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The key protein of endosomal mRNP transport Rrm4 binds translational landmark sites of cargo mRNAs

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Review timeline:

Submission date:	14 June 2018
Editorial Decision:	17 July 2018
Revision received:	18 October 2018
Editorial Decision:	7 November 2018
Revision received:	9 November 2018
Accepted:	9 November 2018

Editor: Martina Rembold

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 17 July 2018

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that the manuscript reports interesting and overall solid findings but they also point out that further clarification of experimental details and data interpretation is required.

In particular, it will be essential to provide data showing the results from the affinity tag purification that led to the identification of Grp1. Please contact me in case you want to keep some of the data for a future publication and we can discuss this aspect further. Moreover, it will be important to provide further details on the iCLIP protocol. The referees are also concerned if the reported association of Rrm4 with the start codon is a by-product of the presence of an AUG at that position rather than truly an enrichment over other UAUG motifs in the genome. Please also be more cautious in drawing conclusions on binding strength based on the number of cross-linking events, as indicated by the referees. In addition, referee 1 pointed out that the functional significance of the reported association of Rrm4 with start and stop codons has not been investigated.
Upon further discussion with the referees we conclude that functional data on these mRNAs would certainly strengthen the manuscript and could be added if easy to obtain (e.g., by mining earlier obtained proteomics data or by testing some candidate mRNAs). However, these experiments are not mandatory.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page with page numbers, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

Regarding data quantification, please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied. Please also include scale bars in all microscopy images.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision). Please insert page numbers in the checklist to indicate where the requested information can be found.
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted figure files in high resolution
(In order to avoid delays later in the publication process please check our figure guidelines before preparing the figures for your manuscript:
http://www.embopress.org/sites/default/files/EMBOPress_Figure_Guidelines_061115.pdf)
- a separate PDF file of any Supplementary information (in its final format)
- all corresponding authors are required to provide an ORCID ID for their name. Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines (http://embor.embopress.org/authorguide).

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if

you have questions or comments regarding the revision.

REFEREE REPORTS
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Referee #1:

This manuscript describes investigations into the nature of RNA-protein interactions during endosome-associated mRNP transport in Ustilago. This is a valuable model system for studying mRNA transport and therefore the findings are likely to be of broad appeal. The manuscript covers an impressive amount of ground and the technical quality of the study is generally very high.

The advances described in the manuscript include:

- the identification of Grp1 as a novel RNA-binding constituent of endosomal/mRNP transport complexes, and the demonstration that this protein is required for the co-ordination of hyphal growth
- the genome-wide analysis of RNA sequences bound by Grp1 and the key RNA binding protein for mRNP transport Rrm4, leading to the realization that these proteins often bind in close proximity
- the discovery of a consensus RNA motif bound by Rrm4, and the occupancy of start codons and stop codons of many RNAs with this protein.

Major points:
1. My major criticism of the paper is that the potential function(s) of the very interesting association of Rrm4 with start and stop codons is not investigated experimentally. For example, have the authors tested if RNAs that have such a mode of Rrm4 binding have altered levels of protein product when Rrm4 is disrupted? The authors describe some candidates that could be tested (Sui1, Dyn1/2, F0F1-ATPase components).
2. Is the enrichment of Rrm4 at start codons versus other UAUG sites in target mRNAs statistically significant? In other words, is the start codon binding simply a by-product of the presence of an AUG?
3. On line 300 the authors write "We therefore conclude that the potential RNA chaperone Grp1 is involved in efficient transport of mRNPs, in particular under suboptimal conditions". I could not find data that support such a specific conclusion. Either this statement should be supported with data (e.g. imaging of mRNP transport in suboptimal conditions) or modified. Couldn't Grp1 be involved in another aspect of post-transcriptional regulation such as translation?
4. Data must be shown from the affinity tag purification experiments that led to the identification of Grp1 as an Rrm4-associated protein. This addition should allow the reader to judge the strength of the evidence for a specific and robust interaction. Was the capture of Grp1 in this experiment dependent on RNA (I gather from the methods that fungal extracts contain RNases but the authors should deal with my question explicitly in the text)? One might expect the interaction to be RNA-dependent as it is abolished by the Rrm4[mR123] mutation. If that is not the case, what do the authors think is going on?

Minor points:
1. Line 178: Gpr1 is written instead of Grp1
2. The authors present an interesting hypothesis that RNAs that bind Grp1 and not Rrm4 might have a perinuclear localization. If they have data to directly support this conclusion they may wish to present it, but this is not essential.
3. Line 227: the authors refer to "stronger relative binding" but presumably they mean more frequent cross-linking? I assume that the number of cross-links is not always a direct readout of affinity.
4. Figure 6. Can the authors rule out that binding of Rrm4 to the different regions of a single mRNA species is not due to RRM3 domains provided by multiple copies of Rrm4. If not, the authors should consider including this caveat in the model.
5. In some figure panels, it would appear that corrections for multiple comparisons should be applied for the tests of statistical significance (e.g. Figure 1C). Was this the case? If not, this should be done and the figures and figure legends edited accordingly.

Referee #2:

The paper deals with the transport of mRNAs in hyphae of Ustilago maydis. The RNAs are transported along microtubules in association with endosomes. Starting from the known protein Rrm4, which binds RNAs and is essential for transport, the authors have identified a second protein involved in the process, Grp1. They have determined transcriptome-wide binding sites for both proteins by iCLIP. Both proteins bind a large fraction of the transcriptome, binding sites for both are primarily in the 3' UTR and are frequently close to each other. For Rrm4, the authors identify a specific tetranucleotide binding motif that is recognized by the protein's third RRM.

In my opinion, this is an interesting paper and technically convincing. It should be published provided that some details are taken care of.

Main comments:

1. The authors start with the identification of Grp1 in some kind of affinity purification using tagged Rrm4. I could not find any information on this procedure, and I could not find any information why or how the authors picked Grp1, presumably from a larger number of proteins found. I can understand if they want to hold back some information for future papers, but some minimum information must be provided here.
Along the same lines, it would obviously be interesting to know if Grp1 and Rrm4 associate with each other directly.

