RNase Y, a novel endoribonuclease, initiates riboswitch turnover in B. subtilis

Karen Shahbabian, Ailar Jamalli, Léna Zig

Corresponding author: Harald Putzer, IBPC-CNRS UPR9073

Review timeline:
- Submission date: 19 March 2009
- Editorial Decision: 20 April 2009
- Revision received: 22 July 2009
- Editorial Decision: 03 August 2009
- Revision received: 20 August 2009
- Accepted: 24 August 2009

Transaction Report:
(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 20 April 2009

Thank you for submitting your manuscript for consideration to The EMBO Journal. As you will see from the enclosed comments, all ref's appreciate the potential importance of identifying an endoribonuclease in a Gram-positive organism that might be responsible for mRNA decay. However, it also becomes apparent that the support for this claim remains currently rather circumstantial and would need to be strengthened according to the critiques outlined below before a final decision on potential publication here can be reached. Specifically, it seems difficult to rationalize the finding of rather specific target sites for some RNA’s with the similarly indicated function in controlling global mRNA stability. Therefore, all three referees demand to more carefully adjust current interpretations with the actual results, beside clearly outlined further experimentation to overall improve the quite interesting study. Given their currently rather positive assessment, and albeit being aware of the in some parts still preliminary state, we still decided to offer you the chance for one round of major revisions. The rather specific comments that all referees provided (and I am not going to repeat these here in very much detail) are a solid basis to eventually convince the referees from the significance of your findings.

I still have to remind you that it is EMBO J policy to allow a single round of revisions only, which means that the final decision on acceptance or rejection entirely depends on the content of the final version of your manuscript that will certainly involve an external assessment from some of our critical referees.

Yours sincerely,

Editor
The EMBO Journal
REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The manuscript of Shahbabian et al. describes a B. subtilis endoribonuclease activity encoded by the ymdA gene, and renamed RNase Y, which is responsible for rapid degradation of terminated riboswitch RNA that is bound by S-adenosylmethionine. A detailed in vitro analysis of RNase Y activity on yitJ riboswitch RNA demonstrates specificity of the endonuclease for the SAM-bound substrate. A strain with an IPTG-inducible ymdA gene showed accumulation of 10 riboswitch RNAs when grown in the absence of IPTG. In addition, the global mRNA half-life increased two-fold when grown under these conditions, suggesting a role for RNase Y in overall mRNA turnover.

The significance of these results is potentially quite high. Recent work in B. subtilis has been energized by the discovery of the RNase J ribonucleases and their dual specificity (endonuclease and 5'-to-3' exoribonuclease activities). However, global mRNA stability has been found in the Putzer laboratory to increase only slightly under conditions where RNase J1 is at very reduced levels. Thus, the discovery of an alternative endonuclease in B. subtilis whose expression affects greatly the overall stability of mRNA would be an important milestone for the study of post-transcriptional regulation in Gram-positive bacteria. It should be noted that there is already a report online (ahead of print) concerning the effect of ymdA expression on endonucleolytic processing of an mRNA - Commichau et al. Mol. Cell Proteomics 2009 Feb 3. This report should be cited.

There are conceptual difficulties in accepting a significant role for RNase Y in global mRNA stability, and these are alluded to, but not sufficiently explored, in the Discussion. Firstly, the authors point out that the purified RNase Y that they worked with was a shortened form of RNase Y, since the full-length protein contains N-terminal sequences that are apparently used for membrane insertion/association in vivo. Affinity purification of full-length RNase Y was contaminated with other membrane-bound proteins. In addition, an earlier study (Hunt et al., 2006) localized RNase Y to the cell periphery. If, in fact, RNase Y is membrane-bound, one must explain how such a protein could have access to bulk mRNA, which could be cytoplasmic, to such an extent that even a reduction in RNase Y concentration (but not elimination) has a profound effect on global mRNA half-life. In this respect, the results and discussion in the paper from the Carpousis lab (Khemici et al. Mol. Microbiol. 70:799-813, 2008) need to be cited. Khemici et al. report that E. coli RNase E, a ribonuclease thought to play a dominant role in E. coli mRNA turnover, is membrane-bound. See discussion there for how this relates to a primary role in turnover bulk mRNA.

Secondly, the specificity of RNase Y cleavage demonstrated here with yitJ riboswitch RNA seems to be at odds with a putative role in general mRNA decay. RNase Y cleavage occurs in a single-stranded region upstream of the highly structured riboswitch RNA, and this cleavage is dependent on binding of SAM to the aptamer. Apparently, RNase Y has some recognition element that discriminates between distinct conformations located downstream of its cleavage site. How does one square away such exquisite specificity with a general role in mRNA cleavage? Thirdly, on the surface the fact that RNase Y activity appears to be insensitive to the 5' phosphorylation state (see below) makes it a logically poor candidate for an enzyme that is responsible for setting a decay rate of many mRNAs. In the cases of RNase E in E. coli and RNase J1 in B. subtilis, one sees the expected sensitivity to 5’ phosphorylation state that would result in protection of initial transcripts bearing a 5’ triphosphate from immediate attack. An enzyme that works efficiently on 5’ triphosphorylated RNA would attack nascent RNA transcripts. However, in this regard the characteristics of RNase J1 may be relevant: Although the 5’ exonuclease activity of RNase J1 is hindered by a 5’ triphosphate, the endonuclease activity is not. As such, there are likely other elements that control access of RNase J1 to nascent transcripts, and this may apply to RNase Y as well.

One wonders whether the global mRNA half-life effect reported here is an indirect consequence of severely reduced RNase Y. In view of these issues, it may be in place to moderate the tone of the paper's conclusions.

Comments in order of the manuscript, which is generally well written but has a number of details that warrant attention. (NB: the lack of pagination and line numbering on the pdf made it difficult to
refer to specific passages in a review):

1. There are several blatant misspellings, starting with "Running titel" on the title page. Spell check is needed.

2. Fig. 1A: Should the arrow for HP1400 face the opposite direction? It is said to be complementary to the 5' sequence.

3. Fig. 1B: Suggest deleting the "AAT" label. In this conformation, there is no antiantiterminator function.

3. Fig. 2A: If the upper and lower bands detected in the absence of Met are 16S and 23S rRNA, as indicated, why are the same bands not detected in the presence of Met? Might the upper band be a yitJ read-through transcript?

4. Fig. 2C: What is "FL" pointing to?

5. page 6: The authors map a transcriptional start site that is significantly upstream from the one that was suggested in earlier reports (Grundy and Henkin, 1998; Tomsic et al., 2008). This should be pointed out explicitly.

6. Fig. 3D: It would be helpful to indicate where nucleotide 196 is in this schematic.

7. page 7, para. 1, line 12: "sensible" should be "sensitive." The experiment in Fig. 3B shows only the end product after a 10-minute incubation. To accept the authors' conclusion that RNase Y activity is insensitive to the 5' phosphorylation state, a time-course with limiting enzyme must be performed.

