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Eukaryotic Ribonucleases P/MRP: the crystal structure of the P3 domain

Anna Perederina, Olga Esakova, Chao Quan, Elena Khanova, Andrey Krasilnikov

Corresponding author: Andrey Krasilnikov, Penn State University

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 02 November 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. We have now received the comments of three reviewers, and I am pleased to inform you that all of them find your study interesting and in principle suitable for publication in The EMBO Journal. We should therefore be happy to consider a manuscript revised to address the various specific points raised by the reviewers, pertaining mostly to aspects of presentation and interpretation. Of special note in this respect is however that the referees ask for the inclusion of the modeling experiments and their results (which are currently only discussed as 'not shown') - I agree that this would be important to strengthen the functional/biological implications of your structural work, and given the current brevity of the paper, inclusion even in the main body of the manuscript would be possible.

I am therefore inviting to prepare a revised version of the manuscript along the lines suggested by the referees' comments. Please be reminded that it is EMBO Journal policy to allow a single round of revision only, and that it is therefore essential that you diligently answer all the points raised at this stage. When preparing your letter of response to the referees' comments, please also bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html. In any case, please do not hesitate to get back to us should you need feedback on any issue regarding your revision.

Thank you for the opportunity to consider your work for publication. I look forward to reading the revised manuscript.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The authors present the first crystal structure pertaining to the eukaryotic ribonuclease P: the co-crystal between two proteins of the RNP, Pop6 and Pop7, together with the P3 domain. The results are striking and important for our perception of RNA-protein interactions: the internal loop of P3 does not contain any base pair and forms complex interactions with the two proteins.
I have a couple of points.
- The authors state, already in the abstract, that the RNA domain P3 is not found in bacteria. There is a P3 helix in bacteria, of various lengths. The difference lies in the presence of the large internal loop in helix P3 (which is not always of the size observed for S. cer.). This should be clarified.
- The authors state that they used for crystallization a circular permutation of the domain P3. This may be confusing. In fact, instead of capping the distal part of the helix, they capped the base of the helix.
- The discussion of Fig.4 is very interesting. It should be complemented by a RNA sequence alignment of domain P3. For example, A37 is also a G (e.g. S. kluyv.), which from the figure could fit.
- The last part of the discussion is potentially of great interest to the whole RNA community. A figure of the modeling exercise illustrating the transition from the RNA components to the proteins would be most welcome.

Referee #2 (Remarks to the Author):

The manuscript by Perederina et al. reports a co-crystal structure of the P3 element of S. cerevisiae RNase MRP RNA in complex with a heterodimer of the RNase MRP/P protein subunits Pop6 and Pop7. The crystal structure provides first insight into the architecture of eukaryal RNases MRP/P, and reveals a variety of interesting features specific to this RNA-protein complex: (i) Pop6, Pop7 and Alba proteins share a similar fold despite little sequence similarity between Pop6 and Pop7; (ii) the internal P3 loop interacts primarily with Pop7; the modes of how Pop6 versus Pop7 interact with the P3 element are surprisingly different despite their similar architecture; (iii) some loop nucleotides (nts) serve as integral components of the structure of the N-terminal domain of Pop7; (iv) there are no base pairing interactions between loop nts; (v) stacking of some loop nts causes loop nts 42-44 to loop out in order to form a network of interactions with Pop6; (vi) the good correlation between footprinting data and the crystal structure makes it unlikely that other protein subunits beyond Pop6/7 make substantial additional contacts to the central part of the P3 domain.

Major comments:
1) p. 2, last line, change to: "The protein moiety is required for the activity of bacterial RNase P in vivo." Add here the following citation: Gˆssringer M, Kretschmer-Kazemi Far R, Hartmann RK. (2006) Analysis of RNase P protein (rnpA) expression in Bacillus subtilis utilizing strains with suppressible rnpA expression. J. Bacteriol. 188, 6816-6823.
2) In the Results section, the authors generally use past tense; with the 2nd paragraph on p. 5, the authors should begin to use present tense when they switch from describing the history of their crystallization setup to description of the features of their crystal structure.

3) p. 5, 1st paragraph: provide information where the zinc ions are located in the structure and discuss what their structural role might be.

4) p. 6, 2nd paragraph, line 1: the "However" does not make sense; rephrase.