2. Modifications of the iCLIP protocol: The authors stress (line 145) the importance of the optimal UV dose. Looking at Fig. S2C, I cannot say why 200 J/cm2 (line 456) is optimal. It is also not clear why not mixing the cells (legend to Fig. S2) saves so much time (probably not so important). Information on p. 20 (lines 456ff) does not include the time of irradiation. I would assume it matters over which time interval the irradiation energy is accumulated. Presumably, it is also relevant in which type of container the 5 ml aliquots were kept during irradiation (spread thin in a Petri dish or as a thick layer in a tube).
In Fig. S2, the asterisks are not explained.
In the description of the method, 'RNase I (1/10 dilution)' (lines 449 and 894) is useless information. Please be more precise.

3. The iCLIP data are presented and discussed in a partially unclear manner. The terms 'crosslink nucleotide' and 'crosslink event' are explained on p. 23, but what is a 'binding site'? Is this is explained in lines 528 ff? Please refer to the definitions when the terms are first used. What is 'the first position' (line 530)? Does this refer to the first position in a read? What are 'binding site clusters'? For example, in Fig. 3C, complex peaks of 'crosslink events per nucleotide' are somehow converted into 'clusters' (black rectangles). This is unclear. In the same figure, color coding is unclear: Is a black rectangle a cluster without UAUG and an orange rectangle a cluster with UAUG? Please explain. Similar question for Fig. 3E, gene cdc10, middle graph: Is orange a 'filtered binding site' with UAUG and red a filtered binding site without UAUG? Choose colors that are more distinct - what I assume is called 'red' looked more brownish in my print-out.

4. Fig. 4 and corresponding text: The definition of 'binding site' (see my questions above) is particularly important when 'overlapping binding sites' are discussed (line 193, Fig. 4B). Does this refer to overlapping sequences of 9 nt (line 531)?
Fig. 4A shows 1783 Rrm4 binding sites in ORFs, the text (line 198) mentions 1315. Please clarify.
Line 200: What is meant by 'selected' target mRNAs?
Line 201: 'Rrm4 binding sites overlapped the start codon in 47 cases.' Again, does this mean that the start codon was in the 9 nt window? The numbers of overlaps between start or stop codons with Rrm4 binding sites mentioned in the text refers to 'cases' - presumably mRNAs. The numbers in Fig. 4 D are 'crosslinking events', presumably including multiple events to the same mRNA. Thus, the numbers are not directly comparable.
Fig. 4C: Obviously, the mRNAs are ordered according to the distribution of binding sites in the 3' UTR. However, in the Grp1 data, there is one RNA at about position 160 on the y axis which does not fit into the distribution. Please clarify.
The authors discuss (line 204) that binding of Rrm4 to a start codon would presumably reflect transport in a translationally silent state. A similar argument would apply to binding the stop codon:

Ribosomes would be expected to be stalled along the RNA.

5. Fig. 6B: As far as I can tell, the authors have no idea of the stoichiometry of protein binding to the RNA. I am not suggesting they change the figure, but they might wish to point this out. I do not find it very appealing that a single protein should simultaneously bind distinct sites spread over a long mRNA molecule, and tight enough to prevent translation. My gut feeling would be that a more complex RNP is built, with additional Rrm4 copies and/or with other proteins.

Minor comments:

Line 37: 'rate of bipolarly growing hyphae increases' - this is unclear: Does the rate of growth increase, or is the fraction of hyphae showing this particular growth pattern increased?

Lines 91, 106 and perhaps elsewhere: This may be a philosophical question, but I think that one should not do an experiment to 'support' or 'verify' a hypothesis, but in order to 'test' or even 'challenge' it - it is an important difference in attitude.

Fig. 2: One can guess what the 'kymographs' are, but a brief explanation would be helpful - this is not standard knowledge.

Lines 113ff: I believe the logic is wrong: If 97% of Grp1-Gfp signals are associated with Pab1-mCherry, this leaves open the possibility that the majority of Pab1-mCherry signals are NOT associated with Grp1-Gfp, so the observation as stated does not justify the conclusion that Grp1 is present on all Rrm4-positive endosomes.

Line 247: I assume the reference should be to Fig. 5G, not 5H.

Line 249: Replace 'into an UAUG motif' by 'in an UAUG motif'?

Line 265: Replace 'provided' by 'provide' to match 'at present'.

Line 274: Delete 'According'.

Line 280: It would be better to say 'IDRs CAN mediate the assembly....'.

Referee #3:

The manuscript "The key protein of endosomal mRNP transport binds translational landmark sites of cargo mRNAs" by Michael Feldbruegge and colleagues describes a comprehensive analysis of the RNA-binding protein Grp1 and its interaction partner Rrm4. Both proteins are involved in endosomal messenger ribonucleoprotein (mRNP) transport in Ustilago maydis. The authors report that both proteins bind a large proportion of all mRNAs in Ustilago with a substantial overlap of target mRNAs. Binding sites of both proteins were preferentially located in the 3' UTR in close proximity to each other. In addition, Rrm4 also bound 50 and 300 transcripts in the vicinity of the start and the stop codon, respectively. The characteristics and binding preferences of Grp1 and Rrm4 suggest a model, in which these proteins bind target mRNAs in their 3' UTRs during endosomal mRNP transport, whereby the translation of these targets is possible during transport. On a subset of targets, Rrm4 also binds in the ORF and/or at the start codon, which might interfere with translation of these mRNAs.
The manuscript contains many interesting data of very good quality. It is certainly an important work for RNA transport in Ustilago maydis and defines a benchmark for future work on RNA-binding proteins in this (or similar) organisms. Particularly the optimization of the iCLIP protocol for Ustilago should be positively mentioned. While this work did not reveal really outstanding findings, it is overall a solid manuscript, which can certainly be published in EMBO reports. I only have a few comments on the work that should be addressed before publication.
1. The most difficult thing for me to understand is how the authors incorporate their data into a model (Figure 6). They write about the UAUG motif that they identified "This motif is recognised by the third RRM domain of Rrm4, mutations of which previously led to strongly reduced overall RNA binding of Rrm4 (Becht et al., 2006)" (p17, lines 371-373). If the third RRM indeed

