nature research

Peer Review File

Manuscript Title: Site-specific RNA methylation by a methyltransferase ribozyme

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1:

The manuscript by Hoebartner and coworkers presents the highly original concept of developing a ribozyme for nucleobase methylation involving the transfer of a methyl group from a heterocyclic cofactor (O6-methylG) to a target base in RNA, generating N1-methylA, and by rearrangement, N6-methylA. What is truly refreshing here is to see tackled an entirely new problem beyond what most ribozymes do, namely hydrolytic cleavage or its reverse. I also appreciated the detective story of sorting out exactly where the methyl group landed on the target RNA. Finally, the application of the ribozyme to install a methyl group on specific sites in tRNA demonstrates the utility of ribozyme.

Overall, this is a brilliant piece of work and worthy of publication in Nature. The experimental work is very thorough, and the conclusions are sound.

I have only very minor questions about the experimental work. (1) I am surprised not to see any degradation of the RNA after treatment at pH 10, 65 °C. Did the authors push the Dimroth rearrangement any further and begin to see strand hydrolysis? (2) 40 mM MgCl2 seems really excessive to me. Is this really necessary to keep the RNA folded? Do the authors imagine any role for Mg2+ in the methyl transfer reaction?

Referee #2:

In this manuscript, Hobartner and coworkers report the in vitro selection of a ribozyme with alkyltransferase activity using a biotinylated benzylguanine as the alkyl donor. The selected cis-acting ribozymes modified specific adenosine residues and could be rationally converted into trans-acting ribozymes. Systematic investigation revealed that neither the biotin nor the benzyl residue were required for activity, and using o-methylguanine as donor, the ribozyme could install a methyl group in a site-specific manner on various RNAs, including tRNAs. Sophisticated chemical and biochemical analysis revealed that ring nitrogen N1 of adenine is specifically methylated, demonstrating that ribozymes are able to install an RNA modification that is relevant in present-day biology.

This is important research, a clever idea, and carried out with the appropriate diligence. The senior author is a leading expert in the field, and has contributed heavily to the development of selection and characterization methods for ribozymes and deoxyribozymes. The story has been nicely told, and I find all claims sufficiently supported. This is certainly work that deserves publication in a journal with double-digit impact factor.

However, I am not fully convinced that the novelty of the work justifies publication in Nature. After all, the alkylation of RNA ring nitrogen atoms was one of the first activities reported for in vitro-selected ribozymes 25 years ago (C. Wilson and J.W. Szostak, Nature 1995, 374:777, not cited). These authors used a similar strategy (involving a biotinylated alkylation substrate), and established the alkylation site (by today's standard one would say tentatively, but at the time this was state of the art) as N7 of a particular guanosine. These authors reported 10^7-fold rate acceleration over the background reaction, and an estimated kcat of 0.01 s^-1. The major difference to the current study is that this ribozyme required the full biotinylated donor as substrate and was not able to transfer smaller groups. Several other papers report N-alkylations of RNA by in vitro selected ribozymes, e.g.

A.K. Sharma, ACS Chem. Biol. 2014, 9:1680, and S. Ameta, Chem. Sci. 2013, 4:957. Of course, I acknowledge that the N1-methylation produces a real biological RNA modification, in contrast to those other examples. However, the manuscript does not report or imply that the ribozymes themselves play a role in present-day biology.

Referee #3:

In this excellent and very interesting study, Scheitl and co-workers describe, for the first time, a methyltransferase (MTase) ribozyme. A ribozyme capable of transferring a methyl group from O6-methylguanosine (m6G) to the N1-position of adenosine in an RNA sequence was evolved by a rather ingenious in vitro selection strategy, and it was shown that similar ribozymes mediated the installation of 1-methyladenosine modifications into tRNA molecules at their naturally occurring positions. The experiments are logically connected and well presented, and the data appear very convincing, with numerous important controls included. The concusions are well supported by the data. The paper is very well written and easy to follow. Clearly, the results have important implications regarding how MTase ribozymes may have functioned in an ancient RNA World, and further developed MTase ribozymes may find important applications as tools to introduce methyl groups at specific locations in nucleic acids.

