EMBO reports

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Genome-wide screen identifies a novel p97/CDC-48-dependent pathway regulating ER stress-induced gene transcription

Esther Marza, Said Taouji, Kim Barroso, Anne-Aurelie Raymond, Leo Guignard, Marc Bonneu, Nestor Pallares, Jean-William Dupuy, Martin E. Fernandez-Zapico, Jean Rosenbaum, Francesca Palladino, Denis Dupuy and Eric Chevet

Corresponding author: Eric Chevet, Inserm

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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.)

Editor: Barbara Pauly

1st Editorial Decision 02 July 2014

Thank you very much for the submission of your research manuscript to our editorial office and for your patience while we were waiting to hear back from the referees. We have now received the full set of reviews on your manuscript.

As the detailed reports are pasted below I will only repeat the main points here. You will see that all reviewers appreciate the interest of your findings and are, in principle, supportive of publication of your study in our journal. However, they are also in agreement that several aspects of your data need to be improved before the paper can be published. For example, all reviewers feel the need to strengthen data on RUVB-2/Reptin degradation by p97 and they suggest possible ways of achieving this. Referee 2 also feels that the data on the RUVB-2/Reptin-mediated repression of endogenous target genes under basal and stress conditions would need to be strengthened and s/he point out that several controls that are currently missing. With regard to the mechanistic insights into how RUVB-2/Reptin counteracts the induction of the UPR response (referee 2), we would not insist on their addition. However, we encourage you to examine whether Reptin enhances degradation of pXBP1(S), as suggested by reviewer 3. It would also be interesting to test whether RUVB-2/Reptin

interacts with p97 and to determine its intracellular localization. The third concern of reviewer 3 also needs to be addressed.

Overall, and given the reviewers' constructive comments, I would like to give you the opportunity to revise your manuscript, with the understanding that the main concerns of the reviewers should be addressed. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

As a minor point, I noticed that on page 9 it says 'The inability of H2O2 to induce YME1L degradation could be attributed to lower levels of eIF2a phosphorylation ....'. Should this not read '...Tim17A degradation...'?

I look forward to seeing a revised form of your manuscript when it is ready. Should you in the meantime have any questions, please do not hesitate to contact me.

REFEREE REPORTS:

Referee #1:

This manuscript by Marza and colleagues provides evidence for a novel relationship between a key regulator of endoplasmic reticulum(ER)-associated degradation (ERAD) and gene transcription in the cellular response to stress in the ER. This response, called the unfolded protein response (UPR), helps cells adjust to unfavorable conditions within the ER by regulating protein synthesis and by increasing expression of genes that promote protein folding as well as genes that promote degradation of misfolded ER proteins (ERAD). Specifically, these investigators used a genetic screen in C. elegans to identify RUVB-2, an AAA ATPase, as a repressor of a subset of UPR genes. The work further links p97/CDC-48, an AAA ATPase that regulates ERAD, to the downregulation of RUVB-2, providing mechanistic insight into previous data implicating p97/CDC-48 to UPR gene expression. Additional data indicate that this relationship also exists in the mammalian UPR as Reptin, the human homolog of RUVB-2, is downregulated during ER stress by a process involving p97. Overall, the work is well-performed and the data support a straightforward story about an interesting regulatory relationship within the UPR involving an ERAD component (p97/CDC-48), RUVB-2/Reptin and induction of certain UPR target genes.

Questions and Comments
1. The authors propose that during ER stress p97 facilitates degradation of RUVB-2/Reptin. Based on the role of p97 in ERAD, this is a reasonable idea. However, while the data presented demonstrate that Reptin decreases during tunicamycin-induced ER stress (Fig. 4D, E), the experiments do not address whether this is due to degradation, reduced synthesis or a combination of these events. In fact, the p97 inhibitor (DBeQ) only partially inhibited the loss of Reptin in tunicamycin-treated cells (Fig. 4E). What is the half-life of Reptin? And, does its half-life shorten during ER stress, as the proposed model would predict? Does Reptin synthesis decrease during ER stress, as would be expected due to translational repression by the PERK pathway in the UPR? The answers to these questions would strengthen the story.

2. The manuscript would benefit from a brief discussion of the Reptin protein (sub-cellular location, structure, functions) and how it might influence UPR gene transcription. Inclusion of such information would broaden the impact of the paper. Specifically, given the interesting potential roles of Reptin as a chaperone or organizer of protein complexes, it seems appropriate in this paper to briefly discuss how such activities might affect UPR signaling mechanisms and/or transcriptional activators.

Referee #2:

Summary
The current manuscript investigates why worms lacking one of the two AAA+ ATPase paralogs fail

to activate the UPRER transcriptional program. The authors used cdc48.2(-/-) worms expressing GFP under the control of ckb-2 promoter to perform a suppressor RNAi screen that eventually led to the identification of ruvb-2 gene. Quantitative proteomics further confirmed that indeed RUVB-2 protein accumulates in cdc48.2(-/-) worms. The authors conclude that "RUVB-2 is a regulator of the ER stress response by repressing the transcription of select UPRER genes in non-stressed conditions and is degraded in a CDC-48-dependent manner in response to ER stress.