8. page 7, para. 2: The authors state that the substrate that was 12 nts longer (i.e., to nt 208) contained sequences required for AT formation. According to Fig. 1B, an RNA ending at nt 208 cannot form the AT structure. Also, the authors state that the default in vitro conformation with a longer RNA would be the antiterminator conformation. Is this proven by secondary structure probing? Reference?

9. page 7, para. 2, line 7: "...physiologically relevant concentrations of the metabolite." Is there a reference for this? The concentration of 80 µM seems quite high.

10. page 7, para. 3, line 3: "occurred" should be "occurring."

11. page 8, para. 1, line 4: The authors state that there was "no effect" of absent/depleted RNase J1/J2 on leader transcript levels. Since RNase J 5' exonuclease activity is expected to cause degradation after RNase Y endonuclease cleavage, one would expect an accumulation of the riboswitch RNA in an RNase J mutant strain. Indeed, in the Discussion, the authors do state that riboswitch degradation intermediates were detected in this strain, although the experiment was apparently messy due to cross-hybridization. In any event, it is misleading to state in the Results that there is "no effect" on leader transcript levels (although technically the authors might be referring to full-length leader RNA). This should be clarified.

12. page 11, para. 3, line 5: "primordial" is the wrong word. The authors mean something like "central" or "essential" or "of primary importance."

Referee #2 (Remarks to the Author):

The authors present an interesting set of data on a new ribonuclease enzyme, which they've coined RNase Y. Depletion of this enzyme, which is essential for viability, results in accumulation of the leader regions for S box-containing transcripts. The authors argue that RNase Y is responsible for promoting turnover specifically of the metabolite-binding region of the leader region (the aptamer) for S box RNAs. This is built upon three claims:

(1) Mixing of RNA substrates with RNase Y in vitro in a single time-point molecular biology-based
assay resulted in a putative cleavage site, just upstream of the aptamer portion.
(2) Cleavage of an RNA substrate, postulated to mimic the antiterminated conformation, was only observed in the presence of SAM.
(3) Full-length mRNAs (i.e., transcripts resulting from antitermination) were not substrates for RNase Y in vivo. This is one of the weaker portions of the manuscript and I remain unconvinced that this statement has been fully demonstrated.

Although the authors do not have direct evidence of cleavage in vivo at the site that is observed in vitro, they argue that these three overall observations demonstrate that RNase Y is specifically required for turnover of S box leader regions. Overall, it is a good manuscript with a novel finding. However, the authors should soften some of their statements to be more conservative with their interpretations. The authors should consider the following before proceeding to publication:

1. The manuscript should be reviewed for multiple misspellings.
2. Page 3, second paragraph. The statements on the RNase J1 structural model should be further clarified. The authors state that the presence of a putative mononucleotide binding pocket at one nucleotide distance from the catalytic site indicates that it cannot accept a triphosphate group. They then claim this indicates that RNase J is likely to switch between endo- to exonuclease modes. These statements should either be dropped or clarified as it will be unclear to the reader as written why the first statement infers the second.
3. Page 5, second paragraph. The claim is made that a rnjA/rnjB double mutant exhibits an 11-fold reduction in RNase J1 from a xylose-inducible promotor. However, it is not clear how this value has been obtained. qPCR? Northern blotting? Was it determined in a previous manuscript?
4. Page 6, second paragraph. The authors wished to determine whether ymdA transcripts contained the full leader region. Therefore, they conducted a primer extension experiment on these transcripts using RNA extracted from methionine-starved cells. They argue for a reverse transcriptase arrest at the terminator, indicating that a portion of the mRNA population is in the terminator conformation. This is reasonable, although in the absence of ladders, it is technically possible that this band results from a cleavage event and not from RT abutting the 3’ side of the terminator helix. They then reason that they do not see evidence for YmdA cleavage, as there are no other bands other than corresponding to the 5’ terminus (again, markers would assist that argument). From the latter, they conclude that the antiterminator conformation is not an "efficient" substrate for YmdA. However, their data seems to suggest that there are populations of both terminated and antiterminated conformations; therefore, it is still unclear why they do not see a cleavage site. One alternative explanation is that cleaved RNAs may simply degrade too rapidly for detection using this assay format.
5. Similarly, the authors argue on the next page (page 7) that the construct corresponding to the terminator conformation is the "transcript on which YmdA is active in vivo." Again, this claim is not fully supported by their data.
6. Within the same paragraph (on page 7): The authors claim that YmdA does not exhibit any preferences for the phosphorylation state at the 5’ terminus. However, the authors should, at minimum, qualify this statement by stating that no preference was observed under the reaction conditions that they have tested. The authors conducted their experiment using saturating levels of enzyme and using an endpoint assay format. Their experiment did not provide any information about potential differences in initial reaction rates. They can only state that the same maximum level of cleavage is achieved for monophosphorylated and triphosphorylated substrates.
7. Same paragraph: It is stated that 25-80 µM S-adenosylmethionine is representative of "increasingly physiologically relevant concentrations." Can the authors include a citation for this statement?
8. Page 8. The authors conclude that their data "indicated that RNase Y is the key enzyme for the turnover of SAM dependent riboswitches in B. subtilis." This may eventually prove to be a true statement but the authors should soften it by saying that their data "suggests" this to be true. There are still other, albeit less likely, scenarios that could explain their observations. For example, isn't it technically possible that depletion of RNase Y could lead to a cellular increase in S-adenosylmethionine through an unknown mechanism, thereby causing increased levels of terminated riboswitches. Couldn't this also explain all of the data in Figure 4 as well as the absence of cleavage sites in Figure 2D?
9. Their data is relatively good. However, their arguments would definitely have been strengthened by additional controls. For example, none of their gels included loading controls. Also, their experiments would have been significantly strengthened if they had tested a construct where
transcription initiated at the base of the aptamer region or, alternatively, contained mutations in the putative cleavage site. Demonstrating that these constructs are stabilized in vivo would provide evidence for in vivo cleavage at the site immediately upstream of the aptamer, something that is currently lacking in the manuscript. In general it is risky to assume that this cleavage is occurring in vivo simply because it occurs with purified RNA substrates under optimized conditions in vitro.

10. Page 10. The authors imply a mechanistic model where RNase Y and RNase J work together for degradation of riboswitch leader RNAs. However, the data presented in their figures suggests that RNase J depletion does not lead to yitJ accumulation, in contradiction to this model. Also, the manuscript by Collins et al., 2007 did not demonstrate exoribonucleolytic activity as is suggested as written in this paragraph. The Collins et al., 2007 manuscript only demonstrated an essential role for RNase J in degradation of glmS transcripts but did not specifically address whether exo- or endonucleolytic activity was involved.

11. Page 11, second full paragraph. I recommend a different word than "primordial" in this section - perhaps it could be replaced with "important". Also, this paragraph should be revisited for clarity.