Minor comments/questions:

a) The MTase ribozyme appears to be rather inefficient; reactions are performed with a large (10-fold) excess of ribozyme relative to substrate, and long incubation times (23 hr) are required for near-complete methylation to be reached. Also, no evidence is presented that more than a single turnover can be achieved, but maybe is this not to be expected as the ribozyme may remain attached/hybridized to its substrate post-methylation.

b) Related to a): When used for tRNA methylation, the ribozyme will have to disrupt the tRNA structure (and, in addition, specific "disruptor RNAs" are used for some tRNAs). It would therefore be interesting to know whether the tRNA regains it structure and functions (e.g. its ability to become aminoacylated) post-methylation, or whether this is precluded by the ribozyme maintaining its disruption of the tRNA structure (this is maybe likely).

c) This study could potentially have been brought to a higher level by demonstrating that one or more of the constructed tRNA MTase ribozymes, when introduced into the relevant organism/cell, could actually complement an inactivating/disrupting mutation of the gene encoding the MTase responsible for the given methylation event (typically by showing that tRNA methylation is restored). However, I realize that this may be challenging, as the unconventional methyl donor m6G would have to be supplied externally, and there may be issues related to its stability and cell permeability.

Author Rebuttals to Initial Comments:

NOTE: Author responses in black

Point-by-point reply to the referees' comments on manuscript 2020-03-04556

We thank the reviewers for their enthusiastic and constructive comments. We address all the queries of the three reviewers as outlined below. The corresponding changes are marked in red in the manuscript. The reviewer's queries are marked in blue and our response is in black.

Referee #1: (expertise: RNA modifications)

The manuscript by Hoebartner and coworkers presents the highly original concept of developing a ribozyme for nucleobase methylation involving the transfer of a methyl group from a heterocyclic cofactor (O6-methylG) to a target base in RNA, generating N1-methylA, and by rearrangement, N6-methylA. What is truly refreshing here is to see tackled an entirely new problem beyond what most ribozymes do, namely hydrolytic cleavage or its reverse. I also appreciated the detective story of sorting out exactly where the methyl group landed on the target RNA. Finally, the application of the ribozyme to install a methyl group on specific sites in tRNA demonstrates the utility of ribozyme.

Overall, this is a brilliant piece of work and worthy of publication in Nature. The experimental work is very thorough, and the conclusions are sound.

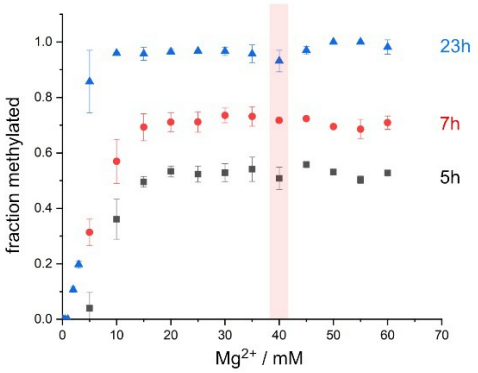
We thank the referee #1 for sharing our excitement about the first methyltransferase ribozyme and appreciate the very supportive comments about our work. We are happy to answer the minor technical questions, and provide some additional data.

I have only very minor questions about the experimental work.
(1) I am surprised not to see any degradation of the RNA after treatment at pH 10, 65 °C. Did the authors push the Dimroth rearrangement any further and begin to see strand hydrolysis?

The reviewer's assumption is correct. Longer incubation times will lead to significant strand degradation. The conditions for our experiment were chosen to achieve a large extent of Dimroth rearrangement and minimal strand cleavage. After 1 hour at 65°C, less than 5% degradation was observed, and the extent of Dimroth rearrangement exceeded 50%. This degree of conversion is consistent with previous reports (Nature 2016, 530, 441). Milder conditions (pH 9, 37°C) require longer incubation times and lead to significantly less conversion (PNAS, 2019, 116, 6784). The pH-dependence of the m^1A-to-m^6A conversion by Dimroth rearrangement is well established (Biochemistry 1968, 7, 3435; Chem. Pharm. Bull 1975, 23, 54).

(2) 40 mM MgCl2 seems really excessive to me. Is this really necessary to keep the RNA folded? Do the authors imagine any role for Mg2+ in the methyl transfer reaction?