CRITIQUE

Overall: The authors in this paper discovered a protein called Reptin to attenuate UPR induction in worms lacking the cdc48 ATPase. The major issue is that they do not provide any data showing how this occurs. Without those experiments, it is pretty much an RNAi screening paper. I feel if the authors add more data to shed light on the mechanism.

1. That RUVB-2 knockdown leads to restoration of the UPRER transcriptional program upon ER stress induction in cdc48 null worms is based entirely on the use of ckb-2::gfp expression. The authors should demonstrate this using endogenous genes. On page 6, the authors say that 'ruvb-2 silencing restored the transcriptional activation of homeostasis regulators CKB-2, F22E6.6, Y719AL.17...'. I have been unable to locate these genes in wormbase. What do they encode? Figure 3D shows that the expression of these genes is restored upon knockdown of ruvb-2. The appropriate control for this experiment is lacking; expression of these genes upon ruvb-2 knockdown in wild type worms should be shown.

2. It appears that knocking down the expression of Reptin in Huh-7 cells results in activation of ER stress, as measured by ERSE::tomato reporter. The authors should discuss this observation.

3. The data shown in figure 4C should be accompanied with data from samples under ER stress to clearly demonstrate the lack of modulation of gene expression upon overexpression of Reptin. Also, a representative blot showing overexpression of Reptin compared to wild type cells should be included.

4. In figure 4D, the authors should probe for UPR markers to clearly demonstrate the upregulation of known proteins such as BiP, GRP94, ERP57 or Chop upon treatment with tunicamycin.

5. The stabilization of Reptin upon treatment with p97 inhibitor DBeQ appears to be minimal. The authors should discuss how a small change in the level of Reptin could bring about significant changes in the transcriptional program upon ER stress.

6. Is Reptin a secretory protein? Where is it localized?

7. What is the connection between Reptin and p97? Do they interact with each other?

8. What is the exact mechanism by which Reptin represses gene expression?

9. What is the half-life of Reptin? Does it get turned over quickly? Pulse chase experiments should be performed in the presence or absence of DBeQ to measure Reptin half-life.

Referee #3:

The endoplasmic reticulum (ER) is a organelle where membrane and secretory proteins are synthesized and folded with the assistance of ER chaperones. Misfolded proteins are extracted by the AAA-ATPase p97/CDC48 from the ER to the cytosol, and degraded by the proteasome, which is called the ER-associated degradation (ERAD). When the synthesis of secretory or membrane proteins is increased and overwhelms the capacity of ER chaperones, most of secretory proteins cannot be folded and accumulated in the ER, which is a dangerous situation since it causes apoptosis, and called ER stress. To cope with ER stress, eukaryotic cells developed a cytoprotective mechanism, the ER stress response (also called the unfolded protein response (UPR) in the ER). The

mammalian ER stress response consists of three response pathways, that is, the ATF6, IRE1 and PERK pathways. The ATF6 pathway regulates expression of ER chaperones with a sensor pATF6(P), a transcription factor pATF6(N) and an enhancer element ERSE. The IRE1 pathway controls expression of ER components with a sensor IRE1, a transcription factor pXBP1(S) and an enhancer UPRE. The PERK pathway transiently attenuates translation to prevent further accumulation of unfolded proteins in the ER.

In C. elegans, the IRE1 and PERK pathways are conserved, whether it does not have the ATF6 pathway. Interestingly, it had been reported that transcriptional activation of XBP1 target genes was attenuated in a cdc48 mutant, suggesting that CDC48 is involved in transcriptional activation upon ER stress.

In this manuscript, the authors tried to identify a novel regulator involved in CDC48-mediated transcriptional regulation through the IRE1 pathway, using C. elegans. They performed a genome-wide screen and isolated RUVB-2, another AAA-ATPase, which restored transcriptional induction in a cdc48 mutant. The effect of RUVB-2 was dependent on IRE1 and XBP1 in C. elegans, suggesting that RUVB-2 is a negative regulator of the CDC48-mediated transcriptional induction of UPR-target genes. The authors also analyzed reptin, a mammalian homolog of RUVB-2, and found that function of reptin is conserved in mammalian cells. Interestingly, they showed that expression of reptin was decreased upon ER stress, and an inhibitor of CDC48 considerably attenuated decrease of reptin expression upon ER stress. From these observations, the authors proposed the following working hypothesis: RUVB-2 represses UPR gene transcription under basal conditions. Upon ER stress, RUVB-2 is degraded through a p97-dependent mechanism, thereby allowing ER stress specific transcription factors such as XBP-1 to activate the transcription of UPR genes.

Since the data that the authors presented in the manuscript are all clear, and their concept is novel and important, the reviewer thinks that the manuscript would become suitable for publication in the journal of EMBO Report with revisions described below.

<Major critiques>
(1) The authors concluded that reptin is degraded by CDC48 upon ER stress, but they merely showed that accumulation of reptin protein was decreased upon tunicamycin treatment. The authors should perform pulse-chase or cycloheximide chase experiments in cdc48-KD cells to show increased degradation of reptin upon ER stress in CDC48-dependent fashion.

(2) The authors described that an AAA-ATPase reptin negatively regulates transcriptional induction of UPR-related genes, but the molecular mechanism is unclear. They should examine whether reptin enhances degradation of pXBP1(S) by cycloheximide chase experiments. It would be possible to screen targets of reptin by 2D-gel electrophoresis of cellular proteins.

