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A Golgi rhomboid protease Rbd2 recruits Cdc48 to cleave yeast SREBP

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1st Editorial Decision 17 February 2016

Thank you again for submitting your manuscript to The EMBO Journal. We have now received feedback from three expert referees, whose reports are copied below for your information. As you will see, the referees consider your findings on fission yeast SREBP cleavage involving Cdc48 and a rhomboid protease, Rbd2, potentially interesting, but they also raise several important caveats with the study. These issues include hesitations regarding the overall conceptual advance (in light of recent studies by Kim et al and Fleig et al) as well as concerns with the decisiveness and depth of analysis in the present version of the manuscript.

Given that Kim et al only provided an initial genetic characterization of Rbd2 roles in SREBP cleavage, we feel that a comprehensive analysis such as this one would in principle still be suitable for EMBO Journal publication. Nevertheless, the conceptual parallels to the earlier study on RHBDL4 would in our view make it essential to strengthen the mechanistic insight in the present work along the lines of the major points raised by referees 1 and 3. In particular, it would be important to follow up on the main comment of referee 3 regarding the role of Cdc48 (which may also help to address related sentiments in referee 1's point 4 and referee 2's point 2). Two other key issues of immediate relevance for the present conclusions are referee 1's point 1 (and related point 4 of referee 2) regarding Rbd2 specificity, and referee 1's point 2, which again should be experimentally addressed. Furthermore, any data to answer the related point 3 of referee 1 would clearly be helpful to additionally increase the impact of this work. Finally, the results of Kim et al have to be introduced upfront, and will also need to be discussed (and differentiated) in the discussion section.

Therefore, should you be able to address these key issues, as well as to satisfactorily clarify the various minor/specific points of the reviewers, then we should be happy to consider a revised manuscript further for consideration. During our regular revision period (which may be extended upon request), it is our policy that publication of any competing work elsewhere would have no negative impact on our final assessment of your own study. I should however point out that we only allow for a single round of major revision, making it essential to diligently respond to all points raised by the referees and editors at the stage of resubmission. Additional information on preparing and uploading a revision can be found below.

Thank you again for the opportunity to consider this work for The EMBO Journal, and please do not hesitate to contact me should you have any comments or questions regarding the referee reports or this decision. I look forward to your revision.

REFEREE COMMENTS

Referee #1:

Hwang et al. report that the fission yeast rhomboid protease Rbd2 cleaves the transcription factor-harboring SREBP homologues (Sre1 and Sre2) in the Golgi. Cleavage requires the AAA-ATPase CDC48, which forms a complex with Rbd2, and very likely substrate ubiquitinylation. In the absence of Rbd2 the proteasome is taking over and degrades the substrate.
The manuscript is very well written, easy to follow, and the outline of the story is clear-cut. The experiments are overall technically very well executed, and with some exceptions detailed below under "Major points", support the conclusions.
One should note that two issues are potentially problematic with respect to the novelty of the data and concepts provided in this study: i) Rbd2 has recently been shown by others to be a protease required for SREBP cleavage (Kim et al., BBRC 2015), and ii) the pathway worked out in this study is, overall, similar to that of the human ER-rhomboid RHBDL4, which has been shown earlier by others to recognize and cleave ubiquitinylated substrates as well as to bind to the human CDC48 homolog p97 (Fleig et al. Mol Cell 2012). Although not identical in detail, as RHBDL4 activity is p97-independent, this may limit somewhat the broader conceptual novelty of this work.

Major points:
1) The authors present data indicating that unlike other rhomboids, Rbd2 cleaves its non-physiological bacterial TatA substrate after large hydrophobic residues. In fact, this is shown only by one mutant substrate and one would need more rigorous mutational analysis to support this claim. As this is sold as one of the highlights of the paper, it would be very important to also show that Rbd2 exerts these properties on its natural fission yeast SREBP Sre1 or Sre2 substrates to further substantiate these data. It could well be that the proposed requirements are not seen for its physiological substrates.

2) Identification of the cleavage sites for the physiological Sre1 or Sre2 substrates would also be important to support the interpretation that Rbd2 acts as analog of S1P and not as S2P as speculated by the authors in the Discussion. Intuitively, one might think that as an intramembrane protease Rbd2 would be acting as S2P in fission yeast and not as S1P. Sre1 or Sre2 cleavage site identification will also show whether there is indeed intramembrane cleavage in TM2 of these substrates as speculated in the Discussion. Actually, I think it would be important to lay out the relevant arguments for the above interpretation at least in part a bit earlier in the text, probably already in the introduction.

3) Related to 2), an obvious and easily testable guess would then be that the fission yeast SPP homologue that is expected to cleave substrates in type II membrane orientation could act as S2P. SPP knock out and overexpression experiments would immediately tell. If positive, adding such data would make the story stronger by more clearly providing an advance over the previous studies mentioned above.

4) The authors suggest that due to the involvement of the Dsc E3 ubiquitin ligase, Rbd2 likely cleaves Sre1 and Sre2 substrates in ubiqitinylated form. Biochemical experiments directly demonstrating ubiquitinylation of Sre1 or Sre2 are not provided so far. To demonstrate that these are indeed ubiquitinylated and to clarify the important question in which form, mono- or poly-ubiquitinylated, Sre1 or Sre2 will become substrates of Rbd2, such experiments should be shown. The model in Fig. 8 is somewhat confusing as it shows that polyubiquitinated Sre can be substrate for both proteases, i.e. Rbd2 and proteasome would compete for substrate raising the question how the access to Rbd2 versus proteasome is regulated, if not by different substrate ubiquitination states.