Mg^{2+} is an essential cofactor for the ribozyme, and may play structural and/or catalytic roles. The concentration of 40 mM Mg^{2+} was used throughout the *in vitro* selection experiment, based on previous experience. Given that other *in vitro* selected ribozymes (e.g. polymerase ribozymes) work at 100 mM Mg^{2+} or more, the concentration of 40 mM Mg^{2+} in our methyltransferase reaction is still moderate. However, we are pleased to report that the MTR1 ribozyme provides equally good yields when the Mg^{2+} concentration is reduced to 10 mM. Below 5 mM Mg^{2+}, the final yield drops significantly. Please see below the methylation yields obtained after incubation with various Mg^{2+} concentrations between 0.5 and 60 mM.



Reply Figure 1. Mg^{2+}-dependence of MTR1-catalysed RNA methylation. The yields after 5, 7, and 23h incubation are given as fraction methylated at different Mg^{2+} concentrations. Almost quantitative methylation after 23 h is observed between 10 mM and 60 mM Mg^{2+}. 5 mM Mg^{2+} still leads to > 80% methylated RNA. These data have been added to Extended Data Fig. 2.

Referee #2: (expertise: RNA catalysis)

In this manuscript, Hobartner and coworkers report the in vitro selection of a ribozyme with alkyltransferase activity using a biotinylated benzylguanine as the alkyl donor. The selected cis-acting ribozymes modified specific adenosine residues and could be rationally converted into trans-acting ribozymes. Systematic investigation revealed that neither the biotin nor the benzyl residue were required for activity, and using o-methylguanine as donor, the ribozyme could install a methyl group in a site-specific manner on various RNAs, including tRNAs. Sophisticated chemical and biochemical analysis revealed that ring nitrogen N1 of adenine is specifically methylated, demonstrating that ribozymes are able to install an RNA modification that is relevant in present-day biology.

This is important research, a clever idea, and carried out with the appropriate diligence. The senior author is a leading expert in the field, and has contributed heavily to the development of selection and characterization methods for ribozymes and deoxyribozymes. The story has been nicely told, and I find all claims sufficiently supported. This is certainly work that deserves publication in a journal with double-digit impact factor.

We thank referee #2 for these kind comments on our manuscript.

However, I am not fully convinced that the novelty of the work justifies publication in Nature. After all, the alkylation of RNA ring nitrogen atoms was one of the first activities reported for in vitro-selected ribozymes 25 years ago (C. Wilson and J.W. Szostak, Nature 1995, 374:777, not cited). These authors used a similar strategy (involving a biotinylated alkylation substrate), and established the alkylation site (by today's standard one would say tentatively, but at the time this was state of the art) as N7 of a particular guanosine. These authors reported 10^7-fold rate acceleration over the background reaction, and an estimated kcat of 0.01 s^-1. The major difference to the current study is that this ribozyme required the full biotinylated donor as substrate and was not able to transfer smaller groups. Several other papers report N-alkylations of RNA by in vitro selected ribozymes, e.g. A.K. Sharma, ACS Chem. Biol. 2014, 9:1680, and S. Ameta, Chem. Sci. 2013, 4:957. Of course, I acknowledge that the N1-methylation produces a real biological RNA modification, in contrast to those other examples. However, the manuscript does not report or imply that the ribozymes themselves play a role in present-day biology.

We are certainly aware of the ground-breaking work by Szostak in the early 1990's, and we regret that we overlooked to include the reference to the first ribozyme that used iodoacetyl-linked biotin for guanine N7-alkylation. This reference should definitely be included, and has now been added in the introduction, together with references to other alkyltransferase ribozymes (refs 18-21).

For the reasons discussed below, we believe that the reviewer's concerns regarding the novelty of our work should be relieved. We elaborate the key innovative aspects of our selection strategy und explain why we respectfully disagree with the reviewer's conclusion that the methyltransferase reaction described in our manuscript is an obvious extension of earlier work.

Wilson and Szostak (and Sharma et al) used iodoacetamide-conjugated biotin (or fluorescein) as substrate, while Ameta and Jäschke used a chloromethylketone-containing peptide. Upon reaction with their ribozymes, iodide/chloride was the leaving group, and the alkylamide/ketone was transferred to the N7 of a guanine nucleobase within the ribozyme's core sequence. In the work by Sharma et al, the position of the reactive nucleotide was not even determined. To the best of our knowledge, transformation of these N7-G-alkylating ribozymes into site-specific methyltransferase ribozymes has not been reported, and in my opinion, this is for a good reason: because it is not feasible. The RNA-catalysts would need to use methyl iodide as the "truncated" alkylation reagent. Methyl iodide is too small for site-specific binding to RNA, and leads to unspecific RNA methylation at multiple sites, rather than at a specific guanine. A reaction with methyl iodide is similar to DMS, which is used as a reagent for RNA structure probing to map accessible nucleobases, but it does not harbour the propensity for site-specific methylation.