(3) The authors examined effect of reptin on ERSE-mediated transcription in Figure 4. However, ERSE-mediated transcriptional induction is regulated by the ATF6 pathway, not by the IRE1-XBP1 pathway. Thus, the authors should use UPRE-tomato instead of ERSE-tomato. In addition, they examined target genes of the ATF6 pathway including BiP, GRP94 and ORP150, which seems inappropriate. Is it possible that reptin degrades pATF6(N) as well as pXBP1(S)?

<Minor critiques>
(1) Figure 4A and 4D: the authors should evaluate effect of CDC48-KD.

(2) The authors used only tunicamycin to induce ER stress. They should use another ER stress inducer, such as thapsigargin or DTT.

<Typographical errors>
(1) Page 4, line 8: "Fig. 1C-I" should be "Fig. 1C".

(2) Page 6, line 3: "(Fig. 3D)" may be missed.

Point-by-point response to the reviewers' comments

Editorial comments

1) For example, all reviewers feel the need to strengthen data on RUVB-2/Reptin degradation by p97 and they suggest possible ways of achieving this.

We now show a new set of data, presented in the revised Figure 4, that demonstrate that p97 binds to Reptin. We have also demonstrated that the ubiquitin modified form of Reptin is enriched in p97 immunoprecipitates (Fig 4D, and E). This indicates that Reptin might be an ubiquitin dependent substrate of p97 as previously shown for many p97 clients (Meyer H, Weihl CC. J Cell Sci. 2014 Sep 15;127(Pt 18):3877-83). In support of these findings, we also observe that pharmacological inhibition of p97 leads to the stabilization of Reptin. Together, these results led us to propose a model in which ubiquitin dependent p97-Reptin association might control Reptin degradation.

2) Referee 2 also feels that the data on the RUVB-2/Reptin-mediated repression of endogenous target genes under basal and stress conditions would need to be strengthened and s/he point out that several controls that are currently missing.

We agree with the reviewer that the heatmap representation of our expression data was sub-optimal. In the revised version of our manuscript we now present the data as bargraph for 4 endogenous UPR responsive genes. These data show that BiP, CHOP and EDEM1 expression is in part under the control of Reptin degradation. Interestingly, the expression of ORP150, which is a genuine target of ATF6 did not seem affected by the silencing of Reptin, thus suggesting that the ATF6 pathway would be independent of Reptin. Moreover we have now shown a gene expression analysis comparing the expression of UPRER target genes in cells expressing FLAG-tagged Reptin and control vector transfected cells under basal conditions and tunicamycin treatment (new Fig S5). These results show that the expression of Reptin is sufficient to decrease the basal expression of UPR^{ER} genes but under stress the effect observed is much less significant (Fig S5B, C). This might be due to the fact that FLAG-tagged reptin expression impacts on endogenous reptin as previously reported (Rousseau et al., Hepatology 2007) and seen in Fig S5A, thereby leading to a global expression (FLAG reptin + endogenous reptin) not that much higher that in untransfected cells.

3) With regard to the mechanistic insights into how RUVB-2/Reptin counteracts the induction of the UPR response (referee 2), we would not insist on their addition. However, we encourage you to examine whether Reptin enhances degradation of pXBP1(S), as suggested by reviewer 3.

As suggested we tested whether Reptin interacted with XBP1s protein (revised Fig 4H) and found that there is no interaction between these two proteins. In contrast we show in the revised Figure 4F that Reptin silencing enhances the splicing of XBP1 mRNA under basal and tunicamycin-induced stress. Moreover these data were confirmed using DBeQ which led to the stabilization of Reptin and therefore reduced XBP1 mRNA splicing (Fig. 4G). Altogether, these results suggest that Reptin act as a repressor of XBP1 mRNA splicing, however the precise underlying mechanisms remain to be defined.

4) It would also be interesting to test whether RUVB-2/Reptin interacts with p97 and to determine its intracellular localization. The third concern of reviewer 3 also needs to be addressed.

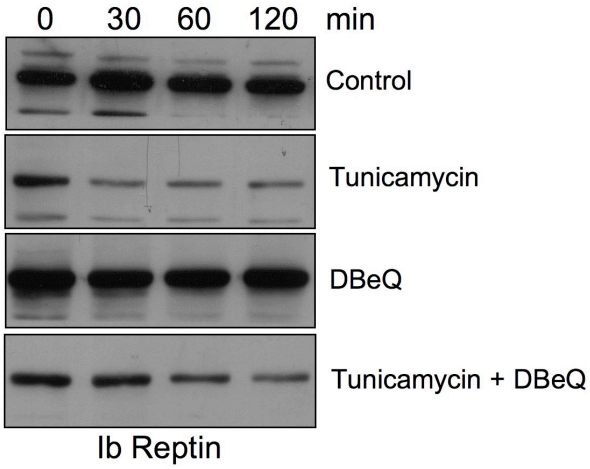
We completely agree and thank you for this remark. We have investigated the presence of p97 and Reptin in a complex using immunoprecipitation. These new data are now presented in Fig 4C and D showing that p97 and Reptin interact and that the interaction is modulated by tunicamycin-induced ER stress and the inhibition of p97. In addition, we show that in the p97 immunoprecipitate, a modified form of Reptin, identified probably as a ubiquitylated form of the protein, was enriched (Fig 4D, E). Together these interaction experiments led us to demonstrate that Reptin and p97 are in a complex and that also this interaction might be dependent on Reptin ubiquitination.

