properties, it also makes it more difficult to unambiguously narrow down one conformation as the 'correct' one. Regarding the cleavage site, our NMR data can clearly identify stacking interactions of rU_{-1} with rU_{-2}, and we observe that the base-pairing of rU_{-1} is very important for activity. The observed *syn* conformation of rU_{-1} further induces a kink of the RNA backbone, which is similar to the cleavage site formed by the 8-17 DNAzyme, and which potentially promotes an in-line attack arrangement (*vide infra*). The resulting structure may sterically favor the smaller size of pyrimidines at his position as e.g., seen in Extended Data Fig. 8f. While potentially interesting, these aspects are, however, either rather speculative or have already been shown before. We, therefore, believe that they will not strengthen our manuscript.

Nevertheless, and although our work cannot provide a complete answer, we find evidence from the MD simulations that G6 in the catalytic loop could play a role. Substrate specificity, particularly the role of the nucleotides at the cleavage site in DNAzymes or other RNA-splicing macromolecules, can be different or opposite to the preferred R|Y junction in the 10-23 DNAzyme (e.g., "UA" is preferred in the env22 twister ribozyme) and can be fostered by specific interactions (Dahm & Uhlenbeck, 1991; Liu et al., 2017; Ren et al., 2014). We identified aromatic stacking interactions of G6 to rG_0 of the cleavage site in 18.4% of the MD simulations time. Aromatic stacking between purine bases is energetically more favorable at broader angles (Elstner et al., 2001) and could therefore favor the presence of a purine base on the 5'-end of the cleavage site to induce specific backbone conformations at the cleavage site.

This aromatic stacking between G6 and rG_0 is fostered by the presence of Mg^{2+}-ions in metal-ion binding site II, with twice as frequent stacking interactions than in the absence of Mg^{2+}-ions in binding site II. This correlates with our results that Mg^{2+} ions interacting with binding site II induce a conformation in the catalytic loop and substrate necessary for cleavage. Additionally, the interaction between a nucleobase of the cleavage site and G6 of the catalytic loop may explain why chemical modification of G6 but not its neighboring residues results in the loss of catalytic activity of the 10-23 DNAzyme, as e.g. reviewed in (Rosenbach, Victor, et al., 2020). We included a respective discussion in the manuscript (lines 205-210).

We have additionally rephrased the respective opening statement in the summary section.

b) It is well established that the DNA-catalyzed cleavage involves in-line attack of the 2'-OH of R on the scissile phosphate, with 5'-OH of Y as leaving group and generation of a cyclic phosphate on R (here G0). Any relevant structure needs to allow for such an in-line orientation, but this aspect is not considered in the manuscript. The authors should search for relevant conformational states in their MD ensembles and include an appropriate discussion. Along these lines, the authors should more clearly address the long-standing question if 10-23 catalyzes RNA cleavage by a general acid-base mechanism and/or an metal ion-assisted mechanism (with M2+ facilitating 2'-OH deprotonation and coordinating to the the 5'-leaving group). There are some hints in the section that discusses the involvement of C13/G14 "strongly suggesting a direct involvement of C13 and/or G14 in catalysis" (but how?), and the MD data that "corroborate interactions of most functional groups of G14 with hexa-hydrated Mg2+". But how these two statements come together remains unclear from Fig. 3f, where the caption says that Mg2+ also interact with the cleavage site. Also, the authors should compare and contrast their new insights on the structure and mechanism of 10-23 with the previously available structure of the 8-17 deoxyribozyme and its mechanistic implications.

We thank the reviewer for stimulating a more thoroughly analysis/discussion of our data in respect to the cleavage mechanism. Following the reviewer's suggestion, we comprehensively analyzed our MD simulations. In brief, our data suggest that the 10-23 DNAzyme catalyzes RNA cleavage by a general acid-base mechanism. During the simulations, G14 interacts with the O2' of rG_0 in 12.5% of the time, whereas Mg^{2+} only interact with O2' of rG_0 in 2.7% of the time. We could not identify bridging Mg^{2+} between G14 and O2'. This suggests the importance of a direct G14-to-rG_0 O2' interaction for the deprotonation of O2'. In addition, we identify interactions between Mg^{2+} and the O5' of rU_{-1} in 5.9% and G14 in 6.8% of the time. The hexa-hydrated Mg^{2+} could act as a proton donor to O5' to facilitate the cleavage in the intermediate state of the reaction, similar to the Pb^{2+} ion in the 8-17 DNAzyme crystal structure and the general mechanism proposed for RNA cleavage (Breslow, 1993; Liu et al., 2017). At physiological pH, guanine can act both as a proton donor and acceptor and has been implicated in fulfilling either role in the VS ribozyme (Wilson et al., 2007). In an analogous manner G14 in the 10-23 DNAzyme might first deprotonate O2' and then protonate O5' at the cleavage site.