5) p. 10, 1st paragraph: rewrite: "RNase MRP nucleotides U67, A68, and A69 are replaced with A74, U75, A76, and U77 in RNase P; the nucleotides U35 and U36 are replaced with U37, U38, and

U39 in RNase P, while U43 is missing in RNase P. All these deletions and additions are in bulged out segments of the P3 loop (Figures 2A, 4A) and thus might be accommodated without causing significant changes in the RNA fold or RNA-protein interactions, consistent with available results of mutational studies [Ziehler et al. 2001]."
However, be cautious here. In the absence of a crystal structure with the RNase P P3 element, it cannot be ruled out that the RNase P P3 element has developed a mode of interaction with Pop6/7 which differs in some aspects from that of MRP P3.

6) p. 12, 3rd paragraph: the authors state here that the apical stem of P3 (nt 45-64) is not involved in protein contacts, except for G45 & A46. However, according to Fig. 4 A, nt 61-64 form contacts with Pop6. Please clarify.

7) p. 13, line 2, rephrase: "...the protein moiety, thus indicating that the bulged configuration of G66 and, by inference, of G73 in RNase P, is maintained in the ..."

8) in Figs. 3 D and S1, sequence alignments: use lighter colours than the dark magenta and dark red in order to make the lettering readable.

Minor comments:

9) use "bacterial" instead of "eubacterial" which is outdated (e.g. p. 2, line 2 from bottom; p. 3, lines 1 and 2; p. 14, line 1).
10) p. 6, last line, change to: "... thus ordering this N-terminal region of Pop7 (Figure 2A)."
11) p. 7, 1st paragraph, line 2 from bottom, rewrite: "The positioning of the additional N-terminal beta-strand E0 ...
12) p. 7, 2nd paragraph, line 5: beta-strand.
13) p. 8, 2nd paragraph, last line: "genuine" instead of "proper".
14) p. 10, lines 1 & 2 from bottom, change to: " ... the P3 RNA domain will be involved in only very limited interactions with the P3 RNA domain (with the possible exception of the proximal (right in Figures 1A, B) helical stems)."
15) p. 11, 1st paragraph, line 5: "... showed complete protection ..."
At several locations in the following discussion, the use of past tense (instead of present tense) is appropriate (when discussing results of other studies).
16) p. 12, 3rd paragraph, line 1: showed / line 5: are protected.
17) p. 13, line 5: "... in the RNase P holoenzyme."
18) Legend to Fig. 4, line 2: orange, not yellow.
19) Legend to Fig. 4 E: indicate which residue belongs to which beta-strand.

Referee #3 (Remarks to the Author):

The manuscript by Perederina et al. describes the crystal structure of an RNA-protein complex that forms a domain of eukaryotic RNase P. RNase P is the universally conserved enzyme that is responsible among other things, for processing the 5' terminus of all tRNAs. Although RNase P is a ribozyme, its catalytic RNA component is found associated with proteins. In eukaryotes and archaea, the protein complement of RNase P is quite elaborate. Eukaryotic RNase P RNA has an RNA domain called the P3 domain that associates with several proteins; this domain is absent from bacterial RNase P. Thus, the available structures of bacterial RNase P do not indicate what the structure or function of the P3 domain is. The structure by Perederina et al. shows that the two proteins that bind the core of the P3 domain RNA are homologs, and reveals the basis for their specificity. Moreover, molecular modeling suggests how an additional protein may interact with the P3 domain, and how this domain may participate in RNase P function. Overall, the work appears technically sound. This referee thinks that the manuscript could be improved by making explicit some of the model-based speculations, so that they can serve as starting points for future work in the field. Some suggestions follow.

1. One of the main conclusions of the paper is that the P3 domain would be placed so that it may functionally replace P15.1 and P15.2 of the Bacillus ribozyme (page 13, last line). Rather than being "data not shown" the results of this modeling experiment should be shown at least as a supplementary figure. The authors should also elaborate in the text as to the functional significance

of this, beyond simply "laying the foundation for the evolutionary transition ..."

2. Along the same lines, the abstract should say explicitly what the structure suggests, rather than the uninformative "... structure suggests roles of the P3 domain in the structure ..."

3. Are the affinities of Pop6/Pop7 for each other and for the RNA known? Are they stated somewhere in the MS? And is the affinity unchanged by the circular permutation of the RNA domain?