determines the RNA binding of Rrm4, why does the model suggest that RRM1 and RRM2 bind to the most important binding sites in the 3' UTRs of Rrm4 target mRNAs? Also, the fact that "more than one third of all Rrm4 binding sites harboured a UAUG motif precisely at the centre of the binding site" (p.11, lines 22-225) does not seem to fit to this model. This inconsistency requires clarification. In this context it would also be interesting to know whether Grp1 enhances binding of the RRM1 of Rrm4 to RNA.
2. How many UAUG motifs are there in Ustilago (in total and at the start codon) and how many of them are bound according to the iCLIP by Rrm4?
3. The authors write that "Notably, the Rrm4 binding sites with UAUG showed stronger relative binding than those lacking the motif (Fig. 5C), suggesting a tight interaction of Rrm4 with the UAUG-associated binding sites". If I understand it correctly, Figure 5C shows a higher number of "relative crosslinking events of Rrm4 binding sites". Although this indicates stronger binding, I'm not sure if this can be written explicitly.
4. I do not understand the connection between cell wall synthesis/integrity, hyphal growth and the mRNA binding/mRNP transport function of Grp1. Perhaps this can be explained in more detail.

1st Revision - authors' response 18 October 2018

Point-by-Point Response to the comments of the Referees.

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Referee #1:

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The advances described in the manuscript include:

- the identification of Grp1 as a novel RNA-binding constituent of endosomal/mRNP transport complexes, and the demonstration that this protein is required for the co-ordination of hyphal growth

- the genome-wide analysis of RNA sequences bound by Grp1 and the key RNA binding protein for mRNP transport Rrm4, leading to the realization that these proteins often bind in close proximity

- the discovery of a consensus RNA motif bound by Rrm4, and the occupancy of start codons and stop codons of many RNAs with this protein.

Major points:

1. My major criticism of the paper is that the potential function(s) of the very interesting association of Rrm4 with start and stop codons is not investigated experimentally. For example, have the authors tested if RNAs that have such a mode of Rrm4 binding have altered levels of protein product when Rrm4 is disrupted? The authors describe some candidates that could be tested (Sui1, Dyn1/2, F0F1-ATPase components).

We agree with the Reviewer that these are very valuable experiments. However, since this analysis would require to generate and carefully test a number of new reporter constructs, we believe that it is beyond the scope of the current manuscript.

Following the Editor's suggestion, we revisted previous proteomics data from our lab. In an earlier study, we performed a differential proteomics approach comparing wild type and *rrm4D* hyphae 6 h.p.i. (2D-DIGE technology; Koepke et al, Mol Cell Proteomics, 2011). We focused at the time on membrane-associated proteins, because in pilot experiments, we oberserved most differences in this protein fraction. We were able to identify ten protein spots exhibiting significantly different amounts (>2.5fold differences). These included, for instance, chitinase Cts1, an enzyme whose unconventional secretion depends on Rrm4 (Koepke et al, Mol Cell Proteomics, 2011; Stock et al, J Biotechnol, 2012), underlining that the analysis provided valuable insights. Interestingly, one differential protein spot corresponded to Atp4, one of the nuclear-encoded subunits of the F_oF_1-ATPase, which are all encoded by Rrm4 target mRNAs. The differential proteomics analysis

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indicated that the protein amount was reduced in *rrm4D* hyphae. In line with a direct impact of Rrm4 binding on protein abundance, the *atp4* mRNA shows Rrm4 crosslink events at the stop codon (Fig EV 4A), though not enough to be called a binding site (see peak calling and filtering in Materials and methods). Nevertheless, this observation supports the hypothesis that Rrm4 might influence translation of target mRNAs encoding subunits of the mitochondrial ATPase.

We show the revisted proteomics data in the new Fig EV4D. Since we did not verify this observation with additional experiments, we present the result very cautiously.

2. Is the enrichment of Rrm4 at start codons versus other UAUG sites in target mRNAs statistically significant? In other words, is the start codon binding simply a by-product of the presence of an AUG?

In order to address this question, and following also the suggestion by Reviewer #3, we first counted all UAUG motifs and overlapping Rrm4 binding sites. Within transcripts that carry Rrm4 binding sites in our iCLIP analysis and are hence sufficiently expressed, we find a total of 14,748 UAUG motifs, of which 15.2% are bound by Rrm4. In general, UAUG motifs are less abundant around the translational start site, because AUG's in the 5'UTR and at the beginning of the open reading frame might interfere with translational initiation. Out of 282 UAUG motifs directly at start codons, 14.5% are bound by Rrm4. This is only marginally more than for 215 and 483 UAUG motifs within 100 nt upstream and downstream of the start codons, out of which 13.0% and 11.0% are bound, respectively. Thus, there is no significant increase in binding to UAUG when it occurs in the context of a start codon. Rather, as the Reviewer pointed out, Rrm4 binds these start codons as its recognition motif UAUG overlaps with AUG. Nevertheless, it is conceivable that by recognising a motif, which can incorporate the start codon and thereby results in binding in 47 cases precisely at the start codon of the transcript, Rrm4 might have an impact on translational initiation.

To address this point in the manuscript, we now include an additional Fig EV5E which summarises Rrm4 binding to UAUG motifs around start codons. Since we did not find evidence for a particular preference for UAUG motifs at start codons, we toned down the respective section in the text and exchanged the example gene shown in Figure 4. We now shows the Rrm4 binding sites within the *rrm4* gene rather than the start codon binding in the *sui1* gene (now Fig EV3D). Nevertheless, it holds that Rrm4 recognises 47 mRNAs at the start codon, with a potential to regulate translational initiation, which is still mentioned in the discussion.

3. On line 300 the authors write "We therefore conclude that the potential RNA chaperone Grp1 is involved in efficient transport of mRNPs, in particular under suboptimal conditions". I could not find data that support such a specific conclusion. Either this statement should be supported with data (e.g. imaging of mRNP transport in suboptimal conditions) or modified. Couldn't Grp1 be involved in another aspect of post-transcriptional regulation such as translation?

We absolutely agree with the Reviewer that this was an exaggeration and rephrased the sentence. It now reads: „We therefore propose that the potential RNA chaperone Grp1 most likely constitutes an accessory component of endosomal mRNPs. Its function could be particularly important under suboptimal conditions. Alternatively, Grp1 might regulate stability and/or translation of mRNAs encoding proteins involved in hyphal growth independent of endosomal mRNA transport."