The main point that the authors are presenting in this portion of the text is unclear.

12. Page 14, under "Assay of RNase Y activity". Include description of the RNA ladders that were used in this experiment.

13. Figure 4 legend. I recommend changing "gene specific" to "leader-specific".


15. Page 1: Since the UUUU sequence is shown in panel A, it would be helpful to also show where it is in the conformation shown in panel B. This portion seems to disappear in panel B as shown.

16. Figure 2, panel C: change "xylose" to "IPTG". Also, what is indicated by "FL" in this panel?

Referee #3 (Remarks to the Author):

1. This is an interesting ms that describes a new ribonuclease, YmdA now named RNase Y, that is widely distributed in bacteria but hitherto uncharacterized.

2. Page 5, first paragraph, second last sentence. The authors comment that they failed to detect the terminated transcript. The experiment is not definitive as I doubt that they could detect it even if it were present. First, the gel is low resolution and the terminated fragment would be small and diffuse. Second, only 69 nt of the probe is complementary to the expected product, so the signal intensity would be low. I recommend that experiment be repeated with higher resolution methods combined with positive and negative controls.

3. Hyphens are missing in most places where they are needed: e.g., strand-specific; base-paired, IPTG-inducible, etc.

4. Page 6, line 4: "gel separated RNA" is lab jargon.

5. Page 6, starting 10 lines from the bottom: The authors attribute a partial cDNA to stalling of reverse transcriptase by a terminator structure. But two sentences later, they state that the riboswitch would normally be in the anti-terminator conformation in order to explain absence of a cleavage product. If conformational switches are to be invoked, then direct evidence for the two conformations should be shown.

6. Page 7 and Fig. 3. To demonstrate that YmdA is an endonuclease, the authors should use uniformly labelled RNA as substrate and demonstrate that both 5' and 3' products can be recovered from the same incubation. The yield of the two products should be identical. The experiments shown in Fig 3 b and c don't address the latter point. In addition, there is considerable background in Fig. 3c suggesting that the substrate was not very clean. Finally, the actual fraction of substrate cleaved is not high; can cleavage be driven to completion?

7. Page 8 and Fig. 6. There appears to be partial cleavage in lane 4 (D369A) as well as a smaller product. The authors should comment.

8. Page 9 (middle of the page): what is the "pseudoknot interaction" that is mentioned in the text?

9. Page 9. Part of the evidence in favour of preferential of the anti-terminated transcript is negative:
failure to detect products. Have the authors done a side-by-side experiment in vitro to test this directly, especially because they state that the two conformations can be interconverted (page 6)?

10. Page 10 (middle). Why doesn't the downstream intermediate accumulate when J1/J2 are inactivated?

10. The authors don't discuss the implications of the TM segment on YmdA/RNase Y. If this enzyme is tethered to the inner membrane, why does it act on mRNAs with SAM-responsive riboswitches?

11. Figure 2C. The "FL" label in the right margin is misplaced.

12. Several of the references to RNase E are very dated (e.g., Cohen & McDowall, 1997; Mudd et al, 1990). The Callaghan et al Nature paper from 2005 would be more suitable.

Referee #1 (Remarks to the Author):

The manuscript of Shahbabian et al. describes a B. subtilis endoribonuclease activity encoded by the ymdA gene, and renamed RNase Y, which is responsible for rapid degradation of terminated riboswitch RNA that is bound by S-adenosylmethionine. A detailed in vitro analysis of RNase Y activity on yitJ riboswitch RNA demonstrates specificity of the endonuclease for the SAM-bound substrate. A strain with an IPTG-inducible ymdA gene showed accumulation of 10 riboswitch RNAs when grown in the absence of IPTG. In addition, the global mRNA half-life increased two-fold when grown under these conditions, suggesting a role for RNase Y in overall mRNA turnover.

The significance of these results is potentially quite high. Recent work in B. subtilis has been energized by the discovery of the RNase J ribonucleases and their dual specificity (endonuclease and 5'-to-3' exoribonuclease activities). However, global mRNA stability has been found in the Putzer laboratory to increase only slightly under conditions where RNase J1 is at very reduced levels. Thus, the discovery of an alternative endonuclease in B. subtilis whose expression affects greatly the overall stability of mRNA would be an important milestone for the study of post-transcriptional regulation in Gram-positive bacteria. It should be noted that there is already a report online (ahead of print) concerning the effect of ymdA expression on endonucleolytic processing of an mRNA - Commichau et al. Mol. Cell Proteomics 2009 Feb 3. This report should be cited.

This manuscript is now cited and discussed with respect to our own data (page 14, second paragraph).

There are conceptual difficulties in accepting a significant role for RNase Y in global mRNA stability, and these are alluded to, but not sufficiently explored, in the Discussion. Firstly, the authors point out that the purified RNase Y that they worked with was a shortened form of RNase Y, since the full-length protein contains N-terminal sequences that are apparently used for membrane insertion/association in vivo. Affinity purification of full-length RNase Y was contaminated with other membrane-bound proteins. In addition, an earlier study (Hunt et al., 2006) localized RNase Y to the cell periphery. If, in fact, RNase Y is membrane-bound, one must explain how such a protein could have access to bulk mRNA, which could be cytoplasmic, to such an extent that even a reduction in RNase Y concentration (but not elimination) has a profound effect on global mRNA half-life. In this respect, the results and discussion in the paper from the Carpousis lab (Khemici et al. Mol. Microbiol. 70:799-813, 2008) need to be cited. Khemici et al. report that E. coli RNase E, a ribonuclease thought to play a dominant role in E. coli mRNA turnover, is membrane-bound. See discussion there for how this relates to a primary role in turnover bulk mRNA.

A section in the discussion is now dedicated to this aspect and we clearly point out the similarities to RNase E (page 15, top)

Secondly, the specificity of RNase Y cleavage demonstrated here with yitJ riboswitch RNA seems to be at odds with a putative role in general mRNA decay. RNase Y cleavage occurs in a single-
stranded region upstream of the highly structured riboswitch RNA, and this cleavage is dependent on binding of SAM to the aptamer. Apparently, RNase Y has some recognition element that discriminates between distinct conformations located downstream of its cleavage site. How does one square away such exquisite specificity with a general role in mRNA cleavage? Thirdly, on the surface the fact that RNase Y activity appears to be insensitive to the 5' phosphorylation state (see below) makes it a logically poor candidate for an enzyme that is responsible for setting a decay rate of many mRNAs. In the cases of RNase E in E. coli and RNase J1 in B. subtilis, one sees the expected sensitivity to 5' phosphorylation state that would result in protection of initial transcripts bearing a 5' triphosphate from immediate attack. An enzyme that works efficiently on 5' triphosphorylated RNA would attack nascent RNA transcripts. However, in this regard the characteristics of RNase J1 may be relevant: Although the 5' exonuclease activity of RNase J1 is hindered by a 5' triphosphate, the endonuclease activity is not. As such, there are likely other elements that control access of RNase J1 to nascent transcripts, and this may apply to RNase Y as well.