4. Although the authors point out the structural homology with the Alba proteins, they do not state if there are other structural similarities of Pop6/Pop7 to other proteins. At least a DALI search should be performed, and the most similar protein domains listed and discussed.

5. The solvent accessible surface area occluded in the Pop6/Pop7 interface and in the RNA-protein interface (for each) needs to be stated.

6. What is the estimated purity of the proteins? How was the RNA purified? What is its purity? How was the RNA stored and treated (i.e. was it annealed?) previous to complexation with the proteins?

7. The methods section states that Supplementary Figure S3 shows the density-modified SAD map. The legend to that figure says it is a composite anneal omit map. Which is it? What should be shown is the density modified SAD map.

8. Table 1 needs to list some phasing statistics (FOM, phasing power, Rcullis), and also the coordinate precision (cross-validated sigmaA).

9. A comparison of the secondary structure of eukaryal RNase P/MRP (Fig 1a) with the bacterial form would be useful, perhaps as a supplementary figure.

10. A supplementary figure with the degree of conservation mapped to the solvent-accessible surfaces of the proteins would be useful.

1st Revision - authors' response 17 November 2009

Reviewer 1

- The authors state, already in the abstract, that the RNA domain P3 is not found in bacteria. There is a P3 helix in bacteria, of various lengths. The difference lies in the presence of the large internal loop in helix P3 (which is not always of the size observed for S. cer.). This should be clarified.

We are replacing the sentence referring to the unique role of the P3 domain in eukaryotic vs bacterial enzymes with the following (Abstract, line 6): "The eukaryotic RNases P/MRP have acquired an essential helix-loop-helix protein-binding RNA domain P3 that plays a key role in eukaryotic enzymes and distinguishes them from bacterial and archaeal RNases P."

In addition, we are modifying the first two sentences of the last paragraph on page 3. Now they read: "The transition from the RNA-rich bacterial to the more complex protein-rich eukaryotic enzymes of the RNase P/MRP family was accompanied by the appearance of a new essential and highly conserved RNA element: a helix-loop-helix P3 RNA domain [Lindahl et al. 2000, Ziehler et al. 2001, Li et al. 2002]. The P3 RNA domain is a universal characteristic feature of eukaryotic RNases P/MRP [Piccinelli et al. 2005]."

- The authors state that they used for crystallization a circular permutation of the domain P3. This may be confusing. In fact, instead of capping the distal part of the helix, they capped the base of the helix.

References to the circular permutation are removed. Now the first two sentences of the Results section (page 5, par. 1) read: "The crystallization construct contained proteins Pop6 (18.2 kDa), Pop7 (15.8 kDa), and a modified P3 domain of the RNA component of RNase MRP (46 nucleotides, Figure 1C). The modification changed only distal parts of the helical stems in the P3 RNA domain and did not affect the regions involved in interaction with Pop6/Pop7."

- The discussion of Fig.4 is very interesting. It should be complemented by a RNA sequence alignment of domain P3. For example, A37 is also a G (e.g. S. kluyv.), which from the figure could fit.

We are including a sequence alignment for the P3 RNA domain loops in S. cerevisiae RNase MRP and RNase P (Supplementary Figure S5). Also, we are adding requested sequence data for various P3 domains. We found that in this case secondary structure diagrams are more useful than simple sequence alignments. Accordingly, we are including such diagrams in lieu of the suggested alignments (new Supplementary Figure S1). In addition, we are extending the discussion of Figure 4 and adding the following sentences (Page 7, Par. 1):"These three nucleotides are conserved in a wide range of eukaryotes as an ACR triad (Supplementary Figure S1), suggesting a conserved structural organization of this part of the complex. At the same time, in some cases (such as S. pombe, Supplementary Figure S1) the ACR triad is absent in P3 RNA domains of RNases P/MRP, possibly indicating an alternative structural organization."

The new Supplementary Figure S1 legend reads:" Secondary structure diagrams for P3 RNA domains of RNases MRP/P from various eukaryotes. The ACR triad corresponding to A37, C38, A39, which are an integral part of the Pop7 protein fold in S. cerevisiae RNase MRP, is shown in red. In S. pombe, the ACR triad appears to be replaced by a UCA triad (shown in green). It is not clear whether S. kluyveri RNase P possesses an unusually positioned ACA triad (red) or an alternative GCA triad (underlined in orange). The diagrams are based on [Tranguch & Engelke 1993, Ziehler et al. 2001, Li et al. 2002, Piccinelli et al. 2005, Lopez et al. 2009]."