Response to the reviewers' critiques

1. The authors propose that during ER stress p97 facilitates degradation of RUVB-2/Reptin. Based on the role of p97 in ERAD, this is a reasonable idea. However, while the data presented demonstrate that Reptin decreases during tunicamycin-induced ER stress (Fig. 4D, E), the experiments do not address whether this is due to degradation, reduced synthesis or a combination of these events. In fact, the p97 inhibitor (DBeQ) only partially inhibited the loss of Reptin in tunicamycin-treated cells (Fig. 4E). What is the half-life of Reptin? And, does its half-life shorten during ER stress, as the proposed model would predict?

To address this point we performed cycloheximide (CHX) pulse-chase experiments (8h cycloheximide treatment followed by a 30, 60, 120 min treatment with tunicamycin, DBeQ or the combination of both). The amounts of Reptin were analyzed by immunoblot and we then calculated an estimated half-life for this protein in our experimental conditions as indicated in the Figure below.

A



B

	Reptin half-life (h)	SD
Control	9.7	±1.8
DBeQ	11.3	±0.4
Tun	2.5	±0.2
DBeQ + Tun	10.1	±2.2

Figure 1 : Determination of Reptin's half-life under ER stress - A- western blots ; B- calculated half-lifes based on western blot experiments.

Although we observe an effect of the treatments to which the cells were exposed, that are in accordance with our model. These experiments are sub-optimal as a long-term CHX treatment needed to overcome Reptin's quite long half-life) exerts also some toxicity on the cells but we believe they address the reviewer's question and further evaluation of the modulation of Reptin's half life falls outside the scope of this publication. To avoid this side effect, we are currently performing 35S-methionine labelling pulse-chase experiments coupled to Reptin immunoprecipitations (as previously done for other proteins of interest in the laboratory (e.g. Cameron et al. 2009 ; Higa et al. 2011). These experiments should provide us with much more precise data on the modification of Reptin half-life upon ER stress.

Does Reptin synthesis decrease during ER stress, as would be expected due to translational repression by the PERK pathway in the UPR? The answers to these questions would strengthen the story.

To properly address this question we need to perform thorough 35S-methionine pulse-chase experiments that are currently ongoing but could not be carried out in the time frame allowed for revision of the manuscript. Moreover, to evaluate the impact of PERK activation we treated HuH7 cells with the PERK inhibitor GSK2606414 (Hetz et al. Nat Rev Drug Discov. 2013 Sep;12(9):703-19) and did not observe any rescue of Reptin expression although PERK phosphorylation was properly shut down. However again, these experiments should be repeated with 35S-methionine metabolic labelling of the cells.

2. The manuscript would benefit from a brief discussion of the Reptin protein (sub-cellular location, structure, functions) and how it might influence UPR gene transcription. Inclusion of such information would broaden the impact of the paper. Specifically, given the interesting potential roles of Reptin as a chaperone or organizer of protein complexes, it seems appropriate in this paper to briefly discuss how such activities might affect UPR signaling mechanisms and/or transcriptional activators.

Two sentences were added in the discussion section about the localization and function of Reptin. However due to limitations in the text length and due to the amount of material we added to the original version of the manuscript we were not able to develop further this concepts.

3. That RUVB-2 knockdown leads to restoration of the UPRER transcriptional program upon ER stress induction in cdc48 null worms is based entirely on the use of ckb-2::gfp expression. The authors should demonstrate this using endogenous genes. On page 6, the authors say that 'ruvb-2 silencing restored the transcriptional activation of homeostasis regulators CKB-2, F22E6.6, Y719AL.17...'. I have been unable to locate these genes in wormbase. What do they encode?

The expression of the endogenous genes mentioned below was measured in our work and in presented in Fig. 3D. There were two typos in the original version of the manuscript which were corrected in the revised version (F22E5.6 and Y71F9AL.17. We thank this reviewer for pointing them.
Ckb-2 description can be found on wormbase by following the link : http://www.wormbase.org/species/c_elegans/gene/WBGene00000512?from=http://www.wormbase.org/db/gene/gene?name=WBGene00000512#06cbe-9d81-3. Y71F9AL.17 also named copa-1 description can be found on wormbase at this address http://www.wormbase.org/species/c_elegans/gene/WBGene00022119?query=copa-1#01-9e376-3. F22E5.6 description can be found on wormbase by following the link:http://www.wormbase.org/species/c_elegans/gene/WBGene00017705?query=F22E5.6#01-9e376-3

4. Figure 3D shows that the expression of these genes is restored upon knockdown of ruvb-2. The appropriate control for this experiment is lacking; expression of these genes upon ruvb-2 knockdown in wild type worms should be shown.

This was done and has been added in Fig S4.

5. It appears that knocking down the expression of Reptin in Huh-7 cells results in activation of ER stress, as measured by ERSE::tomato reporter. The authors should discuss this observation.

This is now addressed and commented in point 13.

6. The data shown in figure 4C should be accompanied with data from samples under ER stress to clearly demonstrate the lack of modulation of gene expression upon overexpression of Reptin. Also, a representative blot showing overexpression of Reptin compared to wild type cells should be included.