We thank the reviewer for these comments. It actually led us to thoroughly reinvestigate the 5' end dependence and we can now say that RNase Y is a 5' end dependent enzyme. The data showing that RNase Y prefers a 5' monophosphorylated substrate are summarized in a new Figure 4. The experiments were carried out with uniformly and 3' end labeled substrates and also a time course experiment was performed using about two-fold less enzyme than in the initial single end-point experiment. We now estimate the initial cleavage rate of RNase Y to be about 20 times faster on a 5' P than a 5' PPP substrate. The new results on the 5' end dependence are summarized in a new Fig. 4. The 5' end dependence and the sensitivity to conformational changes downstream of the cleavage site are discussed (page 14, top and page 16).

The reviewer also points out that the high specificity of RNase Y is difficult to reconcile with a general role in mRNA cleavage. As we discuss on page 14 (top), the two cleavage sites in the yitJ riboswitch RNA are A or AU rich sequences located upstream of a secondary structure. The requirement for a downstream secondary structure would explain why cleavage at site 1 only occurs in the presence of SAM: the lower stem of the aptamer domain (Fig. 3C) only forms upon binding of the metabolite. This suggested that cleavage site selection by RNase Y is extremely specific.

However, SAM might only be needed to form a largely unspecific secondary structure 3' to the cleavage site, similar to the terminator structure downstream of cleavage site 2. In that case, the cleavage specificity of RNase Y would be compatible with a general role in mRNA cleavage.

One wonders whether the global mRNA half-life effect reported here is an indirect consequence of severely reduced RNase Y. In view of these issues, it may be in place to moderate the tone of the paper's conclusions.

We have moderated our conclusions and we cannot completely rule out this possibility. At the same time we now provide evidence that RNase Y is a 5' end dependent enzyme and added a completely new set of data that confirmed that RNase Y functions as an endoribonuclease in vivo (pages 8 and 9 and the new Fig. 5.) Together these data now provide strong evidence for the proposed function of RNase Y.

Comments in order of the manuscript, which is generally well written but has a number of details that warrant attention. (NB: the lack of pagination and line numbering on the pdf made it difficult to refer to specific passages in a review.):

1. There are several blatant misspellings, starting with "Running titel" on the title page. Spell check is needed.
   Ok

2. Fig. 1A: Should the arrow for HP1400 face the opposite direction? It is said to be complementary to the 5' sequence.
   Yes, it should. This has been corrected.

3. Fig. 1B: Suggest deleting the "AAT" label. In this conformation, there is no antiantiterminator function.
   Ok, has been corrected.

3. Fig. 2A: If the upper and lower bands detected in the absence of Met are 16S and 23S rRNA, as indicated, why are the same bands not detected in the presence of Met? Might the upper band be a
yitJ read-through transcript?
The Northern blot has been performed again by increasing prehybridization time. This improved the background and now shows that there is no difference in the level of non specific hybridization to the ribosomal RNA from preparations grown in the presence or absence of methionine. The new Northern is now shown in Fig. 2A.

4. Fig. 2C: What is "FL" pointing to?
«FL» was not positioned correctly and should have pointed to the band representing the +1 transcription start. This has been corrected and «FL» has been replaced with «+1».

5. page 6: The authors map a transcriptional start site that is significantly upstream from the one that was suggested in earlier reports (Grundy and Henkin, 1998; Tomsic et al., 2008). This should be pointed out explicitly.
We now point this out clearly in the results section (page 6, second paragraph).

6. Fig. 3D: It would be helpful to indicate where nucleotide 196 is in this schematic.
This has been done (now Fig. 3C).

7. page 7, para. 1, line 12: "sensible" should be "sensitive." The experiment in Fig. 3B shows only the end product after a 10-minute incubation. To accept the authors' conclusion that RNase Y activity is insensitive to the 5' phosphorylation state, a time-course with limiting enzyme must be performed.
We fully agree and this has been done. We now show that RNase Y is 5' end dependent. A full comment on this matter is given above where this point was raised for the first time.

8. page 7, para. 2: The authors state that the substrate that was 12 nts longer (i.e., to nt 208) contained sequences required for AT formation. According to Fig. 1B, an RNA ending at nt 208 cannot form the AT structure.
The position corresponding to nucleotide 208 was wrongly indicated in Fig. 1B. This has been corrected.

Also, the authors state that the default in vitro conformation with a longer RNA would be the antiterminator conformation. Is this proven by secondary structure probing? Reference?
This conformational switch has been analysed and confirmed by RNase H probing for the yitJ riboswitch studied here and another member of the S-box family. We now cite both studies (page 7, line 23).

9. page 7, para. 2, line 7: "...physiologically relevant concentrations of the metabolite." Is there a reference for this? The concentration of 80 µM seems quite high.
The SAM concentrations used were 25 µM and 80 µM as indicated in the legend to Fig. 3. The physiologically relevant intracellular SAM concentrations observed in the presence or absence of methionine were found to be in the range of < 25 µM to 300 µM (in the presence of methionine). An appropriate reference in which these values were determined is now included (page 7, line 28).

10. page 7, para. 3, line 3: "occurred" should be "occurring."
This has been corrected

11. page 8, para. 1, line 4: The authors state that there was "no effect" of absent/depleted RNase J1/J2 on leader transcript levels. Since RNase J 5' exonuclease activity is expected to cause degradation after RNase Y endonuclease cleavage, one would expect an accumulation of the riboswitch RNA in an RNase J mutant strain. Indeed, in the Discussion, the authors do state that riboswitch degradation intermediates were detected in this strain, although the experiment was apparently messy due to cross-hybridization. In any event, it is misleading to state in the Results that there is "no effect" on leader transcript levels (although technically the authors might be referring to full-length leader RNA). This should be clarified.
We clarified this by writing (page 10, top): Using gene-specific probes we observed no effect of the absence/depletion of RNases J1/J2 on the expected full-length leader mRNAs levels of any of the S-box genes (data not shown).

12. page 11, para. 3, line 5: "primordial" is the wrong word. The authors mean something like
"central" or "essential" or "of primary importance."
We agree, but this part of the discussion is not anymore present as such in the revised version.

Referee #2 (Remarks to the Author):

The authors present an interesting set of data on a new ribonuclease enzyme, which they've coined RNase Y. Depletion of this enzyme, which is essential for viability, results in accumulation of the leader regions for S box-containing transcripts. The authors argue that RNase Y is responsible for promoting turnover specifically of the metabolite-binding region of the leader region (the aptamer) for S box RNAs. This is built upon three claims:

1. Mixing of RNA substrates with RNase Y in vitro in a single time-point molecular biology-based assay resulted in a putative cleavage site, just upstream of the aptamer portion.
2. Cleavage of an RNA substrate, postulated to mimic the antiterminated conformation, was only observed in the presence of SAM.
3. Full-length mRNAs (i.e., transcripts resulting from antitermination) were not substrates for RNase Y in vivo. This is one of the weaker portions of the manuscript and I remain unconvinced that this statement has been fully demonstrated.