Similarly, the epoxide-reactive ribozyme reported by Liu and coworkers that alkylates N7 of guanine (McDonald et al. Nat. Chem. Biol. 2014) would be impossible to be transformed into a methyltransferase ribozyme. Besides the fact that an epoxide may not be properly positioned by an RNA pocket (due to the lack of sufficient H-bonding and stacking options), such an alkylation would transfer a two-carbon moiety at minimum.

Therefore, other methyl group donors are required, that can make specific non-covalent contacts to the RNA (H-bonding, stacking, etc). This is in analogy to binding of SAM in the active site of protein methyl transferases, which recognize the nucleoside and the amino acid side chain of SAM, in order to position the reactive methyl group for site-specific transfer.

Indeed, SAM was considered as a cofactor for RNA-catalysed RNA methylation: The search for a methyltransferase ribozyme has been discussed in the literature since the 1990s. A report by Burke and Gold in 1997 stated: "As a first step toward isolating methyltransferase ribozymes, we used SELEX to isolate SAM-binding RNA aptamers." (Nucl. Acids. Res. 1997, 25, 2020-2024) However, the attempt failed and no methyltransferase activity was found.

Since then, no ribozyme with the desired methyltransferase activity was found. We are excited now being able to report this coveted catalytic activity of an in vitro selected RNA. Our ribozyme is a real "methyl transferase" that transfers the methyl group from the free nucleobase analog m^6G site-specifically to the N1 of adenine at a defined position within the target RNA. Our selection strategy was designed for the ribozyme to develop a binding site for the "leaving group", i.e. guanine, which is well established as an RNA ligand, even in natural riboswitches.

Upon activation of m^6G, the methyl group is transferred, and unmodified guanine is released. We strongly believe that this strategy was the key to success.

Moreover, our MTR1 ribozyme achieves additional milestones that were not viable with the previously reported alkyltransferase ribozymes. First, the MTR1 ribozyme can easily be used in an intermolecular setup (i.e. in *trans*). This was enabled by the design of our selection strategy (the structure of the random pool and the nested two-step RT-PCR amplification strategy). Second, the ribozyme presents a broad substrate scope and can be addressed to a wide variety of target RNAs simply by changing the sequence of the binding arms to maintain Watson-Crick complementarity to the desired target. This enabled the application of MTR1 for site-specific methylation of in vitro transcribed RNAs, and has now been extended to address native tRNAs.

To further underline our reservation against the referee's critique, we point out a recent example from organic synthetic methodology that demonstrates the distinction of methylation vs alkylation. The C(sp3)-H activation for site-specific alkylation reactions next to heteroatoms is a well-established reaction type, with a broad substrate scope (Chem Soc Rev 2007, 36, 1069). However, the seemingly analogous methylation reaction is not a trivial extension of established alkylation reactions. This challenge was solved only recently by an advanced method for late-stage oxidative C(sp3)-H methylation, that enables the exploration of the "magic methyl" reaction (Nature, 2020, 580, 621; N&V April 29, 2020).

The MTR1 ribozyme catalyses N-methylation at N1 of adenine and is the first methyl-transferase ribozyme discoverd. With its activity to make m^1A, it is a true milestone for RNA-catalysed synthesis of native RNA modifications. We strongly believe that the implications of our work reach far beyond the known N7-guanine alkylations with iodoacetamides or epoxides. Our work reveals the principal requirements for the methylation cofactor and also paves the way to search for ribozymes that catalyse chemically demanding C-alkylation reactions, as would be necessary for the synthesis of m^5C (as an example of another very important natural methylated nucleoside).

However, the manuscript does not report or imply that the ribozymes themselves play a role in present-day biology.