This is now shown in Fig. S5.

7. In figure 4D, the authors should probe for UPR markers to clearly demonstrate the upregulation of known proteins such as BiP, GRP94, ERP57 or Chop upon treatment with tunicamycin.

To address this, we used RT-qPCR data from select genes whose expression has already been shown to be under the control of the IRE1/XBP1 axis (BiP, CHOP, EDEM1) or genes that are exclusively under the control of ATF6 such as ORP150. We find that the expression of BiP, CHOP and EDEM1 is in part dependent upon Reptin under stress whereas that of ORP150 is not, thus providing an additional evidence for the role of Reptin in xbp1-mediated gene expression.

8. The stabilization of Reptin upon treatment with p97 inhibitor DBeQ appears to be minimal. The authors should discuss how a small change in the level of Reptin could bring about significant changes in the transcriptional program upon ER stress.

A sentence was added on p7 of the manuscript following the observation that Reptin stabilization (through inhibition of p97) led to decreased splicing of XBP1 mRNA and thus significant effect on UPRER gene expression.

9. Is Reptin a secretory protein? Where is it localized?

Reptin is not a secretory protein and localizes in the cytoplasm and nucleus as previously reported (Kim et al., Mol Cell. 2013 Jan 10;49(1):172-85 ; Sigala et al., Exp Cell Res. 2005 Nov 1;310(2):357-69 ; Uniprot analysis - http://www.uniprot.org/uniprot/Q9Y230#section_comments)

10. What is the connection between Reptin and p97? Do they interact with each other?

These experiments were performed as suggested and are now shown in the revised Fig 4. See also response to Editorial comment 4.

11. What is the exact mechanism by which Reptin represses gene expression?

We found that Reptin silencing enhances the splicing of XBP1 mRNA under basal and tunicamycin-induced stress (Fig 4F). Moreover these data were confirmed by the use of DBeQ that lead to the stabilization of Reptin and therefore reduced XBP1 mRNA splicing (Fig. 4G). Altogether, these results suggest that Reptin might act as a repressor of XBP1 mRNA splicing, however the precise underlying mechanisms remain to be defined. This might represent a way for Reptin to control UPRER gene expression but we cannot exclude at the moment that other mechanisms, for instance involving chromatin remodelling might also be implicated.

12. What is the half-life of Reptin? Does it get turned over quickly? Pulse chase experiments should be performed in the presence or absence of DBeQ to measure Reptin half-life.

See response to comment 1.

13. The authors concluded that Reptin is degraded by CDC48 upon ER stress, but they merely showed that accumulation of Reptin protein was decreased upon tunicamycin treatment. The authors should perform pulse-chase or cycloheximide chase experiments in

cdc48-KD cells to show increased degradation of Reptin upon ER stress in CDC48-dependent fashion.

See response to comment 1.

14. The authors described that an AAA-ATPase Reptin negatively regulates transcriptional induction of UPR-related genes, but the molecular mechanism is unclear. They should examine whether Reptin enhances degradation of pXBP1(S) by cycloheximide chase experiments. It would be possible to screen targets of Reptin by 2D-gel electrophoresis of cellular proteins.

Following the reviewer's suggestion, we initially tested whether reptin interacted with XBP1s protein (revised Fig 4H) and found that there is no interaction between these two proteins. In contrast we show in the revised Figure 4F that reptin silencing enhances the splicing of XBP1 in both, basal conditions and tunicamycin-induced stress. These data were confirmed using the CDC48 pharmacological inhbitor DBeQ. The data included in Figure 4G show the stabilization of reptin and therefore reduced XBP1 mRNA splicing in DBeQ-treated condition. Altogether, these results suggest that Reptin acts as a repressor of XBP1 mRNA splicing, however the precise underlying mechanisms remain to be defined.

15. The authors examined effect of Reptin on ERSE-mediated transcription in Figure 4. However, ERSE-mediated transcriptional induction is regulated by the ATF6 pathway, not by the IRE1-XBP1 pathway. Thus, the authors should use UPRE-tomato instead of ERSE-tomato. In addition, they examined target genes of the ATF6 pathway including BiP, GRP94 and ORP150, which seems inappropriate. Is it possible that Reptin degrades pATF6(N) as well as pXBP1(S)?

To address this question, we used data from select genes whose expression has already been shown to be under the control of the IRE1/XBP1 axis, exclusively under the control of ATF6 (e.g. ORP150) or not (BiP, CHOP, EDEM1). We find that the expression of BiP, CHOP and EDEM1 is in part dependent upon reptin under stress whereas that of ORP150 is not, thus providing an additional evidence for the role of reptin in xbp1-mediated gene expression. Furthermore, it was demonstrated that in C. elegans, the IRE1/XBP1 axis is the major UPR signaling arm (Shen et al. PLoSGenetics, 2005), thus supporting our model.

16. Figure 4A and 4D: the authors should evaluate effect of CDC48-KD.

Unfortunately these experiments could not be performed in the imparted time for revision due to delays in obtaining radiolabelled ^{35}S-Methionine and methionine-cystein free culture media. Nonetheless, we believe that data presented in Fig S6 obtained using DBeQ support the role CDC48 in the regulation Reptin.