4. Data must be shown from the affinity tag purification experiments that led to the identification of Grp1 as an Rrm4-associated protein. This addition should allow the reader to judge the strength of the evidence for a specific and robust interaction. Was the capture of Grp1 in this experiment dependent on RNA (I gather from the methods that fungal extracts contain RNases but the authors should deal with my question explicitly in the text)? One might expect the interaction to be RNA-dependent as it is abolished by the Rrm4[mR123] mutation. If that is not the case, what do the authors think is going on?

As indicated, Grp1 was found in pilot affinity purification experiments using Rrm4-GfpTT as bait. With two independent experimental approaches (by performing mass spectrometry in a complex mixture in solution or by cutting individual bands from an SDS-PAGE gel), we identified Grp1 as a potential interaction partner of Rrm4. Both experiments were considered pilot studies and were only performed once. Instead, we verified the interaction of Grp1 with Rrm4 using *in vivo* dynamic live cell imaging.

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The proteomics experiments at the time did not include an RNase treatment to test for RNA dependency. However, as pointed out by the Reviewer, since endosomal shuttling of Grp1 was no longer detectable with Rrm4-mR123, we believe that the interaction between both proteins is dependent on mRNA. Based on our analysis, we propose that both proteins bind in close proximity in the 3'UTRs of their target mRNAs. Whereas Rrm4 is linked to endosomes via its specific interaction with the FYVE domain protein Upa1, Grp1 is mainly associated to endosomes via its binding to cargo mRNAs.

We now included the inital list of candidate Rrm4 interaction partners from the mass spectrometry approach as well as the gel image of the preliminary tandem affinity purification in Fig EV1C-D.

Minor points:

1. Line 178: Gpr1 is written instead of Grp1

We thank the Reviewer for spotting this mistake.

2. The authors present an interesting hypothesis that RNAs that bind Grp1 and not Rrm4 might have a perinuclear localization. If they have data to directly support this conclusion they may wish to present it, but this is not essential.

Unfortunately, we do not have currently further data to support this hypothesis.

3. Line 227: the authors refer to "stronger relative binding" but presumably they mean more frequent cross-linking? I assume that the number of cross-links is not always a direct readout of affinity.

We agree with the Reviewer that the number of crosslink events should not be used to compare binding sites between transcripts, as it strongly depends on the abundance of the underlying transcript. In order to correct for this, we had normalised the crosslink events within a binding site to the background crosslink events in the surrounding sequence ('signal-over-background', SOB). The underlying assumption is that the background crosslinking of an RBP to a given mRNA can be used as a proxy for the mRNA's expression level. We previously developed this procedure to compare in vivo iCLIP measurements to in vitro binding assays, in which we could determine dissociation constants (Sutandy et al, Genome Res, 2018).

We apologise that this procedure had not been clear in the previous version of the manuscript. In response to the Reviewer's comment, we revised the respective sections in the main text and also give a more detailed explanation in Materials and methods.

4. Figure 6. Can the authors rule out that binding of Rrm4 to the different regions of a single mRNA species is not due to RRM3 domains provided by multiple copies of Rrm4. If not, the authors should consider including this caveat in the model.

Our model is based on the following observations: (1) RRM3 of Rrm4 binds UAUG, (2) UAUG is clearly enriched in the ORF and hardly present in 3´ UTR (see e.g. Fig 3C,E), and (3) transcripts with Rrm4 binding site in the 3´ UTR were significantly enriched for a second Rrm4 binding site in the ORF.

However, we agree with the Reviewer that we cannot discriminate whether one or multiple copies of Rrm4 are present on the same mRNA. To account for this, we simplified the model and indicate in the figure legend that higher-order mRNP structures are most likely formed.

5. In some figure panels, it would appear that corrections for multiple comparisons should be applied for the tests of statistical significance (e.g. Figure 1C). Was this the case? If not, this should be done and the figures and figure legends edited accordingly.

Following the Reviewer's suggestion, we performed a multiple testing correction (Benjamini-Hochberg) to the p-values from all tests in Fig 1C. Figure and legend have been updated accordingly. According to the author checklist, we changed Figure 1F, Figure 2B, 2D and 2F from bar diagrams to scatter plots, since the data points were smaller than five. We also verified the statistical tests and included the information in the figure legends.

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Referee #2:

The paper deals with the transport of mRNAs in hyphae of Ustilago maydis. The RNAs are transported along microtubules in association with endosomes. Starting from the known protein Rrm4, which binds RNAs and is essential for transport, the authors have identified a second protein involved in the process, Grp1. They have determined transcriptome-wide binding sites for both proteins by iCLIP. Both proteins bind a large fraction of the transcriptome, binding sites for both are primarily in the 3' UTR and are frequently close to each other. For Rrm4, the authors identify a specific tetranucleotide binding motif that is recognized by the protein's third RRM.

In my opinion, this is an interesting paper and technically convincing. It should be published provided that some details are taken care of.

Main comments:

1. The authors start with the identification of Grp1 in some kind of affinity purification using tagged Rrm4. I could not find any information on this procedure, and I could not find any information why or how the authors picked Grp1, presumably from a larger number of proteins found. I can understand if they want to hold back some information for future papers, but some minimum information must be provided here. Along the same lines, it would obviously be interesting to know if Grp1 and Rrm4 associate with each other directly.

This relates directly to main point 4 of Reviewer #1. We now include the results of the preliminary study in Figure EV1C-D.

2. Modifications of the iCLIP protocol: The authors stress (line 145) the importance of the optimal UV dose. Looking at Fig. S2C, I cannot say why 200 J/cm2 (line 456) is optimal.

We observed that increasing the UV dose resulted in unspecific RNA/protein complexes with the negative control Gfp. Since Gfp/RNA complexes were detected using 300 or 400 mJ/cm^2, we chose 200 J/cm^2 as optimal for our experiments.

To clarify this point, we now mention this consideration in the figure legend of Fig EV2: "We chose 200 mJ/cm^2 as optimal UV-C irradiation dose, since the amount of unspecific Gfp-RNA complexes increased at higher doses."

It is also not clear why not mixing the cells (legend to Fig. S2) saves so much time (probably not so important).