In our opinion this statement is ambiguous. A "freshly" *in vitro* selected ribozyme would not be expected "to play a role in present-day biology", in the sense of "occurring in present-day nature" or "fulfilling a natural function". In contrast, it may become a synthetic "player" in molecular cell biology, and it may mimic an ancient RNA function that may have been lost during evolution. These thoughts are supported by the comment of referee #3, who said: "Clearly, the results have important implications regarding how MTase ribozymes may have functioned in an ancient RNA World, and further developed MTase ribozymes may find important applications as tools to introduce methyl groups at specific locations in nucleic acids." Indeed, our work demonstrate that the MTR1 ribozyme is a useful tool for fundamental research on biological RNA modifications. It allows the site-specific installation of m^1A in tRNAs and other RNAs, which represents a significant synthetic challenge for RNAs of biologically relevant size. Moreover, the ribozyme is active and highly specific for a given target in the context of total cellular RNA, and it can also be produced by the cellular transcription machinery. The additional data presented below (in response to the suggestions by referee #3) may help to relieve the doubts of referee #2, and demonstrate now even more convincingly the novelty and significance of our work to justify publication in Nature.

Referee #3: (expertise: methyltransferases)

In this excellent and very interesting study, Scheitl and co-workers describe, for the first time, a methyltransferase (MTase) ribozyme. A ribozyme capable of transferring a methyl group

from O6-methylguanosine (m6G) to the N1-position of adenosine in an RNA sequence was evolved by a rather ingenious in vitro selection strategy, and it was shown that similar ribozymes mediated the installation of 1-methyladenosine modifications into tRNA molecules at their naturally occurring positions. The experiments are logically connected and well presented, and the data appear very convincing, with numerous important controls included. The conclusions are well supported by the data. The paper is very well written and easy to follow. Clearly, the results have important implications regarding how MTase ribozymes may have functioned in an ancient RNA World, and further developed MTase ribozymes may find important applications as tools to introduce methyl groups at specific locations in nucleic acids.

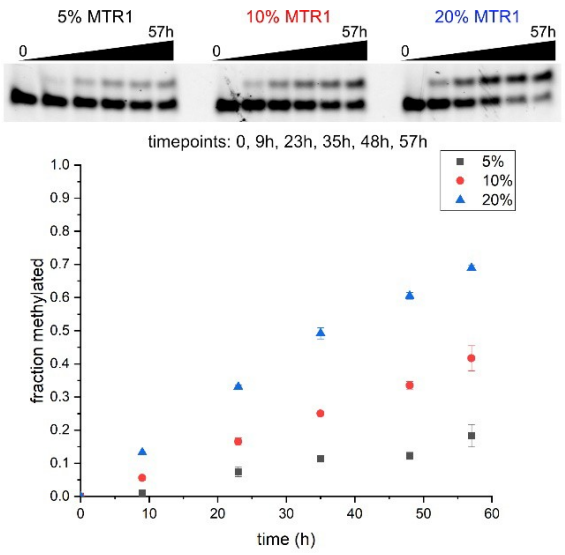
We thank the referee #3 for the very supportive remarks on our work.

Minor comments/questions:

a) The MTase ribozyme appears to be rather inefficient; reactions are performed with a large (10-fold) excess of ribozyme relative to substrate, and long incubation times (23 hr) are required for near-complete methylation to be reached. Also, no evidence is presented that more than a single turnover can be achieved, but maybe is this not to be expected as the ribozyme may remain attached/hybridized to its substrate post-methylation.

Efficiency is not defined in absolute terms, and thus a matter of perspective. From the perspective of a protein MTase enzyme, the MTR1 ribozyme is indeed slow. However, this is not an unusual observation. The majority of RNA-catalysed reactions are significantly slower than their protein-catalysed counterparts (if analogous enzymes evolved in Nature, for example for the catalysis of RNA transesterification, RNA aminoacylation, RNA phosphorylation, etc). From the perspective of RNA-modifying ribozymes, the MTR1 ribozyme is indeed a very efficient ribozyme, that achieves near-complete conversion even for the slower reaction with m^6G (note that the reaction is significantly faster with BG, as shown in manuscript Figure 1). Many other ribozymes never reach such high yields, even after prolonged incubation times. For example, the ribozymes mentioned above in the reply to referee #2, using epoxides for N7G-alkylation (McDonald et al, Nat Chem Biol 2014), used reaction times up to 48 hours and reached yields below 50%.