17. The authors used only tunicamycin to induce ER stress. They should use another ER stress inducer, such as thapsigargin or DTT.

Some of our experiments were also performed with thapsigargin and similar results for instance in UPR^{ER} gene expression in mammalian cells silenced for Reptin (Figure 2). These data were not generated for all experiments performed in the manuscript and therefore we believe that it might just complexify the message of the paper at this stage.

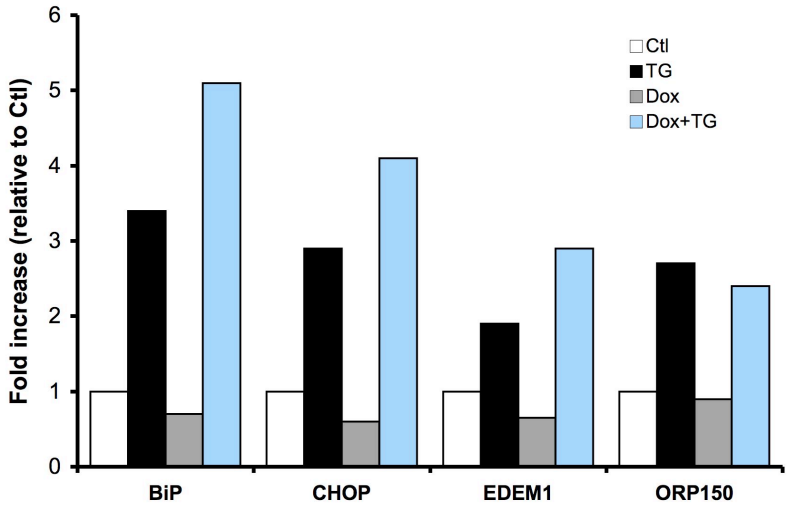


Figure 2: RUVB-2 is a transcriptional repressor inactivated by CDC-48 upon thapsigargin induced ER stress - RT-qPCR analysis of four ER homeostasis control genes under basal conditions or upon thapsigargin treatment (0.5 nM, 16 hours) in HuH7 cells subjected or not to doxycycline-induced Reptin silencing.

<Typographical errors>
(1) Page 4, line 8: "Fig. 1C-I" should be "Fig. 1C".

This was corrected in the revised version

(2) Page 6, line 3: "(Fig. 3D)" may be missed

This was corrected in the revised version

2nd Editorial Decision 06 November 2014

Thank you very much for submitting your revised manuscript to EMBO reports. I would like to apologize for the unusual delay in getting back to you with a decision, but we have just now received the enclosed reports on it.

While referee 1 is satisfied with the way in which you have addressed his/her concerns, both referees 2 and 3 still raise some issues that relate to concerns that were already brought up in the first round of review.

Specifically, referee 3 is not convinced that the current data set is strong enough to support the idea that Reptin specifically affects the XBP1-dependent arm of the UPR in mammalian cells, as s/he states that BiP and Chop are mainly regulated by ATF6 and PERK, respectively, and not by XBP1 in mammals. This reviewer therefore feels that it should be investigated whether ATF6 cleavage and/or PERK phosphorylation are affected by Reptin. I discussed this issue also with referee 2 and he essentially agrees with reviewer 3 on this point. Therefore, I would kindly ask you to follow this reviewer's suggestions on how to strengthen the data so that you can conclude that both in worms an humans, RUVB-2/Reptin specifically blocks the Xbp1 arm of the UPR.

In addition, reviewer 2 also feels that pulse-chase experiments instead of CHX assays should be

used to determine the half-life of Reptin and since you indicated that this experiment is already underway in your lab, I would suggest you include it when submitting the final version of your study.

I thank you in advance for your cooperation and apologize again for the delay in getting back to you on your manuscript.

REFEREE REPORTS:

Referee #1:

In this revised manuscript, Marza and colleagues provide stronger evidence for a relationship between a component of the ERAD system and ER stress-induced gene expression. Using a genetic screen in C. elegans, the investigators identified RUVB-2, an AAA ATPase, as a repressor of certain ER stress-inducible genes (genes regulated by the unfolded protein response). They then linked p97/CDC-48, an AAA ATPase that regulates ERAD, to the downregulation of RUVB-2, and these data provide new insight into previous data implicating p97/CDC-48 in UPR gene expression. This relationship also exists in the mammalian UPR as Reptin, the human homolog of RUVB-2, is downregulated during ER stress by a process involving p97. Importantly, in this revised manuscript, the authors have included additional data indicating an interaction between p97 and RUVB-2/Reptin and supporting a model in which p97 promotes degradation of RUVB-2/Reptin (revised Fig. 4). Interestingly, the data are consistent with RUVB-2/Reptin being a negative regulator of UPR-mediated Xbp1 mRNA splicing. A reasonable model is that during ER stress p97 facilitates degradation of RUVB-2/Reptin so that UPR-mediated Xbp1 mRNA splicing can maximally increase. This would then contribute to maximal synthesis of the XBP1s transcription factor, a major regulator of UPR gene expression. This model remains to be further explored, and the mechanism by which RUVB-2/Reptin affects Xbp1 mRNA splicing remains to be determined. Overall, the revised manuscript provides a stronger story about an interesting regulatory connection between an ERAD component (p97/CDC-48), RUVB-2/Reptin and expression of certain UPR target genes.