We agree that omiting this step seems like a minor improvement. However, mixing the cells thoroughly still takes some time, and any possible shortcut was included to minimise the loss of material.

Information on p. 20 (lines 456ff) does not include the time of irradiation. I would assume it matters over which time interval the irradiation energy is accumulated.

The time interval depends on the age of the UV tubes. The irradiation device (Biolink UV-Crosslinker, Vilber-Lourmat, Eberhardzell, Germany) adjusts the irradiation time automatically to obtain the targeted UV dose (about 60 seconds for 200 J/cm^2).

Presumably, it is also relevant in which type of container the 5 ml aliquots were kept during irradiation (spread thin in a Petri dish or as a thick layer in a tube).

This is correct. We used square petri dishes with an area of 10 cm^2. We now included this information in the protocol description in Materials and methods.

In Fig. S2, the asterisks are not explained.

We thank the Reviewer for spotting this. The asterisks mark putative degradation products of the purified RBPs. We now include this information in the legend of Fig EV2.

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In the description of the method, 'RNase I (1/10 dilution)' (lines 449 and 894) is useless information. Please be more precise.

In response to this comment, we revised the chapter "iCLIP experiments" in Materials and methods. Instead of the degree of dilution, we now specify the applied concentration in Units. The figure legend now refers to this chapter.

3. The iCLIP data are presented and discussed in a partially unclear manner. The terms 'crosslink nucleotide' and 'crosslink event' are explained on p. 23, but what is a 'binding site'? Is this is explained in lines 528 ff? Please refer to the definitions when the terms are first used. What is 'the first position' (line 530)? Does this refer to the first position in a read? What are 'binding site clusters'? For example, in Fig. 3C, complex peaks of 'crosslink events per nucleotide' are somehow converted into 'clusters' (black rectangles). This is unclear. In the same figure, color coding is unclear: Is a black rectangle a cluster without UAUG and an orange rectangle a cluster with UAUG? Please explain. Similar question for Fig. 3E, gene cdc10, middle graph: Is orange a 'filtered binding site' with UAUG and red a filtered binding site without UAUG? Choose colors that are more distinct - what I assume is called 'red' looked more brownish in my print-out.

We apologise that the analysis of the iCLIP data had not been clear (see also Minor Point 3 of Reviewer #1). As suggested by the Reviewer, we introduce the term binding site and explain in more detail the different steps in the analysis pipeline. In brief, we first used ASpeak to detect windows with significantly increased crosslink event frequency ("peak calling"). In order to ease comparisons, we then resized the initial peak predictions into uniform 9-nt windows that were further filtered according to their reproducibility between replicates and resolved overlapping windows. In this context, 'first position' meant that when determining the position with highest number of crosslink events within the peak, the most upstream position was taken if two or more positions showed the same count (now explained more precisely in Materials and methods).

In order to focus on the top 25% of binding sites, we included an additional processing step, in which we used the ratio of crosslink events within the binding site over the crosslink events in the surrounding sequence (termed 'signal-over-background', SOB) as a proxy of binding site strength. In the original version of the manuscript, we initially showed binding sites also before this processing step in the first genome browser view (refered to as 'clusters' to distinguish from the finallly filtered binding sites). To avoid confusion, we now only use the finally filtered binding sites throughout the manuscript.

In the revised version, we improved the description of the iCLIP data in the text and explain more carefully in Materials and methods how binding sites were determined. We removed the mention of 'clusters' from the figures and the text and changed the orange colour for better visibility.

4. Fig. 4 and corresponding text: The definition of 'binding site' (see my questions above) is particularly important when 'overlapping binding sites' are discussed (line 193, Fig. 4B). Does this refer to overlapping sequences of 9 nt (line 531)?

Binding sites of Rrm4 and Grp1 were considered as overlapping if at least one nucleotide was shared within the 9-nt binding site. We added this information to the description of the iCLIP data analysis in Materials and methods.

Fig. 4A shows 1783 Rrm4 binding sites in ORFs, the text (line 198) mentions 1315. Please clarify.

Figure 4A shows the number of Rrm4 binding sites in ORFs, whereas the mentioned text passage refers to the number of target mRNAs with Rrm4 binding sites in the ORF. In order to clarify this, we rephrased the text, now specifying „1,315 mRNAs with 1,783 ORF binding sites".

Line 200: What is meant by 'selected' target mRNAs?

The sentence meant to say that not all target mRNAs show this binding pattern. We rephrased to „of a subset of target mRNAs".

Line 201: 'Rrm4 binding sites overlapped the start codon in 47 cases.' Again, does this mean that the start codon was in the 9 nt window? The numbers of overlaps between start or stop codons with Rrm4 binding sites mentioned in the text refers to 'cases' - presumably mRNAs. The numbers in Fig.

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4 D are 'crosslinking events', presumably including multiple events to the same mRNA. Thus, the numbers are not directly comparable.

The Reviewer is correct that the numbers in Fig 4D are not directly comparable, since the plots displays total Rrm4 (and Grp1) crosslinking at these sites. In order to clarify this and also the other questions raised by Reviewer, we rephrased the respective sentence.

Fig. 4C: Obviously, the mRNAs are ordered according to the distribution of binding sites in the 3' UTR. However, in the Grp1 data, there is one RNA at about position 160 on the y axis which does not fit into the distribution. Please clarify.

The mRNAs in Fig 4C are not ordered by binding site locations but by decreasing 3' UTR length. We apologise that this information was missing in the figure legend and corrected this in the revised version. The same order of mRNAs underlies both panels in Fig 4C.

Since the *Ustilago maydis* gene annotation only includes the open reading frames of genes, we manually annotated transcript ends by visual inspection of RNA-seq data from wt AB33 hyphae. A more detailed description of the manual 3' UTR annotation can be found in the Methods section. Since standard RNA-seq coverage usually drops before the actual transcript end, this procedure harbours a certain inaccurracy, in particular for lowly expressed genes. This may explain the one RNA which does not seem to fit. Moreover, the depicted region extends up to 350 nt behind the stop codon, which may reach into the next transcribed gene, especially for genes with short 3' UTRs.

The authors discuss (line 204) that binding of Rrm4 to a start codon would presumably reflect transport in a translationally silent state. A similar argument would apply to binding the stop codon: Ribosomes would be expected to be stalled along the RNA.