Although the authors do not have direct evidence of cleavage in vivo at the site that is observed in vitro, they argue that these three overall observations demonstrate that RNase Y is specifically required for turnover of S box leader regions. Overall, it is a good manuscript with a novel finding. However, the authors should soften some of their statements to be more conservative with their interpretations. The authors should consider the following before proceeding to publication:

1. The manuscript should be reviewed for multiple misspellings. This has been done.
2. Page 3, second paragraph. The statements on the RNase J1 structural model should be further clarified. The authors state that the presence of a putative mononucleotide binding pocket at one nucleotide distance from the catalytic site indicates that it cannot accept a triphosphate group. They then claim this indicates that RNase J is likely to switch between endo- to exonucleolytic modes. These statements should either be dropped or clarified as it will be unclear to the reader as written why the first statement infers the second.
   We decided to drop the statement on the activity switch rather than going into more details since RNase J is not the major focus of this manuscript.
3. Page 5, second paragraph. The claim is made that a rnjA/rnjB double mutant exhibits an 11-fold reduction in RNase J1 from a xylose-inducible promoter. However, it is not clear how this value has been obtained. qPCR? Northern blotting? Was it determined in a previous manuscript?
   We have determined this value by Western blotting in a previous manuscript (Mäder et al., 2008) that we now cite at the appropriate position in the manuscript. The values have also been corrected: the J1 level is 20-fold lower in the absence of xylose (page 5, last paragraph).
4. Page 6, second paragraph. The authors wished to determine whether ymdA transcripts contained the full leader region. Therefore, they conducted a primer extension experiment on these transcripts using RNA extracted from methionine-starved cells. They argue for a reverse transcriptase arrest at the terminator, indicating that a portion of the mRNA population is in the terminator conformation. This is reasonable, although in the absence of ladders, it is technically possible that this band results from a cleavage event and not from RT abutting the 3' side of the terminator helix. They then remark that they do not see evidence for YmdA cleavage, as there are no other bands other than corresponding to the 5' terminus (again, markers would assist that argument). From the latter, they conclude that the antiterminator conformation is not an "efficient" substrate for YmdA. However, their data seems to suggest that there are populations of both terminated and antiterminated conformations; therefore, it is still unclear why they do not see a cleavage site. One alternative explanation is that cleaved RNAs may simply degrade too rapidly for detection using this assay format.
   We agree with the reviewer and have repeated the primer extension experiment and included a sequence ladder (Fig. 2B). The reverse transcriptase arrest could be mapped to the first base-paired
nucleotide on the 3’ side of the terminator structure. This strongly supports the notion that this band resulted from an arrest at the stable terminator structure and not from a cleavage event.

Our data do suggest that in the primer extension experiment there is a significant amount of yitJ mRNA in the terminator conformation. However, it is likely that at the time when the mRNA was isolated from the cells most if not all of this mRNA was actually in the favored antiterminator conformation which is required to obtain full-length transcripts. The denaturing step carried out on the isolated RNA prior to the primer extension and the subsequent refolding is the most likely cause for creating a conformational mixture of yitJ leader mRNA. We cannot completely rule out that a small part of the full-length mRNA was cleaved and then rapidly degraded. However, if this cleavage was as efficient as on the terminated transcript then we would not even expect to detect read-through transcripts originating at the original transcription start. In addition, if efficient cleavage was followed by a very rapid degradation of the mRNA, then yitJ expression level under methionine starvation conditions should be measurably increased in an RNase Y mutant. This was not the case.

5. Similarly, the authors argue on the next page (page 7) that the construct corresponding to the terminator conformation is the "transcript on which YmdA is active in vivo." Again, this claim is not fully supported by their data.

See also comments immediately above. We have changed the wording to: This configuration corresponds to that of the terminated leader transcript, which is the most likely substrate for YmdA in vivo.

6. Within the same paragraph (on page 7): The authors claim that YmdA does not exhibit any preferences for the phosphorylation state at the 5’ terminus. However, the authors should, at minimum, qualify this statement by stating that no preference was observed under the reaction conditions that they have tested. The authors conducted their experiment using saturating levels of enzyme and using an endpoint assay format. Their experiment did not provide any information about potential differences in initial reaction rates. They can only state that the same maximum level of cleavage is achieved for monophosphorylated and triphosphorylated substrates.

We thank the reviewer for these comments. It actually led us to thoroughly reinvestigate the 5’ end dependence and we can now say that RNase Y is a 5’ end dependent enzyme. The data showing that RNase Y prefers a 5’ monophosphorylated substrate are summarized in a new Figure 4. The experiments were carried out with uniformly and 3’ end labeled substrates and also a time course experiment was performed using about two-fold less enzyme than in the initial single end-point experiment. We now estimate the initial cleavage rate of RNase Y to be about 20 times faster on a 5’ P than a 5’ PPP substrate. The 5’ end dependence and the sensitivity to conformational changes downstream of the cleavage site are discussed (page 14, second paragraph and page 16).

7. Same paragraph: It is stated that 25-80 µM S-adenosylmethionine is representative of "increasingly physiologically relevant concentrations." Can the authors include a citation for this statement?

The SAM concentrations used were 25 µM and 80 µM. The physiologically relevant intracellular SAM concentrations observed in the presence or absence of methionine were found to be in the range of < 25 µM to 300 µM (in the presence of methionine). The appropriate reference (Tomsic et al, 2008) in which these values were determined is now included.

8. Page 8. The authors conclude that their data "indicated that RNase Y is the key enzyme for the turnover of SAM dependent riboswitches in B. subtilis." This may eventually prove to be a true statement but the authors should soften it by saying that their data "suggests" this to be true. There are still other, albeit less likely, scenarios that could explain their observations. For example, isn’t it technically possible that depletion of RNase Y could lead to a cellular increase in S-adenosylmethionine through an unknown mechanism, thereby causing increased levels of terminated riboswitches. Couldn’t this also explain all of the data in Figure 4 as well as the absence of cleavage sites in Figure 2D?

We replaced « indicates » with « suggests ». We cannot completely rule out the scenario proposed by the reviewer. However, we believe that the new data that we have added (5’ end dependence and in vivo data) make for a much stronger argument now that RNase Y acts as an endoribonuclease in vitro and in vivo.