- The last part of the discussion is potentially of great interest to the whole RNA community. A figure of the modeling exercise illustrating the transition from the RNA components to the proteins would be most welcome.

We are including the requested illustration as Figure 6.

Reviewer 2

Major comments:
1) p. 2, last line, change to: "The protein moiety is required for the activity of bacterial RNase P in vivo." Add here the following citation: Gossringer M, Kretschmer-Kazemi Far R, Hartmann RK. (2006) Analysis of RNase P protein (rnpA) expression in Bacillus subtilis utilizing strains with suppressible rnpA expression. J. Bacteriol. 188, 6816-6823.

This sentence ("The protein moiety is required for the activity of RNase P in vivo.", now pg. 3, first line) was meant to convey that the protein moiety was required for both bacterial and eukaryotic RNase P; changing the sentence as suggested would limit the meaning to just bacterial RNase P. At the same time, we agree with Reviewer 2 that proper references here are needed. We are adding several references pertinent to both bacterial and eukaryotic enzymes, including the suggested reference. The sentence now reads: "The protein moiety is required for the activity of RNase P in vivo [Kirsebom et al. 1988, Chamberlain et al. 1998, Gossringer et al. 2006 and references therein]."

2) In the Results section, the authors generally use past tense; with the 2nd paragraph on p. 5, the authors should begin to use present tense when they switch from describing the history of their crystallization setup to description of the features of their crystal structure.

Changed as suggested.

3) p. 5, 1st paragraph: provide information where the zinc ions are located in the structure and discuss what their structural role might be.

The following paragraph is added (page 8, par. 2): "Two zinc-binding sites were found to be associated with Pop7 in the crystal structure. One zinc ion is coordinated by two symmetry-related histidines 18 in Pop7. While playing a role in crystal formation, this zinc ion is unlikely to be of physiological relevance. The second, highly anisotropic zinc-binding site (modeled as two partially occupied zinc sites) is formed due to the close positioning of His26 and His30 in Pop7. The two histidines belong to the short -helix H0 (Figure 3B); the pair is not well conserved phylogenetically (Supplementary Figure S2) and the importance and physiological relevance of this zinc-binding site are not clear."

4) p. 6, 2nd paragraph, line 1: the "However" does not make sense; rephrase.

Corrected. The paragraph now reads: "Pop6 and Pop7 are basic proteins (pI 9.28 and 9.34, respectively). The crystal structure reveals that positively charged residues are concentrated mainly in the regions directly involved in interactions with the P3 domain RNA (Figure 5)."

5) p. 10, 1st paragraph: rewrite: "RNase MRP nucleotides U67, A68, and A69 are replaced with A74, U75, A76, and U77 in RNase P; the nucleotides U35 and U36 are replaced with U37, U38, and U39 in RNase P, while U43 is missing in RNase P. All these deletions and additions are in bulged out segments of the P3 loop (Figures 2A, 4A) and thus might be accommodated without causing significant changes in the RNA fold or RNA-protein interactions, consistent with available results of mutational studies [Ziehler et al. 2001]."
However, be cautious here. In the absence of a crystal structure with the RNase P P3 element, it cannot be ruled out that the RNase P P3 element has developed a mode of interaction with Pop6/7 which differs in some aspects from that of MRP P3.

Rewritten as suggested (page 10, par. 3, line 3 and on).

6) p. 12, 3rd paragraph: the authors state here that the apical stem of P3 (nt 45-64) is not involved in protein contacts, except for G45 & A46. However, according to Fig. 4 A, nt 61-64 form contacts with Pop6. Please clarify.

Corrected (now pg. 13, line 1). The sentence now reads: "The results of the holoenzyme footprinting showed that the distal (left in Figure 1B, RNase MRP nucleotides 45-64) helical stem of the P3 RNA domain was not protected by the protein moiety, except for nucleotides G45 and A46, which have their phosphate backbones protected in the presence of proteins."

7) p. 13, line 2, rephrase: "...the protein moiety, thus indicating that the bulged configuration of G66 and, by inference, of G73 in RNase P, is maintained in the ..."

Rephrased as suggested (now pg. 13, par. 2, line 8).