We also identify the O2' atom of rG_0 and the P and O5' atom of rU_{-1} in an in-line attack angle in 3.3% of the time, whereas we identify this conformation between rA_{+7} and rU_{+6}, a similar base pair in the helical region, in 0.0% of the time. The in-line attack configuration of the cleavage site occurs three times more often if Mg^{2+} is present in metal-ion binding site II, again suggesting that Mg^{2+} presence at this site conformationally primes the enzyme and substrate for cleavage. If we combine the interactions of heteroatoms of G14 to rG_0 O2' and the in-line attack angle of the cleavage site, we identify an interaction between O2' and G14 with a simultaneous in-line attack angle in 0.8% of the time, whereas the interaction between Mg^{2+} and O2' is four times less likely. This corroborates our above findings of an acid-base mechanism for the 10-23 DNAzyme similar to the 8-17 DNAzyme, and the low percentage of such configurations correlates with the comparatively slow reaction kinetics of the 10-23 DNAzyme. In this line, Mg^{2+} binding to G14 could sterically hinder the interaction to rG_0 O2' and reduce the basicity of G14 by reducing the electron density in the purine ring. Thio-

modification of G14 consequently would reduce Mg^{2+} binding and, thus, foster O2' deprotonation by G14, in line with the increased catalytic activity of thio-G14 in the presence of Mg^{2+}. Additionally, we could identify interactions between G14 and rG_0 O2' with a simultaneous in-line attack angle exclusively in cluster 1, which we consider to represent the active conformation of the complex.
Taken together, we suggest an acid-base mechanism for the 10-23 DNAzyme dependent on an in-line attack angle in the cleavage site with G14 of the catalytic loop acting as the base and hexa-hydrated Mg^{2+} or G14 protonating O5' of rU_{-1} to foster the cleavage reaction. This is very similar to the mechanism proposed for the 8-17 DNAzyme. Furthermore, we find that the Mg^{2+} binding to the metal-ion binding site II acts as a conformational switch priming the structure for cleavage. We included this point by additional discussions and Figures in the manuscript.

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c) How can the results help to rationalize the faster cleavage rate of the Dz5A vs Dz5C variant? All studies were performed with Dz5C in order to avoid a potentially problematic palindromic sequence motif in the catalytic loop. However, the sequence containing the palindrome cleaves RNA faster. Please also include rate constants to allow for a more quantitative description.

To be able to reliably answer this interesting question about the origin of the faster cleavage rate of Dz^{5A} vs Dz^{5C}, we have designed and carried out additional experiments that also include the inactivating mutation A5G. Our data strongly indicate that the 5C variant, unlike the 5G variant, adopts a similar structure as the 5A variant and that the reduced cleavage rates are a consequence of moderately reduced Mg^{2+} affinities for the identified metal-ion binding site II (in line with its role for conformational activation). Our data further supports that the results obtained using Dz^{5C} are to a very large extent representative also of Dz^{5A}.
We have included two new subfigures (Fig. 2g,h) and a respective discussion in the main text.

We have also included the rate constants of the reactions shown in the respective Figures (also see point 5 in "Further considerations for a revision").

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d) It is surprising that the authors did not comment on or use any information from from 31P-NMR. Previous phosphorothioate substitution and rescue experiments with thiophilic Mn2+ ions have identified functionally important phosphate groups in the 10-23 DNAzyme (e.g. Nawrot et al., FEBS J., 2007, 274:1062). The same work also used 6-thioguanosine (s6G) and its metal ion interactions in the 10-23 DNAzyme, and this earlier work should be cited and discussed, especially because the authors also performed studies using Mn2+ and s6G.

We agree with the reviewer that ^{31}P may offer additional useful information about the system. Indeed, we had carried out a series of ^{31}P NMR experiments in the early phase of the project. However, in contrast to several reported NMR studies carried out on smaller nucleic

acid (model) systems at very high concentrations (typically in the range of 2 mM), the respective spectra on the Dz·RNA complex were severely limited by low signal-to-noise levels and limited peak resolution [**REDACTED]** We have included a short discussion in the NMR assignmentparagraph in the Methods section.
Regarding the very useful work of Nawrot et al., we thank the reviewer for bringing this oversight to our attention. In fact, the mentioned thioguanosine modification, in particular at loop position 6, served as a valuable control experiment in our study and hence certainly should be cited (also see reviewer 2, point 1). We have corrected this oversight in the revised manuscript.