Referee #2:

The comments for the original submission were very similar from all of the reviewers. The authors have addressed all of them in their resubmission; two questions were not addressed satisfactorily.

One comment was to determine the half life of Reptin by pulse labeling method. They have used CHX chase assays instead. The authors state they are performing metabolic pulse-chase experiments which should be required to support their conclusion.

Other question was how Reptin represses gene expression? The authors suggest that it might prevent Xbp1 splicing but do not further investigate the precise mechanism. Since this is a short paper in EMBO Reports, the authors think those experiments are beyond the scope of this paper. I think the Reptin story is of interest but in the current shape, the manuscript is quite descriptive.

Referee #3:

*** Reviewer's previous comment ***

15. The authors examined effect of Reptin on ERSE-mediated transcription in Figure 4. However, ERSE-mediated transcriptional induction is regulated by the ATF6 pathway, not by the IRE1-XBP1 pathway. Thus, the authors should use UPRE-tomato instead of ERSE-tomato. In addition, they examined target genes of the ATF6 pathway including BiP, GRP94 and ORP150, which seems inappropriate. Is it possible that Reptin degrades pATF6(N) as well as pXBP1(S)?

*** Authors' response ***

To address this question, we used data from select genes whose expression has already been shown to be under the control of the IRE1/XBP1 axis, exclusively under the control of ATF6 (e.g. ORP150) or not (BiP, CHOP, EDEM1). We find that the expression of BiP, CHOP and EDEM1 is in part dependent upon reptin under stress whereas that of ORP150 is not, thus providing an additional evidence for the role of reptin in xbp1-mediated gene expression. Furthermore, it was demonstrated that in C. elegans, the IRE1/XBP1 axis is the major UPR signaling arm (Shen et al. PLoSGenetics, 2005), thus supporting our model.

*** Reviewer's new comment ***

The reviewer slightly disappointed that the authors did not realize the importance of my suggestion that they should clearly demonstrate that the effect of reptin knock down is specific to the IRE1-XBP1 pathway. It is possible that reptin is essential to degradation of unfolded proteins accumulated in the endoplasmic reticulum (ER) by the mechanism of the ER-associated degradation (ERAD) as well as p97/cdc48. Knock down of reptin would result in accumulation of unfolded proteins in the ER, that is, ER stress. It is quite natural that ER stress evoked by reptin knock down activates all branches of the mammalian ER stress response pathways (the ATF6, IRE1 and PERK pathways), including splicing of XBP1(U) mRNA. In that case, the findings of the authors described in the manuscript would indicate that they merely identified another protease of which function is similar to p97/cdc48, and they should retract the conclusion that "In the present work, we have uncovered a novel regulatory mechanism of UPRER genes expression in response to ER stress conserved throughout metazoan evolution involving two AAA+ ATPases, RUVB-2 (or Reptin) and CDC-48 (or p97)."

Since transcriptional induction of BiP and CHOP is mainly regulated by the ATF6 and PERK pathways respectively, these markers are inappropriate to demonstrate that the effect of reptin knock down is specific to the IRE1-XBP1 pathway. Thus, the Reviewer thinks that the authors should examine whether the ATF6 and PERK pathways are not affected by reptin knock down by examining cleavage of pATF6(P) and phosphorylation of PERK and eIF2 alpha. Otherwise, the conclusion of the manuscript should be changed. Without this revision, the manuscript would not be appropriate for the journal of EMBO report.

The data of ERSE-tomato in Figure 3E seems very strange for UPR people, because it indicates that the ATF6 pathway is activated by knock down of reptin, suggesting the general activation of the mammalian stress and indirect activation of XBP1 splicing, which contradicts to the authors' conclusion. This data should be replaced by the data of UPRE-tomato or deleted.

2nd Revision - authors' response 08 December 2014

Point-by-point response to the reviewers' comments

Referee #1:

We thank Referee#1 for his/her constructive comments. No additional experiments were requested by this reviewer.

Referee #2:

Comment #1: Determine the half life of Reptin by pulse labeling method. They have used CHX chase assays instead. The authors state they are performing metabolic pulse-chase experiments which should be required to support their conclusion.

We have now added a supplementary table (Table S6) summarizing the reptin's half-life studies using both a cycloheximide-based method and 35S-methionine pulse-chase. In both methods, DBeQ stabilizes the protein whereas Tunicamycin-induced ER stress prompts its degradation.

Comment #2: Other question was how Reptin represses gene expression? The authors suggest that it might prevent Xbp1 splicing but do not further investigate the precise mechanism. Since this is a short paper in EMBO Reports, the authors think those experiments are beyond the scope of this paper. I think the Reptin story is of interest but in the current shape, the manuscript is quite descriptive.

We respectfully disagree with this reviewer regarding the descriptive nature of our work. Here, we have established a previously unreported molecular link between p97 and UPR signaling. This mechanism involved p97-dependent degradation of reptin, and we provide molecular evidences on how this occurs. Often studies of this nature, like ours, raise questions regarding novel molecular events regulating the newly identified pathway. We strongly feel that these questions are beyond the scope of this manuscript and we really hope the reviewer understands our position on this matter.