8) in Figs. 3 D and S1, sequence alignments: use lighter colours than the dark magenta and dark red in order to make the lettering readable.

We agree with Reviewer 2 that for some printer/monitor settings the parts of the alignments highlighted with darker colors can be difficult to read. In order to make lettering more readable we are replacing black letters with white letters for the fields highlighted in dark colors (now Figs. 3D and S2). We tested this combination and found it preferable to using additional lighter colors (which appear too similar to already present colors). In addition, we are replacing magenta with brown, and violet with dark blue for clarity.

Minor comments:

9) use "bacterial" instead of "eubacterial" which is outdated (e.g. p. 2, line 2 from bottom; p. 3, lines 1 and 2; p. 14, line 1).

Changed as suggested.

10) p. 6, last line, change to: "... thus ordering this N-terminal region of Pop7 (Figure 2A)."

Changed. Now this part of the sentence reads: "Öthus ordering the N-terminal region of Pop7 (Figure 2A)."

11) p. 7, 1st paragraph, line 2 from bottom, rewrite: "The positioning of the additional N-terminal beta-strand E0 ...

Corrected as suggested (now pg. 7, par.2, line 3 from bottom).

12) p. 7, 2nd paragraph, line 5: beta-strand.

Corrected as suggested (now pg. 7, line 2 from bottom).

13) p. 8, 2nd paragraph, last line: "genuine" instead of "proper".

Changed as suggested (now pg. 9, line 1).

14) p. 10, lines 1 & 2 from bottom, change to: " ... the P3 RNA domain will be involved in only very limited interactions with the P3 RNA domain (with the possible exception of the proximal (right in Figures 1A, B) helical stems)."

Changed as suggested (now pg. 11, par. 2, last 2 lanes).

15) p. 11, 1st paragraph, line 5: "... showed complete protection ..."
At several locations in the following discussion, the use of past tense (instead of present tense) is appropriate (when discussing results of other studies).

Changed as suggested.

16) p. 12, 3rd paragraph, line 1: showed / line 5: are protected.

Changed as suggested.

17) p. 13, line 5: "... in the RNase P holoenzyme."

Corrected as suggested (now pg. 13, par. 2, last line).

18) Legend to Fig. 4, line 2: orange, not yellow.

Corrected as suggested.

19) Legend to Fig. 4 E: indicate which residue belongs to which beta-strand.

Changed as suggested. Now the legend reads "Ö(E) A stack of hydrophobic residues (Ile33 (-strand E1), Val65 (-strand E2), Val92 (-strand E3), and Leu136 (-strand E4)) helps stabilize the -sheet in Pop7."

Reviewer 3

1. One of the main conclusions of the paper is that the P3 domain would be placed so that it may functionally replace P15.1 and P15.2 of the Bacillus ribozyme (page 13, last line). Rather than being "data not shown" the results of this modeling experiment should be shown at least as a supplementary figure. The authors should also elaborate in the text as to the functional significance of this, beyond simply "laying the foundation for the evolutionary transition ..."

We are including the requested illustration as Figure 6.

The functional significance of the positioning of the P3 domain and the bound proteins is that it allows, in principle, for the replacement of bacterial RNA elements lost in eukaryotes by protein components, as stated in the last paragraph of the Discussion section. We feel that further elaboration would be overly speculative and premature at the current level of our understanding of these enzymes.

2. Along the same lines, the abstract should say explicitly what the structure suggests, rather than the uninformative "... structure suggests roles of the P3 domain in the structure ..."

Unfortunately the Abstract length limitation (175 words) does not permit any additions to the current text (173 words).

3. Are the affinities of Pop6/Pop7 for each other and for the RNA known? Are they stated somewhere in the MS? And is the affinity unchanged by the circular permutation of the RNA domain?

In this manuscript we did not discuss the binding constants for the Pop6/Pop7 interaction with RNA. These constants were previously estimated to be in the 150 nM range in the presence of a 100 fold excess of a competitor [Perederina et al. 2007]. The measured affinities for the full-length RNase MRP RNA and an isolated P3 domain were practically identical [Perederina et al. 2007]. We did not measure the affinity for the permutated P3 domain since footprinting assays indicated that the regions affected by the permutation were not interacting with Pop6/Pop7 [Perederina et al. 2007, Esakova et al. 2008], consistent with the crystal structure. As for the affinity of Pop6 and Pop7 for each other, it cannot be determined because Pop7 is not soluble in the absence of Pop6 [Perederina et al. 2007]. To indicate this fact, we are adding the following sentence: "Co-expression of Pop6 and Pop7 was required for the solubility of Pop7 [Perederina et al. 2007]." (The second sentence of the Methods/Crystallization section, pg. 14, last paragraph.)