[REDACTED]

Further considerations for a revision:

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1. The "unexpected but effective fold" needs a better structural description. Fig. 2d and the description in the text that says "a condensed core region involving an additional turn of the Dz's loop around the RNA", is not sufficient to understand and appreciate the complexity of the 30 fold. Also, the authors need to explain more precisely how the DNA architecture with the RNA threaded through a structured DNA loop is compatible with the multi-state scheme in Fig. 3a, which suggests that both binding arms are hybridized before the core loop folds in the activated state. However, the "additional turn" mentioned above is absent in Fig. 3a, and the supplementary discussion describes a pre-structured DNA loop even in the absence of the RNA.

We have added additional figures to the manuscript to highlight the features of the fold, which comprise the view of the structure from the 'other' side (Fig. 1g, right), a schematic model explicitly highlighting the turn of the loop around the substrate (Fig. 1h), as well as a stereo view of the structure (Supplementary Figure S2).

In general, the inactive complex states mentioned in Fig. 3a could be a result of two scenarios, either i) DNAzymes that have not formed the 'additional turn' around the RNA and would need to dissociate one binding arm to be activated, or ii) DNAzymes that have already formed the additional turn and merely need to be restructured in key regions of the loop by the metal ions. Our data strongly favor the second scenario. Yet, we detect an off-pathway state, which is present in parallel to the on-pathway inactive state (see Extended Data Fig. 9e-g) at low ionic strength. It is tempting to speculate that this off-pathway state represents Dz·RNA complexes that have formed without the additional turn of the loop around the RNA and that this conformation is only found in the absence of metal ions. The presence/addition of metal ions could then favor a rehybridization of the active fold. Notably, the schematic illustration of the inactive complex (state B1) represents the correctly hybridized state containing the additional turn.
We have included a brief discussion in the manuscript (lines 247- 251) and in the caption of Extended Data Fig. 9 to make this point clear.

In the discussion of the single-stranded Dz, we describe a 'minimally structured central region' in the Dz that resembles features found for the same region after the complex formation. We further describe these features as T-stacking and similar chemical environment for distinct regions of the loop. We believe that this description is well in line with the features found after complex formation including the additional turn of the loop around the substrate. However, to avoid potential overinterpretation of the preformed character of the single-stranded Dz, we included a remark about the transient and locally restricted character of this aspect in the Supplementary Discussion.

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2. On what basis were the six 2'F positions chosen for the Dz6xF variant used for 19F-STD experiments? The effect of 2'-F substitution in the catalytic core should be compared with previously available data on 2'-OMe derivatization (Schubert et al., NAR, 2003, 31:5982), which was found to be tolerated at six positions in the catalytic core.

We thank the reviewer for bringing the missing discussion of the 6xF selection to our attention. In this respect, the two ^{19}F modifications in the arm region were designed to provide a reference for the respective distance in ^{19}F,^{19}F NOESY experiments. However, neither the anticipated cross peak nor any other ^{19}F-^{19}F NOE peak could be detected, establishing a distance detection limit of this approach (data not shown) and promoting the ^{19}F-STD approach as a useful alternative.
The selection of the four additional ^{19}F labels in the catalytic loop was carried out using a combination of previously published positions, which could be modified in different ways

without affecting the activity, and a good distribution over the loop region. In this respect the work by Schubert et al. is indeed an important reference showing that (single) 2'-OMe modifications at all the 4 positions that we used in the catalytic loop of Dz^{6xF} did not considerably affect the activity of Dz^{5A}.
We included a discussion of the basis for the Dz^{6xF} design in the respective ^{19}F-NMR Methods section and included the above-mentioned reference.

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3. Some figure panels and labels are too tiny for good readability at 100% (on screen and on printout). The figure captions need to more precisely describe what is shown. For example, Fig. 2a and ED Fig. 1d mention "nucleotide-specific resonances", but it remains unclear which resonances for each nucleotide were in fact analyzed (or sum or average?).

We have adjusted a large set of subfigures to increase readability.
Concerning the Figure captions, the respective caption of Fig. 2a (now Fig. 1f) has been rewritten to:
Nucleotide-specific dynamic measurements of the 1'-CH moieties in uniformly ^{13}C-labeled Dz (T_1, black and HetNOE, turquoise).

The caption of Extended Data Fig. 1d (now Extended Data Fig. 1e) now reads:
To enable a reliable comparison between different nucleotides, the changes of the cross-peak intensities of the correlation between H1' and H6/H8 are shown for each Dz nucleotide.