We agree with the Reviewer that Rrm4 binding at start and stop codons may interfere with translation at the level of initation and termination. Since we toned tone the start codon binding we deleted the sentence on transport in a translationally silent state from the Results section. However, in the discussion we mention that Rrm4 binding at start and stop codons might modulate translation at different stages of translation, namely translational initiation and termination.

5. Fig. 6B: As far as I can tell, the authors have no idea of the stoichiometry of protein binding to the RNA. I am not suggesting they change the figure, but they might wish to point this out. I do not find it very appealing that a single protein should simultaneously bind distinct sites spread over a long mRNA molecule, and tight enough to prevent translation. My gut feeling would be that a more complex RNP is built, with additional Rrm4 copies and/or with other proteins.

We agree with the Reviewer that we lack information about the stoichiometry of the mRNP and cannot discriminate whether one or multiple copies of Rrm4 are present on the same mRNA (see also Minor Point 4 of Reviewer #1). As pointed out above, we simplified our model and removed the very speculative RNA loop. We also indicate in the figure legend that higher-order mRNP structures are most likely formed.

Minor comments:

Line 37: 'rate of bipolarly growing hyphae increases' - this is unclear: Does the rate of growth increase, or is the fraction of hyphae showing this particular growth pattern increased?

We clarified this. The text now reads: "the fraction of hyphae growing bipolarly increases".

Lines 91, 106 and perhaps elsewhere: This may be a philosophical question, but I think that one should not do an experiment to 'support' or 'verify' a hypothesis, but in order to 'test' or even 'challenge' it - it is an important difference in attitude.

We agree with the Reviewer and revised the manuscript accordingly.

Fig. 2: One can guess what the 'kymographs' are, but a brief explanation would be helpful - this is not standard knowledge.

We added the following explanation to the figure legend: "To visualise directed movement of signals (distance over time) within a series of images, kymographs were generated by plotting the position of signals along a defined path (x-axis) for each frame of the corresponding video (y-axis)."

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Lines 113ff: I believe the logic is wrong: If 97% of Grp1-Gfp signals are associated with Pab1-mCherry, this leaves open the possibility that the majority of Pab1-mCherry signals are NOT associated with Grp1-Gfp, so the observation as stated does not justify the conclusion that Grp1 is present on all Rrm4-positive endosomes.

We revised the sentence accordingly.

Line 247: I assume the reference should be to Fig. 5G, not 5H.

Line 249: Replace 'into an UAUG motif' by 'in an UAUG motif'?

Line 265: Replace 'provided' by 'provide' to match 'at present'.

Line 274: Delete 'According'.

We thank the Reviewer for spotting these mistakes, which we corrected accordingly.

Line 280: It would be better to say 'IDRs CAN mediate the assembly....'.

We edited the sentence accordingly.

****

Referee #3:

The manuscript "The key protein of endosomal mRNP transport binds translational landmark sites of cargo mRNAs" by Michael Feldbruegge and colleagues describes a comprehensive analysis of the RNA-binding protein Grp1 and its interaction partner Rrm4. Both proteins are involved in endosomal messenger ribonucleoprotein (mRNP) transport in Ustilago maydis. The authors report that both proteins bind a large proportion of all mRNAs in Ustilago with a substantial overlap of target mRNAs. Binding sites of both proteins were preferentially located in the 3' UTR in close proximity to each other. In addition, Rrm4 also bound 50 and 300 transcripts in the vicinity of the start and the stop codon, respectively. The characteristics and binding preferences of Grp1 and Rrm4 suggest a model, in which these proteins bind target mRNAs in their 3' UTRs during endosomal mRNP transport, whereby the translation of these targets is possible during transport. On a subset of targets, Rrm4 also binds in the ORF and/or at the start codon, which might interfere with translation of these mRNAs.
The manuscript contains many interesting data of very good quality. It is certainly an important work for RNA transport in Ustilago maydis and defines a benchmark for future work on RNA-binding proteins in this (or similar) organisms. Particularly the optimization of the iCLIP protocol for Ustilago should be positively mentioned. While this work did not reveal really outstanding findings, it is overall a solid manuscript, which can certainly be published in EMBO reports. I only have a few comments on the work that should be addressed before publication.

1. The most difficult thing for me to understand is how the authors incorporate their data into a model (Figure 6). They write about the UAUG motif that they identified "This motif is recognised by the third RRM domain of Rrm4, mutations of which previously led to strongly reduced overall RNA binding of Rrm4 (Becht et al., 2006)" (p17, lines 371-373). If the third RRM indeed determines the RNA binding of Rrm4, why does the model suggest that RRM1 and RRM2 bind to the most important binding sites in the 3' UTRs of Rrm4 target mRNAs? Also, the fact that "more than one third of all Rrm4 binding sites harboured a UAUG motif precisely at the centre of the binding site" (p.11, lines 22-225) does not seem to fit to this model. This inconsistency requires clarification. In this context it would also be interesting to know whether Grp1 enhances binding of the RRM1 of Rrm4 to RNA.

Important for the understanding of our model are the following results that were obtained previously (Becht et al., 2006):

(i) Mutations in RRM1 and RRM3 reduced the RNA binding activity of Rrm4 in *in vivo* UV crosslinking experiments. The strongest reduction was observed for RRM3.

(ii) Mutations in RRM1 lead to loss-of-function, i.e. the respective strains exhibit the same mutant bipolar growth phenotype as *rrm4D* strains. The mutant Rrm4 variant is still shuttling on endosomes like wild type protein, indicating that the phenotype is linked to loss of RNA binding.

(iii) Mutations in RRM3 do not cause a mutant phenotype. Growth of the respective hyphae is indistinguishable from wild type. Thus, this domain is not essential for the function of Rrm4. As expected, this variant is also shuttling on endosomes like wild type.

Combined with the new findings, we propose the following model: RRM3 binds to the UAUG sequence mainly within the ORFs (Figs 5F & 5G). Rrm4 binding at these sites is consistently stronger than at the 3'UTR binding sites (Fig 5C), consistent with the strong reduction in overall RNA binding upon mutation of RRM3 (Becht et al., 2006). Since the RRM3-mediated binding at the UAUG-containing binding sites appears to be not crucial for unipolar hyphal growth (Becht et al., 2006), we hypothesised that the interaction is used for fine tuning. In contrast, the other two RRMs most likely bind at non-UAUG sites, present mainly in the 3´ UTRs (Fig 5G), which are likely to be functionally relevant (Becht et al., 2006).