4. Although the authors point out the structural homology with the Alba proteins, they do not state if there are other structural similarities of Pop6/Pop7 to other proteins. At least a DALI search should be performed, and the most similar protein domains listed and discussed.

We did perform a DALI search when we were analyzing the structure. The search yielded a variety of proteins, but they were similar to Pop6 and Pop7 only by the virtue of sharing the well known and well characterized ALBA fold [Wardleworth et al. 2002, Aravind et al. 2003]. Therefore, we limited the discussion of the structural similarities to the structural similarity with the canonical ALBA proteins: we felt that a discussion of structural similarities and differences between individual ALBA-related proteins, while interesting and important (given the abundance of ALBA-related proteins), was beyond the scope of this work.

5. The solvent accessible surface area occluded in the Pop6/Pop7 interface and in the RNA-protein interface (for each) needs to be stated.

The following sentence is added to the Results/Overview of the structure section (pg. 5, par. 3, line 3): "The formation of the Pop6/Pop7 heterodimer buries 1760 $Å^2$ of the proteins' solvent accessible area, while the interaction of the heterodimer with the P3 RNA domain buries 900 $Å^2$ and 1830 $Å^2$ in Pop6 and Pop7, respectively." In addition, the following sentence is added to the Methods section (pg. 17, par. 2, last sentence): "The surface area calculations were performed using AREAIMOL [Lee & Richards 1971]."

6. What is the estimated purity of the proteins? How was the RNA purified? What is its purity? How was the RNA stored and treated (i.e. was it annealed?) previous to complexation with the proteins?

The proteins were purified to be RNase-free as described in the provided reference [Perederina et al. 2007]. The protein and RNA preparations' purity was estimated to be better than 98%. To address this comment of Reviewer 3, the beginning of the first paragraph of the Methods/Crystallization section is rewritten and now reads (pg. 14, last paragraph): "The Pop6/Pop7 heterodimer was overexpressed in E. coli strain BL-21. Cloning, expression, and purification of the Pop6/Pop7 heterodimer were described earlier [Perederina et al. 2007]. Co-expression of Pop6 and Pop7 was

required for the solubility of Pop7 [Perederina et al. 2007]. The modified RNase MRP P3 RNA domain was produced by run-off transcription with T7 RNA polymerase [Milligan et al. 1987], followed by purification on 15% denaturing (8M urea) polyacrylamide gels as previously described [Perederina et al. 2007]. The P3 RNA domain-Pop6/Pop7 complexes were produced by incubating the Pop6/Pop7 heterodimer with refolded RNA domain in a 1:1 molar ratio as described in [Perederina et al. 2007]."

7. The methods section states that Supplementary Figure S3 shows the density-modified SAD map. The legend to that figure says it is a composite anneal omit map. Which is it? What should be shown is the density modified SAD map.

The originally used map was a composite omit electron density map as was stated in the figure legend. We are replacing that map with the requested density modified SAD map (now Supplementary Figure S6).

8. Table 1 needs to list some phasing statistics (FOM, phasing power, Rcullis), and also the coordinate precision (cross-validated sigmaA).

We are including phasing statistics and coordinate error data (Table 1).

9. A comparison of the secondary structure of eukaryal RNase P/MRP (Fig 1a) with the bacterial form would be useful, perhaps as a supplementary figure.

These comparisons are very common and can be found in most reviews on RNase P, including several reviews cited in the manuscript (Altman & Kirsebom 1999, Walker & Engelke 2006, Gopalan 2007, Walker & Engelke 2008). We feel that the manuscript probably would not benefit from the addition of this diagram (considering that the revised manuscript already contains 6 main body figures and 6 supplementary figures).

10. A supplementary figure with the degree of conservation mapped to the solvent-accessible surfaces of the proteins would be useful.

We are including this figure as Supplementary Figure S3.