Referee #3:

Comment #1: The effect of reptin knock down is specific to the IRE1-XBP1 pathway should be demonstrated. It is possible that reptin is essential to degradation of unfolded proteins accumulated in the endoplasmic reticulum (ER) by the mechanism of the ER-associated degradation (ERAD) as well as p97/cdc48. Knock down of reptin would result in accumulation of unfolded proteins in the ER, that is, ER stress. It is quite natural that ER stress evoked by reptin knock down activates all branches of the mammalian ER stress response pathways (the ATF6, IRE1 and PERK pathways), including splicing of XBP1(U) mRNA. In that case, the findings of the authors described in the manuscript would indicate that they merely identified another protease of which function is similar to p97/cdc48, and they should retract the conclusion that "In the present work, we have uncovered a novel regulatory mechanism of UPRER genes expression in response to ER stress conserved throughout metazoan evolution involving two AAA+ ATPases, RUVB-2 (or Reptin) and CDC-48 (or p97)."

We agree with this reviewer, this should have been addressed in the previous round. We have now tested whether the three arms of the UPR changed upon Reptin knockdown, we investigated the activation of ATF6 (as measured by its cleavage) and phosphorylation of eIF2alpha in response to tunicamycin treatment in the presence/abscence of reptin silencing. Our results indicate that beyond the activation of xbp1s observed under reptin silencing conditions, we also observe the activation of ATF6 but not of the PERK pathway (as assessed by the phosphorylation of eIF2alpha) (Figure 5 B,C). We have now revised our conclusion to describe that both IRE1 and ATF6 arms of the UPR appear to be affected, and that we reptin could act as a repressor of these arms without affecting the PERK arm.

To further evaluate the levels of protein misfolding in the ER upon reptin silencing, we immunoblotted alpha1 antitrypsin (AAT) and ApoB100, two major secretory proteins in HuH7 cells, in the supernatant and pellet of cells transfected with siReptin or control siRNA. No difference between the two conditions was observed (data not shown). We acknowledge that this immunoblot-based method for detecting protein misfolding in the ER is not very sensitive, but if protein misfolding was induced after reptin silencing, this event does not lead to a stress intense enough to cause a significant induction of UPR signals and therefore cannot explain the selective effects on xbp1s and atf6 activation.

Comment #2: Since transcriptional induction of BiP and CHOP is mainly regulated by the ATF6 and PERK pathways respectively, these markers are inappropriate to demonstrate that the effect of reptin knock down is specific to the IRE1-XBP1 pathway. The authors should examine whether the ATF6 and PERK pathways are not affected by reptin knock down by examining cleavage of pATF6(P) and phosphorylation of PERK and eIF2 alpha. Otherwise, the conclusion of the manuscript should be changed. Without this revision, the

manuscript would not be appropriate for the journal of EMBO report.

Following the reviewers suggestion, we have evaluated the activation of the three arms of the UPR in mammalian cells upon reptin silencing. This is now presented in the new Figure 5. We found that although eIF2alpha phosphorylation remains unaffected upon reptin silencing, the cleavage of ATF6 is increased as if reptin acted as a repressor of this pathway as well as for the XBP1 pathway. As Reptin has been shown to stabilize/chaperone proteins from the PIKK family we could therefore postulate a role for this family of proteins or a similar function of reptin on the UPR sensors as for the PIKKs in the phenomenon observed in our study.

Comment #3: The data of ERSE-tomato in Figure 3E seems very strange for UPR people, because it indicates that the ATF6 pathway is activated by knock down of reptin, suggesting the general activation of the mammalian stress and indirect activation of XBP1 splicing, which contradicts to the authors' conclusion. This data should be replaced by the data of UPRE-tomato or deleted.

Considering our new data showing an activation of ATF6 under reptin silencing conditions (Figure 5D), we believe that the experiments with the ERSE-tomato reporter should be left in the revised manuscript as they are supportive of the new data included in the manuscript.

3rd Editorial Decision 18 December 2014

Thank you for your patience while we have reviewed your revised manuscript. As you will see from the reports below, the referee that was asked to assess the revised version now recommends publication in EMBO reports. I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor issues/corrections have been addressed, as follows.

1. By going through the manuscript one more time myself, I noticed that there was a strange-looking band in Fig 4E (basically, a band runs over two different lanes, lanes T and D. Could you please clarify what this is and ideally provide a new image for this figure?

2. Please send us the following:
* a short, two-sentence summary of the manuscript
* two to three bullet points highlighting the key findings of your study
* a schematic figure (in jpeg or tiff format with the exact width of 550 pixels and a height of about 400 pixels) that can be used as part of a visual synopsis on our website

You will then receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports.

REFEREE REPORT:

Referee #3:

The manuscript has been extensively improved, and Reviewer thinks that it is now suitable for publication in the journal of EMBO Report.

3rd Revision - authors' response 22 December 2014

On behalf of all of the co-authors, I would like to sincerely thank you for the careful handling of our manuscript and the opportunity to submit a revised version for consideration for publication in EMBO Reports.

As per requested we have now added a Highlights section as well as a 2-sentences summary and a scheme. Moreover, Figure 4E has been revised and a blot of better quality is now shown.

Thank you again for considering of our revised manuscript, and I hope that with these revisions and added results, you will now find our study appropriate for publication in EMBO Reports.

4th Editorial Decision 02 January 2015

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