In a revised version, we now modified the discussion accordingly. The text now reads

"At start codons and within the ORF, the Rrm4 binding sites frequently harboured UAUG. This motif is recognised by the third RRM domain of Rrm4. In accordance with its ELAV-type domain organisation, we therefore propose that Rrm4 binds UAUG-containing binding sites via its third RRM to influence translation (Fig 6B), while the two tandem RRMs (RRM1/2) bind the target mRNAs in the 3´ UTR, possibly together with Grp1 (Fig 6B). In a previous study, we observed that mutations in RRM1 and RRM3 led to strongly reduced overall RNA binding of Rrm4, although mutations in RRM3 did not show a mutant phenotype with respect to hyphal growth [16]. In contrast, mutations in RRM1 affected hyphal growth strongly [16], accompanied by reduced endosomal shuttling of Pab1 as well as cdc3 mRNA [6]. Therefore, the potential translational regulation during endosomal mRNP transport mediated by RRM3 may be an additive and used for fine tuning. The tandem RRM domains RRM1 and RRM2 of Rrm4 might mediate the recognition of target mRNAs for transport, possibly via binding in the 3´ UTR."

We agree that it is important to investigate the binding of Rrm4 in the absence of Grp1. However, such differential iCLIP experiments including the bioinformatics analysis are challenging and beyond the scope of the current study.

2. How many UAUG motifs are there in Ustilago (in total and at the start codon) and how many of them are bound according to the iCLIP by Rrm4?

To address this question, we counted all UAUG motifs and overlapping Rrm4 binding sites within the expressed transcripts. In total, we find 14,748 UAUG motifs, of which 15.2% are bound by Rrm4. The binding frequency is similar for UAUG motifs at start codons, indicating that UAUG motifs are not preferencially bound in this context.

For more details, please see Major point 2 of Reviewer #1.

3. The authors write that "Notably, the Rrm4 binding sites with UAUG showed stronger relative binding than those lacking the motif (Fig. 5C), suggesting a tight interaction of Rrm4 with the UAUG-associated binding sites". If I understand it correctly, Figure 5C shows a higher number of "relative crosslinking events of Rrm4 binding sites". Although this indicates stronger binding, I'm not sure if this can be written explicitly.

We perfectly agree with the Reviewer that the number of crosslink events strongly depends on the abundance of the underlying transcript and thereby is not equivalent to binding strength. In order to overcome this obstacle and to estimate the binding strength of Rrm4, we had therefore normalised the crosslink events within each binding site to the background crosslink events in the surrounding sequence ('signal-over-background', SOB). This approach is based on the assumption that the unspecific binding of the RBP to the mRNA can serve as a proxy for transcript abundance. We previously applied this procedure to compare in vivo iCLIP measurements to in vitro binding assays, in which we could determine dissociation constants (Sutandy et al, Genome Res, 2018).

We apologise that this procedure had not been clear in the previous version of the manuscript. We carefully revised the respective sections in the main text and also give a more detailed explanation in Materials and methods. Moreover, we changed the y-axis label in Fig 5C to "Relative binding strength at Rrm4 binding sites (SOB)" to avoid confusion.

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4. I do not understand the connection between cell wall synthesis/integrity, hyphal growth and the mRNA binding/mRNP transport function of Grp1. Perhaps this can be explained in more detail.

At present, we cannot give a detailed mechanistic explanation for the function of Grp1. In general, it is well-studied that active cell wall synthesis at the growth pole is essential for the growth of fungal hyphae. This is achieved by active transport of macromolecules like lipids, proteins and mRNAs along the cytoskeleton. In *U. maydis*, we discovered that endosomal mRNA transport is needed for efficient unipolar hyphal growth.

Our new data clearly identify Grp1 as a novel component of these endosomal transport mRNPs (Fig 2). Nevertheless, as described for small glycine-rich proteins in other organisms, loss of Grp1 does not generally impair mRNP transport, but seems to impact selectively under stress conditions. Consistently, we do not observe reduction of hyphal growth under optimal conditions. However, we observe clear defects when treating the cells with the cell wall synthesis inhibitor Calcoflour White. In the absence of Grp1, the hyphae exhibit aberrant shapes indicative for defects in cell wall synthesis, suggesting a link of Grp1 to cell wall synthesis and hyphal growth.

Combining these results, we hypothesise that Grp1 may be important to regulate mRNA stability, translation or transport under stressed conditions. The phenotype in *grp1D* strains could be due to altered expression of the 1,051 Grp1-unique target mRNAs or the 2,114 shared target mRNAs. Since we know that Rrm4 influences hyphal growth and that Grp1 binds similar targets, we prefer the hypothesis that the deregulation of shared target transcripts is the cause for the altered hyphal growth. However, at present we do not have further evidence for this assumption. Therefore, we decided to discuss the potential function of Grp1 only briefly in the manuscript.

To clarify this and also in response to Comment 3 by Reviewer #1, we rephrased the section about the potential role of Grp1. The text now reads „We therefore propose that the potential RNA chaperone Grp1 most likely constitutes an accessory component of endosomal mRNPs. Its function could be particularly important under suboptimal conditions. Alternatively, Grp1 might regulate stability and/or translation of mRNAs encoding proteins involved in hyphal growth independent of endosomal mRNA transport."

2nd Editorial Decision 7 November 2018

Thank you for the submission of your revised manuscript to EMBO reports. Your manuscript was re-evaluated by referee 1 and 3 and we have now received their reports that are copied below.

As you will see, both referees are positive about the study and support publication in EMBO reports after some changes to text, figure legends and the statistical analysis.

From the editorial side, there are also a few things that we need before we can proceed with the official acceptance of your study.

REFEREE REPORTS
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Referee #1:

The manuscript has been improved as a result of the changes made in response to the reviewers' comments. Provided the following minor points are dealt with the manuscript should be published in EMBO Reports.