2nd Editorial Decision 02 December 2009

Thank you for submitting your revised manuscript. It has now been seen once more by the original referees 2 and 3. I am happy to inform you that both of them consider the manuscript significantly improved and most of their original concerns satisfactorily addressed, with only a few minor issues remaining. I am therefore returning the study to you once more, kindly asking you to incorporate these additional requested changes in a last round of revision. Once we will have received this final version, we should then be able to proceed with the acceptance of your paper.

I am looking forward to receiving your final version.

Yours sincerely,

Editor
The EMBO Journal

Referee #2 (Remarks to the Author):

All comments, except for one, have been addressed satisfactorily.

The remaining point to be clarified pertains to my original comment 6: (p. 12, 3rd paragraph: the authors state here "that the apical stem of P3 (nt 45-64) is not involved in protein contacts, except for G45 & A46. However, according to Fig. 4 A, nt 61-64 form contacts with Pop6. Please clarify.)

On p. 13, 1st paragraph, the authors state :"The results of the holoenzyme footprinting showed that the distal (left in Figure 1B, RNase MRP nucleotides 45-64) helical stem of the P3 RNA domain was not protected by the protein moiety, except for nucleotides G45 and A46, which had their phosphate backbones protected in the presence of proteins. This statement is based on the paper by Esakova et al. (RNA 2008). However, in the X-ray structure depicted in Fig. 4 A, Pop6 forms also contacts to stem nt 61-64 (those are nt identical between the native P3 and the crystallized isolated P3 domain), e.g.Thr20 to the 5'-phosphate of C62, or Asn52 and Lys51 to nt 62-64. Please discuss this apparent discrepancy. Is it because these contacts are weak van der Waals interactions that remained undetected in the probing experiments, or do these contacts represent non-native crystal contacts? Might it be helpful to differentiate (by colour) in Fig. 4 A between van der Waals contacts and H bonds?

Referee #3 (Remarks to the Author):

The revision has improved the manuscript, and except for one issue, should be appropriate for publication. This referee feels that the background information in the abstract could still be streamlined, and the salient conclusions of the paper be stated in the abstract, rather than having the completely uninformative "... suggests roles of the P3 domain in the structure and function of RNase ..." It would be a much better abstract if the roles suggested were stated in the abstract itself.

2nd Revision - authors' response 08 December 2009

Reviewer 2
All comments, except for one, have been addressed satisfactorily.

The remaining point to be clarified pertains to my original comment 6: (p. 12, 3rd paragraph: the authors state here "that the apical stem of P3 (nt 45-64) is not involved in protein contacts, except for G45 & A46. However, according to Fig. 4 A, nt 61-64 form contacts with Pop6. Please clarify.)

On p. 13, 1st paragraph, the authors state :"The results of the holoenzyme footprinting showed that the distal (left in Figure 1B, RNase MRP nucleotides 45-64) helical stem of the P3 RNA domain was not protected by the protein moiety, except for nucleotides G45 and A46, which had their phosphate backbones protected in the presence of proteins. This statement is based on the paper by Esakova et al. (RNA 2008). However, in the X-ray structure depicted in Fig. 4 A, Pop6 forms also contacts to stem nt 61-64 (those are nt identical between the native P3 and the crystallized isolated P3 domain), e.g.Thr20 to the 5'-phosphate of C62, or Asn52 and Lys51 to nt 62-64. Please discuss this apparent discrepancy. Is it because these contacts are weak van der Waals interactions that remained undetected in the probing experiments, or do these contacts represent non-native crystal contacts? Might it be helpful to differentiate (by colour) in Fig. 4 A between van der Waals contacts and H bonds?

In the paper by Esakova et al. (2008), we used hydroxyl ions produced by Fenton reaction (Fe-EDTA) to test for the protein protection of the RNA backbone in the RNase MRP holoenzyme. Hydroxyl ions are believed to mainly attack exposed sugar residues at the C4' and, probably, C1' positions (see, for example, Moine et al. in "RNA structure and function", eds. Simons & Grunberg-Manago, pg. 80 (CSHLP 1998)). The RNA-protein interactions in question do not substantially affect the exposure of the C1' and C4' positions in the crystal structure (please see Illustration 1). Thus it is not surprising that these types of interactions remained undetected in the Fe-EDTA