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4. Fig. 3f: Please label the yellow ball; is it the P atom of the scissile phosphate? Please also include the 2'-OH nucleophile, and indicate from which MD snapshot this image was taken.

We thank the reviewer for this suggestion and have included a new subfigure, originating from point 2.), that includes the respective changes. We have also included information on the MD snapshot in the respective Methods section.

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5. Fig. 3g: The kinetics trace for 6-oxo-G14 is a reference, and should correspond to data shown for Dz(5C) in Fig. 1b, but shows significantly slower cleavage. How are the conditions different? Please include the rate constants.

The difference in the respective cleavage rates of the same Dz^{5C} construct is indeed a consequence of different conditions, that is, the different Mg^{2+} concentrations used in the shown experiments. The data shown in Fig. 1b were recorded in the presence of 3 mM Mg^{2+}, which was selected to match the concentrations used in the shown NMR spectra Fig. 1e. The

data shown in Fig. 3g were recorded in the presence of 1 mM Mg^{2+}, which was the concentration also used in the real-time NMR spectra shown in Fig. 3. We have included the missing information about the Mg^{2+} concentration in the figure panel and caption of Fig. 3g. We have also added the rate constants in the text.

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6. Nomenclature: Syn and anti orientation of the nucleobase describe conformations, and not configurations as stated in the manuscript. (syn and anti conformers can be accessed via rotation around the glycosidic bond; no covalent bonds needs to be broken and reconnected, as it would be the case for syn/anti configurations).

We appreciate the reviewer's correction of the used nomenclature and changed the manuscript accordingly.

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Reviewer Reports on the First Revision:

Referee #1:

The authors have answered all my previous suggestions, including additional experiments. Thus, I strongly recommend acceptance of this manuscript in its current form.

Referee #2:

The authors have revised their manuscript in response to the three reviewers' comments (I was reviewer 2). I am satisfied with all of the changes directly in response to my own comments. The inclusion of the additional 6-thio-G6 data is very helpful for establishing the conclusion from the key 6-thio-G14 experiment. The changes in response to the other two reviewers also appear satisfactory, although for several of the technical NMR comments from reviewer 1, I must leave the assessment to the other reviewer. Therefore I recommend publication of this manuscript in Nature as resubmitted, with two minor modifications to the newly added text as listed below.

1. Line 70: Fix subscript MgCl - either MgCl2 or just Mg, or move "3 mM MgCl2" out of the subscript. Same for line 330.

2. Line 315: "a metal ion transfers a proton" is not the best phrasing of this mechanistic step.

Scott Silverman

Referee #3:

The authors have responded well to the reviewers' concerns and requests and significantly improved the accessibility of their results and the conclusions. The figures and captions are now of high quality and easier to read, and the additional data and analyses included helped to focus the discussion.

I have only few minor remarks.

a) The authors collected supporting arguments for a general acid-base mechanism, involving the residue G14. However, the influence of the s6G variant is only discussed with respect to metal ion coordination, but significant pKa perturbation should also be considered.

b) It remains unclear what the authors mean by "aromatic stacking between purine bases is energetically more favourable at broader angles". Which angels are they referring to? In general, and in the context of stacking between rG0 and G6. Please rephrase.

c) The manuscript describes the 10-23 DNAzyme as "the most effective" and "most efficient" RNA-cleaving DNAzyme, while in the reply letter, when discussing the comparison to the 8-17 DNAzyme, the authors point out the "comparatively slow reaction kinetics of 10-23". Such comparisons always need to take the reaction conditions into account. There is no need for the superlative in the manuscript, and I suggest to remove it.

In summary, I consider the revised version of this study a suitable candidate for publication in Nature.

Author Rebuttals to First Revision:

Referee #1:

The authors have answered all my previous suggestions, including additional experiments. Thus, I strongly recommend acceptance of this manuscript in its current form.

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The authors have revised their manuscript in response to the three reviewers' comments (I was reviewer 2). I am satisfied with all of the changes directly in response to my own comments. The inclusion of the additional 6-thio-G6 data is very helpful for establishing the conclusion from the key 6-thio-G14 experiment. The changes in response to the other two reviewers also appear satisfactory, although for several of the technical NMR comments from reviewer 1, I must leave the assessment to the other reviewer. Therefore I recommend publication of this manuscript in Nature as resubmitted, with two minor modifications to the newly added text as listed below.

Scott Silverman

1. Line 70: Fix subscript MgCl - either MgCl2 or just Mg, or move "3 mM MgCl2" out of the subscript. Same for line 330.

We appreciate the reviewer's comment and fixed the subscript to MgCl2.