Single-turnover kinetics with ribozyme in excess over substrate is the standard experiment in the field for the kinetic characterization of ribozymes. We agree that the question about multiple turnover is also interesting, and we have addressed this question experimentally.
A large excess of ribozyme over substrate is not needed. Nearly complete methylation of the RNA substrate was achieved in 23h with equimolar ribozyme and substrate concentration. In addition, we investigated the methylation reaction under multiple-turnover conditions, with sub-stoichiometric amounts of ribozyme. The reaction was run with 5, 10 and 20 mol% ribozyme, and monitored for 57 hours. The results depicted in Reply Figure 2 show that more than 3–4 turnovers were achieved, clearly indicating that the ribozyme:target RNA complex dissociated under the experimental conditions and new target was hybridized and methylated.



Reply Figure 2. Multiple turnover assay for MTR1 ribozyme. Substrate concentration was 1 µM target RNA, and 50 nM (black), 100 nM (red), 200 nM (blue) ribozyme, 100 µM m^6G, pH 7.5, 40 mM $MgCl_2$, 25°C.

b) Related to a): When used for tRNA methylation, the ribozyme will have to disrupt the tRNA structure (and, in addition, specific "disruptor RNAs" are used for some tRNAs). It would therefore be interesting to know whether the tRNA regains it structure and functions (e.g. its ability to become aminoacylated) post-methylation, or whether this is precluded by the ribozyme maintaining its disruption of the tRNA structure (this is maybe likely).

We cannot exclude that the ribozyme may stay partially associated with the tRNA. However, the multiple-turnover observation reported above is a clear indication that RNA is released from the ribozyme to fold into its functional structure. The hybridization kinetics are strongly dependent on the exact conditions, lengths of the binding arms, etc, and may be modulated by temperature, metal ion concentration etc. tRNA methylation was also detected with sub-stoichiometric ribozyme concentrations, and thus it is expected that the released RNA can be aminoacylated (we don't have an aminoacylation assay established to demonstrate this experimentally). The presence of disruptor oligonucleotides is clearly not essential, as demonstrated by site-specific methylation of one specific tRNA type in total *E.coli* tRNA.

The MTR1 ribozyme with binding arms complementary to the TΨC-stem-loop sequence of *E.coli* tRNA-Asp was applied to total *E.coli* tRNA (MRE 600 strain). *E.coli* does not contain m^1A in any of its tRNAs, therefore the successful artificial methylation of *E.coli* tRNA was detectable by LC-MS after digestion into mononucleosides. Moreover, primer extension experiments with six different tRNA-specific primers clearly established that the methyl group was installed at A58 only in tRNA-Asp, but not in tRNAs-Glu, Gly, Ser, His, which all share highly similar TΨC-stem-loop sequences. The distinctive sequence difference is at position 59, which is G in tRNA-Asp, but A or U in the other tRNAs, resulting in a mismatched base pair in the MTR1 ribozyme:non-target-tRNA complexes, which prevents their methylation. In other words, faithful Watson-Crick base-pairs between the target RNA and the ribozyme flanking the target adenosine ensure the selectivity for target methylation, even if large fractions of the binding arms are complementary to non-target RNAs. These new results on natural tRNA substrates provide additional support for the high potential of the MTR1 ribozyme as useful biochemical tool. These data have been included in Figure 3, new panels c and d, and in Extended Data Figure 6.

a) This study could potentially have been brought to a higher level by demonstrating that one or more of the constructed tRNA MTase ribozymes, when introduced into the relevant organism/cell, could actually complement an inactivating/disrupting mutation of the gene encoding the MTase responsible for the given methylation event (typically by showing that tRNA methylation is restored). However, I realize that this may be challenging, as the unconventional methyl donor m6G would have to be supplied externally, and there may be issues related to its stability and cell permeability.

This suggestion is indeed challenging, and it is considered as a long-term goal to apply new ribozymes in cells. Only very few reports exist in the literature that demonstrate *in vitro* selected ribozymes being active in cells, and even less work has been done on engineering ribozymes for *trans* activity on cellular targets. The differences in metal ion availability in the cell compared to the test tube, folding and stability of the ribozyme are some of the challenges to be overcome. Even for the hammerhead ribozyme, an RNA-cleaving ribozyme that is researched for more than 30 years, an *in vivo* evolved variant for engineered *trans* activity in bacterial and eukaryotic cells was only reported last year (Huang et al, Nucleic Acids Res 2019, 47, 2514).