probing experiments, even though these interactions are not necessarily weak. Therefore there is no discrepancy between the absence of protection of the region in question in Esakova et al. and the interactions observed in the crystal structure.
To address the remaining concern of the Reviewer 2, we are rewriting the first paragraph on page 13 and discussing the similarities and discrepancies between the results of the footprinting studies and the crystal structure of this region. The paragraph now reads:
"The crystal structure shows that Pop6 interacts with several nucleotides of the distal (left in Figures 1, 2, 4A) helical stem of the P3 RNA domain (nucleotides 45, 59, 61-64, Figure 4A); however these interactions do not provide substantial protection of ribose in nucleotides 59, 61-64 (Figure 2A). This is consistent with the results of the Fe-EDTA holoenzyme footprinting, which do not show noticeable protection of ribose in these nucleotides in the presence of proteins [Esakova et al. 2008]. In addition, the holoenzyme footprinting shows a protection of the riboses in nucleotides G45 and A46 [Esakova et al. 2008]. While the protection of nucleotide G45 is consistent with the crystal structure, the protection of nucleotide A46 in the holoenzyme cannot be explained by the observed interactions with the Pop6/Pop7 heterodimer. This discrepancy, as well as the protection of the nucleobase in U43 (above), may indicate an interaction with additional component(s) in the context of the holoenzyme. "

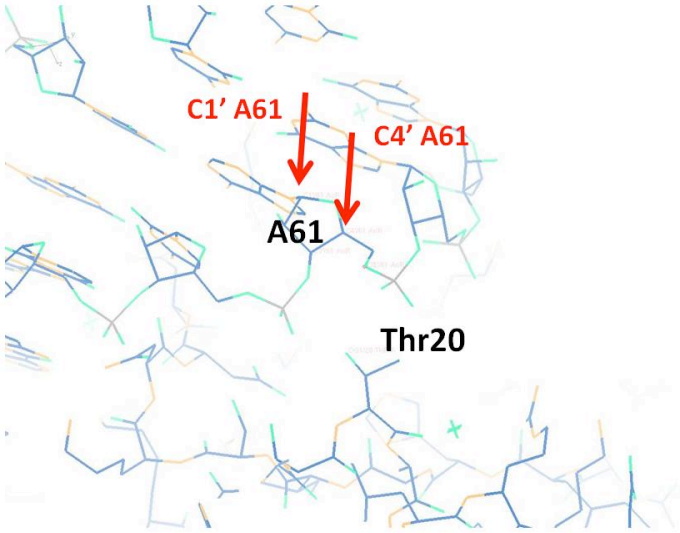

Illustration 1. The interactions between Pop6 and nucleotides 59, 61-64 are not extensive and do not provide significant protection of C1' and C4' positions of ribose (the targets of the cleavage in Fe-EDTA footprinting). Thus they may not be detectable in Fe-EDTA footprinting assays used in [Esakova et al. 2008]. A61 and Thr20 are shown; the other interactions are very similar.

Reviewer 3

The revision has improved the manuscript, and except for one issue, should be appropriate for publication. This referee feels that the background information in the abstract could still be streamlined, and the salient conclusions of the paper be stated in the abstract, rather than having the completely uninformative "... suggests roles of the P3 domain in the structure and function of RNase ..." It would be a much better abstract if the roles suggested were stated in the abstract itself.

To address the remaining concern of Reviewer 3, we are revising the Abstract. Following the Reviewer's suggestion, we are streamlining the introductory part, removing the part which the Reviewer considers uninformative and adding a brief overview of the major conclusions. The revised Abstract now reads:
"Ribonuclease (RNase) P is a site-specific endoribonuclease found in all kingdoms of life. Typical RNase P consists of a catalytic RNA component and a protein moiety. In the eukaryotes the RNase

P lineage has split into two, giving rise to a closely related enzyme, RNase MRP, which has similar components but has evolved to have different specificities. The eukaryotic RNases P/MRP have acquired an essential helix-loop-helix protein-binding RNA domain P3 that plays a key role in eukaryotic enzymes and distinguishes them from bacterial and archaeal RNases P. Here we present a crystal structure of the P3 RNA domain from Saccharomyces cerevisiae RNase MRP in a complex with RNase P/MRP proteins Pop6 and Pop7 solved to 2.7 Å. The structure suggests similar structural organization of the P3 RNA domains in RNases P/MRP and possible roles of the P3 domains and proteins bound to them in the stabilization of the holoenzymes' structures as well as in interactions with substrates. It provides the first insight into the structural organization of the eukaryotic enzymes of the RNase P/MRP family."