9. Their data is relatively good. However, their arguments would definitely have been
strengthened by additional controls. For example, none of their gels included loading controls. Also, their experiments would have been significantly strengthened if they had tested a construct where transcription initiated at the base of the aptamer region or, alternatively, contained mutations in the putative cleavage site. Demonstrating that these constructs are stabilized in vivo would provide evidence for in vivo cleavage at the site immediately upstream of the aptamer, something that is currently lacking in the manuscript. In general it is risky to assume that this cleavage is occurring in vivo simply because it occurs with purified RNA substrates under optimized conditions in vitro.

We agree that the in vitro data should be backed up by more in vivo data. We now have added an extensive data set on the yitJ riboswitch turnover in vivo and propose a detailed degradation pathway. In short, our data suggest that RNase Y cleaves upstream (as in vitro) and downstream of the aptamer in vivo. This allows for the efficient degradation of the aptamer domain by the 3'-5' exoribonucleases and for the 5'-3' exonucleolytic degradation of the downstream cleavage product containing the terminator. A separate section in the Results (pages 8 and 9) describes these experiments which are summarized in a new Figure 5.

10. Page 10. The authors imply a mechanistic model where RNase Y and RNase J work together for degradation of riboswitch leader RNAs. However, the data presented in their figures suggests that RNase J depletion does not lead to yitJ accumulation, in contradiction to this model. Also, the manuscript by Collins et al, 2007 did not demonstrate exoribonucleolytic activity as is suggested as written in this paragraph. The Collins et al., 2007 manuscript only demonstrated an essential role for RNase J in degradation of glmS transcripts but did not specifically address whether exo- or endonucleolytic activity was involved.

The reviewer is correct in pointing out that our data did not sufficiently support the model that RNase Y and RNase J work together for degradation of the riboswitch leader RNAs. In the Northern analysis (Figure 2B, lane 3) we saw no degradation intermediates in an RNase J1 depletion strain. This was expected since the oligonucleotide used as a probe (HP1400) could only detect sequences upstream of the RNase Y cleavage site. We now show in a new primer extension experiment (using oligo HP1134) and an S1 mapping experiment that downstream degradation intermediates following the two endonucleolytic cleavages accumulate when RNase J1 is depleted (Fig. 5A, lane 3 and Fig. 5C, lane 3). In addition, as we now know from the new in vivo data, most of the aptamer domain is actually degraded by the 3'-5' exoribonucleases PNPase and RNase R following endonucleolytic cleavage downstream of the aptamer domain (Fig. 5). All in vivo data are summarized in the new figure 5.

11. Page 11, second full paragraph. I recommend a different word than "primordial" in this section - perhaps it could be replaced with "important". Also, this paragraph should be revisited for clarity. The main point that the authors are presenting in this portion of the text is unclear.

We agree, but this part of the discussion has been taken out in the revised version.

12. Page 14, under "Assay of RNase Y activity". Include description of the RNA ladders that were used in this experiment.

This has been done.

13. Figure 4 legend. I recommend changing "gene specific" to "leader-specific".

This has been done.


This has been done.

15. Figure 1: Since the UUUU sequence is shown in panel A, it would be helpful to also show where it is in the conformation shown in panel B. This portion seems to disappear in panel B as shown.

The UUUU sequence is now also shown in panel B.

16. Figure 2, panel C: change "xylose" to "IPTG". Also, what is indicated by "FL" in this panel?

This has been corrected. « FL « stood for full-length and was misplaced. « FL » has been replaced with « +1 » next to the band corresponding to the +1 position.
Referee #3 (Remarks to the Author):

1. This is an interesting ms that describes a new ribonuclease, YmdA now named RNase Y, that is widely distributed in bacteria but hitherto uncharacterized.

2. Page 5, first paragraph, second last sentence. The authors comment that they failed to detect the terminated transcript. The experiment is not definitive as I doubt that they could detect it even if it were present. First, the gel is low resolution and the terminated fragment would be small and diffuse. Second, only 69 nt of the probe is complementary to the expected product, so the signal intensity would be low. I recommend that experiment be repeated with higher resolution methods combined with positive and negative controls.

The reviewer is correct in that the small fragment would give a diffuse signal, that we could nevertheless see in an RNase Y depletion strain (data not shown). As recommended by the reviewer, we show a high resolution Northern blot with RNA from the wild type and three ribonuclease mutant strains in Figure 2B. The results clearly show that the terminated leader transcript is only present under conditions of RNase Y depletion.

3. Hyphens are missing in most places where they are needed: e.g., strand-specific; base-paired, IPTG-inducible, etc.
This has been corrected.

4. Page 6, line 4: "gel separated RNA" is lab jargon.
This has been changed to: The RNA was separated on a polyacrylamide gel….

5. Page 6, starting 10 lines from the bottom: The authors attribute a partial cDNA to stalling of reverse transcriptase by a terminator structure. But two sentences later, they state that the riboswitch would normally be in the anti-terminator conformation in order to explain absence of a cleavage product. If conformational switches are to be invoked, then direct evidence for the two conformations should be shown.

We have repeated the primer extension experiment and included a sequence ladder. The reverse transcriptase arrest could be mapped to the first base-paired nucleotide on the 3' side of the terminator structure. This strongly supports the notion that this band resulted from an arrest at the stable terminator structure and not from a cleavage event.

Our data do suggest that in the primer extension experiment there is a significant amount of yitJ mRNA in the terminator conformation. However, it is likely that at the time when the mRNA was isolated from the cells most if not all of this mRNA was actually in the favored antiterminator conformation which is required to obtain full-length transcripts. The denaturing step carried out on the isolated RNA prior to the primer extension and the subsequent refolding is the most likely cause for creating a conformational mixture of yitJ leader mRNA. We cannot completely rule out that a small part of the full-length mRNA was cleaved and then rapidly degraded. However, if this cleavage was as efficient as on the terminated transcript in vivo then we would not even expect to detect read-through transcripts originating at the original transcription start. In addition, if efficient cleavage was followed by a very rapid degradation of the mRNA, then yitJ expression level under methionine starvation conditions should be measurably increased in an RNase Y mutant. This was not the case.

The conformational switch invoked has also been extensively analysed and confirmed by RNase H probing for the yitJ riboswitch studied here and another member of the S-box family. We now cite both studies (Epshtein et al, 2003; McDaniel et al, 2003).

6. Page 7 and Fig. 3. To demonstrate that YmdA is an endonuclease, the authors should use uniformly labelled RNA as substrate and demonstrate that both 5' and 3' products can be recovered from the same incubation. The yield of the two products should be identical. The experiments shown in Fig 3 b and c don't address the latter point. In addition, there is considerable background in Fig. 3c suggesting that the substrate was not very clean. Finally, the actual fraction of substrate cleaved is not high; can cleavage be driven to completion?

We now show a cleavage assay using an RNA uniformly labelled with α-[32P]UTP as substrate and identify the two cleavage products (Fig. 4A, lane 4). Quantification of the radioactivity in the two bands confirms that the yield of both products is identical with respect to the number of U residues
present in the respective fragments.
The substrate in figure 3C (now 3B) was clean, but for SAM binding the protocol required to incubate the transcript for 10 min at 70°C in the presence of Mg+2. Under this conditions, some unspecific RNA degradation cannot be avoided.