1. The normalizations that have been performed to produce a 'signal-over-background' value help alleviate my previous concerns about interpreting cross-linking frequency as a readout for binding strength. In general, is the cross-linking probability in CLIP experiments independent of RNA sequence, or do some nucleotides cross-link better than others? If the latter is true, it seems that the authors should tone down (or caveat) the claim that signal-over-background equates to binding strength.
2. Without supporting experimental evidence the section on perinuclear localization of transcripts that bind Grp1 but not Rrm4 should be toned down. This could be done, for example, with the

following small change "Although these mRNAs were expressed and bound by Grp1, they were most likely not transported by the Rrm4 machinery, which we presume would result in perinuclear localization".
3. Figure EV1. The legend or methods should explain what peptide count, total peptide score and best peptide score mean. Why are values for total peptide score and best peptide score different in cases when there is seemingly only one peptide found (the case for Grp1)? In the legend, panel C is incorrectly referred to as panel B. In the table in the figure, hypothetical is misspelled.
4. Multiple testing corrections should be applied for all statistical analyses that involve more than one group, not just for Figure 1C.
5. Line 244: "However, the frequency Rrm4 binding was not increased compared to UAUG motifs...". There is an 'of' missing after 'frequency'.

Referee #3:

The revisions have improved the clarity of the manuscript. I fully support publication in EMBO Reports.

2nd Revision - authors' response 9 November 2018

Referee #1:

The manuscript has been improved as a result of the changes made in response to the reviewers' comments. Provided the following minor points are dealt with the manuscript should be published in EMBO Reports.
1. The normalizations that have been performed to produce a 'signal-over-background' value help alleviate my previous concerns about interpreting cross-linking frequency as a readout for binding strength. In general, is the cross-linking probability in CLIP experiments independent of RNA sequence, or do some nucleotides cross-link better than others? If the latter is true, it seems that the authors should tone down (or caveat) the claim that signal-over-background equates to binding strength.
We agree with the Reviewer that the SOB procedure does not correct for UV crosslinking biases and others confounding effects, and should therefore only be used as a proxy for bulk comparisons between binding sites. We now mention this caveat in the main text and in the methods section.

The text now reads:

Main text:

„The background binding served as a proxy for the abundance of underlying transcript. Since the SOB procedure did not correct for UV crosslinking biases and similar confounding factors, comparisons between binding sites were only performed at a global scale. We observed that the Rrm4 binding sites with UAUG showed stronger relative binding than those lacking the motif (Fig 5C), suggesting a tight interaction of Rrm4 with the UAUG-associated binding sites.“

Methods section:
„We note that while this procedure alleviates the impact of transcript-level differences, it does not correct for UV crosslinking bias and similar confounding factors, and should therefore only be used as a proxy for bulk comparisons of binding sites.“

2. Without supporting experimental evidence the section on perinuclear localization of transcripts that bind Grp1 but not Rrm4 should be toned down. This could be done, for example, with the following small change "Although these mRNAs were expressed and bound by Grp1, they were most likely not transported by the Rrm4 machinery, which we presume would result in perinuclear localization".
We changed the text accordingly.

3. Figure EV1. The legend or methods should explain what peptide count, total peptide score and best peptide score mean.
Why are values for total peptide score and best peptide score different in cases when there is seemingly only one peptide found (the case for Grp1)? In the legend, panel C is incorrectly referred to as panel B. In the table in the figure, hypothetical is misspelled.
We include the information on the peptide scores in the figure legend and in Materials and methods. In the figure legend the text now reads: “Peptide count: number of identified peptides corresponding

to predicted protein; total peptide score: sum of all peptide scores corresponding to predicted protein, excluding the scores of duplicate matches; best peptide score: best score from all identified peptides corresponding to predicted protein. Note, that the difference between total peptide score and best peptide score is a correction of the software depending on how many possible predicted candidates match to the identified peptide mass.“

We correct the citation of panel C and the word hypothetical.

4. Multiple testing corrections should be applied for all statistical analyses that involve more than one group, not just for Figure 1C.
We agree that multiple testing corrections are important for statistical analyses involving more than one group. However, the statistical evaluationin the remaining figures is less complex than in Figure 1C. We compare, for example, wild type with grp1Δmutant in Figure 1D or unstressed versus stressed hyphae in Figure 1F. Therefore, we believe that the statistical tests applied aresufficient.

5. Line 244: "However, the frequency Rrm4 binding was not increased compared to UAUG motifs...". There is an 'of' missing after 'frequency'.
We corrected this.

Referee #3:

The revisions have improved the clarity of the manuscript. I fully support publication in EMBO Reports.
We are grateful to both Reviewers for their support.

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Corresponding Author Name: Michael Feldbrügge & Kathi Zarnack
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2018-46588-T

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures
1. Data
The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

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- an explicit mention of the biological and chemical entity(ies) that are being measured.
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- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
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  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
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Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).
We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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B- Statistics and general methods

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Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	To ensure appropiate sample size we analysed more than 100 endosomes or hyphae in independent experiments. The exact sample sizes are given in the figure legends and the software is given in Materials and methods.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Key results from microscopic images were quantified and checked by at least one other co-worker.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Quantification of microscopical images are displayed as ratiometric data. For sample-to-sample comparison of the means, Student´s t-tests were used (paired or unpaired according to data structure; as specified in the figure legends).
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Application of the Student's t-test is based on the assumption that the data follows a normal distribution. This assumption is fulfilled in the respective analyses (e.g. Figure 1C, 2B etc) as mean measurements of independent biological replicates are compared). For confirmation, the distribution was further assessed using the standard software Prism 5 (Graphpad) using the recommended D'Agostino-Pearson normality test.
Is there an estimate of variation within each group of data?	Standard deviations or standard errors of the mean (as specified in the figure legend) are indicated where applicable.
Is the variance similar between the groups that are being statistically compared?	The variance is expected to be comparable between the tested conditions.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Chromateck antibody GFP-Trap®_MA was used for pull down experiments. Anti-GFP primary antibodies were used from Sigma.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

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D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

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15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	All data has been deposited in GEO. The accession codes are given in the Data Availability section as follows: "The iCLIP and RNASeq dataset are available from GEO under the accession numbers GSE109557 and GSE109560, respectively. The associated SuperSeries is GSE109561."
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	All iCLIP and RNA-seq data has been deposited in GEO (see above).
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No