2. Line 315: "a metal ion transfers a proton" is not the best phrasing of this mechanistic step.

We agree and changed the term to "a metal ion-coordinated water molecule transfers a proton"

Referee #3:

The authors have responded well to the reviewers' concerns and requests and significantly improved the accessibility of their results and the conclusions. The figures and captions are now of high quality and easier to read, and the additional data and analyses included helped to focus the discussion.

I have only few minor remarks.

a) The authors collected supporting arguments for a general acid-base mechanism, involving the residue G14. However, the influence of the s6G variant is only discussed with respect to metal ion coordination, but significant pKa perturbation should also be considered.

We thank the reviewer for her/his suggestion. In general, the lower basicity of 6-thio-G14, i.e., p*K*a from 9.3 (dG) to 8.3 (6-thio-dG), should lead to a reduced activity. The fact that we still observed an increased activity therefore strongly indicates that the metal-ion effects outweigh the p*K*a effect. We included the following modification (marked in red) in the respective section in the manuscript:

Lines 272-284: "In this picture, Mg^{2+} binding to G14 could sterically hinder the interaction with rG_0 O2' and reduce the electron density in the purine ring, lowering the basicity of G14.

A 6-thio modification of G14 offers the opportunity to reduce these potentially catalytically detrimental Mg^{2+} interactions. In general, the substitution of oxygen by sulphur in 6-thio-dG lowers the basicity and strongly decreases Mg^{2+} affinity while still maintaining interactions with Mn^{2+}. Interestingly, the 6-thio-G14 modification indeed leads to an about 6-fold increased cleavage rate in the presence of Mg^{2+} (Fig. 3g, $k_{obs,\ 1mM\ MgCl2} = 7.32 \cdot 10^{-4}\ s^{-1}$ and $4.16 \cdot 10^{-3}\ s^{-1}$ for oxo- and thio-G14, respectively). By contrast, decreased rates are observed in the presence of Mn^{2+} (Extended Data Fig. 10m). This behaviour strongly differs from a 6-thio modification at position G6 used as a control[23] and is in line with an acid-base mechanism involving the nucleobase of G14 that competes with catalytically unfavourable Mg^{2+} interactions of the 6-oxo group of G14. Our data indicate that reducing these Mg^{2+} interactions via a 6-thio modification even outweighs the effects of the associated lowered pK_a."

b) It remains unclear what the authors mean by "aromatic stacking between purine bases is energetically more favourable at broader angles". Which angels are they referring to? In general, and in the context of stacking between rG0 and G6. Please rephrase.

We agree that the used terms could be misleading and rephrased the respective sentence to:

Lines 175-178: "Since, in general, aromatic stacking between purine bases is energetically more favourable over a broader range of twist angles[18], these data may provide a clue on the purine|pyrimidine preference of the cleavage site, the activating role of the Mg^{2+} binding in metal-ion binding site II, and the well-known importance of G6 in the catalytic loop[3]."

c) The manuscript describes the 10-23 DNAzyme as “the most effective” and “most efficient” RNA-cleaving DNAzyme, while in the reply letter, when discussing the comparison to the 8-17 DNAzyme, the authors point out the “comparatively slow reaction kinetics of 10-23”. Such comparisons always need to take the reaction conditions into account. There is no need for the superlative in the manuscript, and I suggest to remove it.

We fully agree that the catalytic rates are strongly dependent on the reaction conditions. To avoid potential misunderstandings, we rephrased the respective sentences, focusing on the clearly outstanding role of the 10-23 DNAzyme as one of the most prominent DNAzymes.

Please note that the sentence about “the comparatively low catalytic activity”, was intended to compare DNAzymes to the manyfold more effective protein-based catalysts. We also modified the respective sentence to make this clearer. The modified sentences now read:

Line 23: “The 10-23 DNAzyme is one of the most prominent catalytically active DNA sequences[1,2]”

Lines 53-55: “Yet, all catalytically relevant states of one of the most prominent RNA-cleaving DNAzymes, i.e., the 10-23 DNAzyme[1], eluded high-resolution characterization attempts.”

Lines 269-272: “The interaction between G14 and O2’ and the in-line attack conformation (Fig. 3f) occur simultaneously during 0.8% of the MD simulations time (Extended Data Fig. 10k), which may be related to the still low catalytic activity of the 10-23 DNAzyme, as compared to protein-based catalysts.”

Lines 289-291: “With the here presented atomic-level and time-resolved mechanistic description of one of the most potent RNA-cleaving DNAzyme, we aim to initiate the knowledge-based development of further enhanced next-generation DNAzymes."

In summary, I consider the revised version of this study a suitable candidate for publication in Nature.