It is true that the actual fraction cleaved is not high. The efficiency of cleavage can vary from one experiment to other, but generally cannot be driven to completion under the conditions used. We do not yet understand the reason for this but it is likely that RNase Y is quite sensitive to the folding of the RNA downstream of the cleavage site. In vivo the interaction with other proteins as has been suggested (eg. degradosome-like complex) might render cleavage more efficient.

7. Page 8 and Fig. 6. There appears to be partial cleavage in lane 4 (D369A) as well as a smaller product. The authors should comment.

The RNA was separated on a polyacrylamide gel.

We say that the enzyme is not inactivated but that its activity is greatly reduced in the mutants.

We now mention that the residual activity of the D369A mutant also cleaves at a second site 2-3 nucleotides upstream which corresponds very closely to the cleavage site in vivo. At present we ignore why this mutant protein behaves like that.

8. Page 9 (middle of the page): what is the "pseudoknot interaction" that is mentioned in the text?
The aptamer structure involves a pseudoknot interaction. Since this is not relevant here we took out this term and refer to this structure simply as the aptamer domain.

9. Page 9. Part of the evidence in favour of preferential of the anti-terminated transcript is negative: failure to detect products. Have the authors done a side-by-side experiment in vitro to test this directly, especially because they state that the two conformations can be interconverted (page 6)?

In vitro, the two conformations can only be interconverted by choosing a transcript capable of forming the favored antiterninator structure and adding SAM to shift the equilibrium towards the terminator conformation. This is the experiment that we show in Figure 3B.

We think that in vivo the read-through transcripts are not cleaved because the antiterminator conformation is maintained to a large extent. For reasons why we also detect the terminator conformation in the primer extension experiment please refer to our response in point 5.

10. Page 10 (middle). Why doesn't the downstream intermediate accumulate when J1/J2 are inactivated?

In the Northern analysis (Figure 2B, lane 3) we saw no degradation intermediates in an RNase J1 depletion strain. This is expected since the oligonucleotide used as a probe (HP1400) could only detect sequences upstream of the RNase Y cleavage site 1. We now show in a new primer extension experiment (using oligo HP1134) and an S1 mapping experiment that downstream degradation intermediates following the two endonucleolytic cleavages accumulate when RNase J1 is depleted (Fig. 5A, lane 3 and Fig. 5C, lane 3). In addition, as we now know from the new in vivo data, most of the aptamer domain is actually degraded by the 3'-5' exoribonucleases PNPase and RNase R following endonucleolytic cleavage downstream of the aptamer domain (Fig. 5). All in vivo data are summarized in the new figure 5.

10. The authors don't discuss the implications of the TM segment on YmdA/RNase Y. If this enzyme is tethered to the inner membrane, why does it act on mRNAs with SAM-responsive riboswitches?

A section in the Discussion is now dedicated to this aspect (page 15, second paragraph) and we discuss membrane binding of RNase Y also with respect to the latest results obtained with RNase E which has also been shown to be a membrane binding protein.

11. Figure 2C. The "FL" label in the right margin is misplaced.

This has been corrected and « FL » has been replaced with « +1 ».

12. Several of the references to RNase E are very dated (e.g., Cohen & McDowall, 1997; Mudd et al, 1990). The Callaghan et al Nature paper from 2005 would be more suitable.

We have also added this reference, but kept most of the old references too because they contain the original data we are referring to.
Your revised manuscript has now been re-assessed by one of the original referees whose comments you will find enclosed.

As you will see essential concerns remain that relate to confidence in the conclusions that can be drawn from the actual experimental data, quality of figures and more careful statements on a putative role of YmdA in global mRNA decay (indeed a concern that had been similarly raised from the original referee#1). Despite these concerns, the overall support for potential publication here remains, pending satisfactorily further revisions. I therefore urge you to take these additional points seriously into consideration and address them in full before submitting an ultimate version of your paper for final assessment.

Yours sincerely,

Editor
EMBO Journal

REFEREE REPORTS:

Referee #3 (Remarks to the Author):

1. The ms is significantly improved in this revision and the comments I raised as reviewer #3 have been addressed. The authors have obviously invested considerable thought in responding to the reviewers. Nonetheless, I still have some concerns.

2. Page 7. First, on line 6 the authors should state that, "An N-terminally truncated form of YmdA was purified ....". Although it's a small point, the N-terminal 25 amino acids were never actually expressed and so weren't removed prior to purification. On line 16, there is a cleavage at position 52 (Fig. 3A) but also at two other positions closer to the 5'-end. Where do they map on the substrate? Third, the background with no enzyme in Fig. 3B is really very high. There is also at least one other band above the product with the scissors that does increase with time, but is not mentioned by the authors. Fourth, I am concerned that the results in Fig. 3 were obtained at very high enzyme to substrate ratios. Fifth, the authors also provide no evidence that the substrates fold as indicated in the figures, something that may explain the low efficiencies. But without having confidence in the structure of the substrates, the reader cannot be certain that the specificity of enzyme can be interpreted as the authors would like.

3. Page 8. On line 5, Fig. 3D should be Fig. 3C. On line 9, the authors should add, "plus many additional products, some in significant yields." to the end of the sentence. This is an example of authors seeing what they want, but ignoring everything else. On line 21, change "depended" to "depends". On line 26, add "full length" just before "leader".

4. Page 9. Figure 5C is of fair quality at best. The model for processing the riboswitch at the bottom of Fig. 5D is not very clear (and frankly, could merit a clear figure in the Discussion on its own).

5. Page 9-10 and Figure 6. The data in the figure raise as many questions as they address. First, do the observed sizes of species in the Northern agree with expectations? Why are the presumed degradative intermediates so variable in recovery and relative intensity compared to the full length species in each case? Compare Met E (no smaller intermediates) with MetK (larger and smaller bands are also visible) and YkrT where the "intermediate" is more intense than the ~240 nt main band.

6. Page 10 and Fig. 8. The gel has been so highly cropped that full length RNA is not visible, so the efficiency of conversion and overall quality is unclear.
7. Page 11, starting on line 18. The authors comment on the sensitivity of YmdA to RNA conformation, but this is speculation because the authors provide very little evidence for the assumed conformations of the substrate.

8. Discussion. I recommend that the authors shorten the Discussion, omit Fig. 7 in the process, and eliminate "and global mRNA decay" from the title. Based on a small sample, one or two sites in yitJ, it also seems premature to discuss sequence specificity of the cleavage (pages 13-14). Rather, the authors might want to focus on the pathway of SAM riboswitch decay and then allude to the possibility that YmdA participates more widely in RNA metabolism.