The reviewer's suggestion raised the additional question, if the MTR1 ribozyme would remain active when introduced into a larger RNA construct for potential future cellular applications. To address this question, we designed two plasmids for the expression of MTR1 ribozyme constructs in *E.coli*, containing either a *cis*-active (i.e. self-methylating) MTR1 construct (FBC-MTR1) or a *trans*-active MTR1 (FBT-MTR1) with binding arms directed against endogenous *E.coli* tRNA-Asp. To monitor the expression of the RNA constructs, a fluorogenic aptamer (the DFHBI-binding Broccoli aptamer) was also included. If folded correctly, the *cis* construct would methylate itself when provided with the appropriate concentrations of m^6G and Mg^{2+}.

Both RNAs were prepared by *in vitro* transcription, and their catalytic activity was successfully confirmed by LC-MS and primer extension experiments. These results are very encouraging and suggest that further *in vivo* evolution may be feasible, but this goes beyond the scope of the current study.

As a first test, the FBC- and FBT-MTR1 constructs were also expressed in *E.coli*. After IPTG induction, *E.coli* BL21(DE3) cells were grown in LB medium supplemented with m^6G and $MgCl_2$. Total RNA was isolated, the expression of MTR1 constructs was confirmed by staining with DFHBI, and potential methylation of the target RNA was probed by primer extension. A series of experiments with varying m^6G and $MgCl_2$ concentrations in the LB medium, switching m^6G for BG, and experimenting with incubation time and temperature provided only weak indication of RNA-catalysed RNA methylation taking place in the cell. However, when total RNA was extracted from IPTG-induced cells, and incubated with m^6G/BG and Mg^{2+} *in vitro*, the expected RT stop signals were clearly observed. This indicates that the limitation is not poor production or poor folding of the ribozyme, but rather the availability of the cosubstrate (m^6G) and/or cofactor (Mg^{2+}) inside the cells. The reviewer's suggestion had already pointed out that the external supply of m^6G may be challenging, and this seems indeed to be the case.

Nevertheless, we would like to include the data on the plasmid-encoded ribozyme constructs and the demonstration of their *in vitro* methylation ability. These data are presented in the new Figure 4 of the manuscript and Extended Data Figures 7 and 8.

Reviewer Reports on the First Revision:

Referee #2:

The only change to the manuscript in response to my comments is the addition of a half-sentence that adds the improperly omitted citations of closely related work without any further comment or comparison. I do not understand why the authors did not use the opportunity of a revision to explain to the readers why a methyltransferase ribozyme is a major breakthrough in the presence four known alkyltransferase ribozymes. The authors have valid arguments, as they indicate in their rebuttal letter, but they do not consider them important enough to be mentioned in the manuscript. I think they could have done a better job revising the manuscript.

Referee #3:

In response to my comments, Scheitl et al. have added several new results to the revised version of their manuscript. Most importantly, they present evidence that strengthen the notion that an MTase ribozyme may be active in vivo, i.e. they show that it specifically can modify its tRNA target in the context of E. coli total tRNA, and they demonstrate the activity of plasmid-encoded, E. coli-expressed ribozymes. They also attempted to demonstrate in vivo methylation by supplying m6G and Mg2+ to E. coli cells expressing plasmid-encoded ribozymes, but this was not so surprisingly unsuccessful, likely due to poor cell permeability of the co-factors.

In summary, the authors have responded well to my concerns, and although they have not directly demonstrated in vivo activity for the MTase ribozyme, the revised version of this study is clearly a strong candidate for publication in Nature.

Author Rebuttals to First Revision:

We are happy to learn that Referee #3 was satisfied by our additional experiments and the data we have included, and did not have any additional suggestions.

We also thank Referee #2 for the time to read our reply letter and revisions, and for appreciating our arguments on the significance of a methyltransferase ribozyme. We understand that a more elaborate discussion in the manuscript may be desirable, but due to space constraints it is not possible to include all considerations discussed in our first response letter. We tried our best to explain that previously known alkyltransferase ribozymes cannot be engineered into methyltransferases, but instead a very different approach for *in vitro* selection was necessary for finding our now reported ribozyme. The corresponding statement in the manuscript now reads:

Self-alkylating ribozymes have been described using reactive iodo- or chloroacetyl derivatives,[18-20] or electrophilic epoxides,[21] but the design of earlier *in vitro* selection strategies prevented the emergence of catalysts capable of transferring a one-carbon unit. Thus, ribozymes that catalyse RNA methylation have so far remained elusive.