2nd Revision - Authors’ Response 20 August 2009

Referee #3 (Remarks to the Author):

1. The ms is significantly improved in this revision and the comments I raised as reviewer #3 have been addressed. The authors have obviously invested considerable thought in responding to the reviewers. Nonetheless, I still have some concerns.

2. Page 7. First, on line 6 the authors should state that, "An N-terminally truncated form of YmdA was purified ...". Although it's a small point, the N-terminal 25 amino acids were never actually expressed and so weren't removed prior to purification.
   > This has been corrected.

On line 16, there is a cleavage at position 52 (Fig. 3A) but also at two other positions closer to the 5'-end. Where do they map on the substrate?
   > We have added the following sentence: « Two minor but not reproducible cleavages were also observed after positions 124 (in the apical loop of the aptamer) and 185 (between the aptamer and the terminator, Fig. 3A)".

Third, the background with no enzyme in Fig. 3B is really very high.
   > The background in the control with no enzyme is the same as in the lanes containing the enzyme. This is because the relatively high background is not caused by the activity of the enzyme but by the 10 min incubation at 70°C in the presence of Mg^{2+} prior to the addition of the enzyme which leads inevitably to some non specific degradation of the RNA. All samples, including the control, were treated in the same way.

There is also at least one other band above the product with the scissors that does increase with time, but is not mentioned by the authors.
   > There is a very faint band corresponding to a cleavage two nucleotides downstream of the major cleavage site. We now mention this signal, too.

Fourth, I am concerned that the results in Fig. 3 were obtained at very high enzyme to substrate ratios. Fifth, the authors also provide no evidence that the substrates fold as indicated in the figures, something that may explain the low efficiencies. But without having confidence in the structure of the substrates, the reader cannot be certain that the specificity of enzyme can be interpreted as the authors would like.

> Yes, the enzyme to substrate ratio is high. We do not yet understand why a high amount of enzyme is necessary to observe reasonable cleavage in vitro. This situation is actually very similar to that of RNase J where we and others have observed that high enzyme to substrate ratios are necessary for endonucleolytic cleavage in vitro. This might be due to a missing partner which is present in vivo but not in vitro, an assumption that is plausible given the recent discovery of a potential degradosome-like complex in B. subtilis. Alternatively, or in addition it could be that the substrate did not fold properly explaining the low cleavage efficiency (as suggested by the reviewer). It is certainly correct that, for example, the structure binding SAM is not folding very efficiently in vitro and requires heating of the RNA. This has been observed for the yitJ riboswitch.
studied here and another member of the S-box family (McDaniel et al., 2003; Winkler et al., 2003). RNase Y cleavage depended nevertheless on the conformational switch of the S-box antitermination system. This switch has been thoroughly analysed and confirmed in vitro by RNase H and inline probing as well as in vivo by mutational studies notably from the T. Henkin and R. Breaker labs.

3. Page 8. On line 5, Fig. 3D should be Fig. 3C. On line 9, the authors should add, "plus many additional products, some in significant yields." to the end of the sentence. This is an example of authors seeing what they want, but ignoring everything else. On line 21, change "depended" to "depends". On line 26, add "full length" just before "leader".

> All these changes have been carried out as requested.

4. Page 9. Figure 5C is of fair quality at best. The model for processing the riboswitch at the bottom of Fig. 5D is not very clear (and frankly, could merit a clear figure in the Discussion on its own)

> In Fig. 5C there is some « streaking » from the marker bands in the first lane caused by the necessary exposure adjustment for the other lanes. The presence of ladder-like background bands notably in the sample with reduced RNase J1 levels was observed reproducibly and probably represents 5'-3' degradation intermediates. Also, the S1 experiment only has a supplementary role for other data.

In Fig. 5D the two mRNAs, for practical reasons, are drawn to a similar length in the cartoon. This actually confusing and we now indicate their real length (132 and 38 nts, respectively). We agree that it would have been ideal to include the model for processing the riboswitch in the Discussion. However, we think that an essential part of this figure is required to indicate the positions of the cleavage sites and the oligonucleotides used in the RACE experiment. A figure only depicting the processing model in the Discussion would then represent almost a duplication of Fig. 5D. We therefore decided to improve Figure 5D but leave it where it is.

5. Page 9-10 and Figure 6. The data in the figure raise as many questions as they address. First, do the observed sizes of species in the Northern agree with expectations?

> Yes, they do correlate well with the transcription initiation sites inferred from a sequence based search for promoters (Grundy and Henkin 1998). We now added this statement in the Discussion.

Why are the presumed degradative intermediates so variable in recovery and relative intensity compared to the full length species in each case? Compare Met E (no smaller intermediates) with MetK (larger and smaller bands are also visible) and YkrT where the "intermediate" is more intense than the ~240 nt main band.

> In order to assure complete specificity when probing all 11 S-box leaders we had to use oligonucleotides that hybridized to different regions of the respective leaders. This obviously led to the detection of different degradation intermediates depending, for example, on whether the probe hybridized to sequences up-or downstream of the putative RNase Y cleavage sites. We therefore do not interpret in detail the various Northern blots. The point we wanted to make here is that the turnover of all S-box riboswitches (except cysH) depends on RNase Y. This is clearly the case since for all incriminated genes the full length leader riboswitch is only detectable or significantly increased when RNase Y is depleted.

6. Page 10 and Fig. 8. The gel has been so highly cropped that full length RNA is not visible, so the efficiency of conversion and overall quality is unclear.

> We have already shown 4 full figures of RNase Y in vitro cleavages (Figs 3A/B and 4A/B) and we think that showing the major cleavage product in this case is sufficient to illustrate that the mutated proteins are much less active than the wild type protein.

7. Page 11, starting on line 18. The authors comment on the sensitivity of YmdA to RNA conformation, but this is speculation because the authors provide very little evidence for the assumed conformations of the substrate.

> As already mentioned above the invoked confirmation switch (for example in the presence or absence of SAM) has been studied extensively. While we cannot conclude on the quantitative formation of a given structure, our in vitro data together with the observation that antiterminated full length mRNAs are not cleaved significantly by RNase Y in vivo are good arguments that in our view justify our rather prudent comments in the Discussion.

8. Discussion. I recommend that the authors shorten the Discussion, omit Fig. 7 in the process, and...
eliminate "and global mRNA decay" from the title. Based on a small sample, one or two sites in yitJ, it also seems premature to discuss sequence specificity of the cleavage (pages 13-14). Rather, the authors might want to focus on the pathway of SAM riboswitch decay and then allude to the possibility that YmdA participates more widely in RNA metabolism.

> We have omitted Fig. 7 and also eliminated « and global mRNA decay » from the title. We shortened the discussion slightly but did not omit completely the discussion on sequence specificity. We believe that the apparent high cleavage specificity (SAM dependence) could simply be linked to the required presence of a secondary structure downstream of the cleavage site. This would be a reasonable explanation reconciling enzyme specificity and a global effect on mRNA metabolism, a point initially raised by reviewer 1.