Minor points:
- The authors frequently switch between Sre1 and Sre2 analyses. May this could be better structured.

- p. 28, the composition of the IP wash buffer should be given.

- in Figure 5B, C and 6A, the labeling of bound fraction is not fully clear (1x, 5x, 25x: in relation to what?)

- in Figure 5B, lower panel, the bands of the GST-fusion proteins should be labeled.

- in Figure 5C, a description of the results and a corresponding interpretation for the Δ200-240 construct that shows no binding is missing.

- in Figure 7B and C, the protein bands should be labeled.

- In Figure EV2, the labeling indicating the presence or absence of oxgene is reverted (there is cleavage in the presence of oxygen).

- In Figure EV3, what is the band in lane 7? Shouldn´t this lane be empty?

Referee #2:

This study by Hwang et al. describes the identification and functional analysis of Rhomboid2 (Rbd2) in the activation pathway of fission yeast SREBPs. They first establish a requirement for Rbd2 in Sre1/2 activation (consistent with a recent report from Kim et al., 2015), then do an admirably thorough job of establishing exactly when and how it acts in the pathway. These experiments indicate that Rbd2 acts after Dsc complex mediated Sre1/2 ubiquitination, and requires both its catalytic activity and Cdc48 recruitment to initiate Sre1/2 processing. The results were convincing, the topic is of wide importance, and the manuscript is very well written. Overall, I have little to criticize here, and can support publication with minimal or no changes. The below comments are provided for the authors' consideration out of my own curiosity, but they need not be addressed for the paper to be accepted.

1) The evidence is most consistent with Rbd2 directly cleaving Sre1 and Sre2, but it formally remains possible that it works indirectly. While the most rigorous demonstration would be in vitro reconstitution of the cleavage in vitro (well beyond the scope of this study), a step in that direction might be to show a physical interaction between Rbd2 (presumably a catalytically inactive mutant) and Sre1 or Sre2. Has this been done?

2) The authors seem to demur on whether Cdc48 recruitment might involve ubiquitinated client. The most sensible model seems to be one where a combination of the SHP domain on Rbd2 and ubiquitin on the client collaborate to mediate optimal Cdc48 binding (i.e., Cdc48 would be a coincidence detector that is only recruited at the right place/time). A comment about this might be worthwhile in the discussion. It is also potentially testable using their APEX assay if they observe that less Cdc48 is recruited to Rbd2 in Dsc mutant cells.

3) Do the authors think that Dsc-dependent precursor degradation occurs directly from the Golgi, or involves trafficking back to the ER where putative dislocation machinery exists? The model seems to indicate the former, but it wasn't clear whether this was demonstrated anywhere.

4) I didn't understand the basis for stating that Rbd2 is a founding member of a new class of rhomboid protease. What makes it different? It's substrate specificity, the role in SREBP activation, or something else? To my eye, these differences didn't warrant its designation as a 'new class' since such fine distinctions might well lead to each rhomboid being in its own class!

Referee #3:

Hwang et al. describe a role of the fission yeast rhomboid protease Rbd2 in SREBP activation. The authors identified Rbd2 in a genetic screen and demonstrate by bioinformatic analyses and cell-based functional assays that it is a novel type of rhomboid protease. They show that Rbd2 is required for the processing of the fission yeast SREBP homologs Sre1 and Sre2 into their N-terminal, active fragments. Furthermore, they show that the Sre1/2 precursor proteins are rapidly degraded in the absence of Rbd2, and that this degradation requires the E3 ubiquitin ligase activity of the Dsc complex and the 26S proteasome. Finally, the authors show that Rbd2 contains a C-terminal SHP box motif that mediates binding to Cdc48, and that Cdc48 binding to Rbd2 is required for Sre1/2 processing. The authors propose a model where Rbd2 cleaves TM2 of Sre1/2 in a Dsc complex- and Cdc48-dependent manner to generate a Golgi membrane-anchored, N-terminal type-II intermediate. This intermediate is proposed to be subsequently released into the cytosol upon cleavage by a further, unknown protease.

This is an interesting paper on an important topic. The experiments are technically sound and well presented, and the conclusions are plausible. However, some key findings of this study are not entirely novel. The processing of Sre1 by Rbd2 and the phenotypic analysis of rbd2 mutants were recently published by Kim et al (BBRC 468, 606-610; 2015). The concept of Cdc48 recruitment to a rhomboid protease is reminiscent of the Rbd2-related human RHBDL4 protease involved in ERAD of membrane proteins (Fleig et al, Mol Cell 47, 558-569; 2012). Given this, the present story would benefit from additional mechanistic insights into the role of Cdc48: Does Cdc48 interact with the (ubiquitinylated?) precursor, with the postulated intermediate, with Sre1/2N, and/or with the C-terminal Sre1/2 cleavage products? Is Cdc48 required for the release of Sre1/2N from the Golgi membrane, in analogy to its role in ERAD and Spt23 processing in budding yeast?

Further points:

1. Fig. 1E: The colocalization of Rbd2 with Golgi markers could be driven by unspecific oligomerization/aggregation of the huge tandem FP tags. Is there any other evidence that Rbd2 localizes to the Golgi and not, for instance, to the ER?

2. Fig. 2B and others: Sre1N runs as multiple bands. What is the identity of these bands? Do they represent distinct cleavage products or posttranslational modifications (ubiquitinylation?)? Are they all soluble forms of Sre1N, or are some of them membrane-associated?

3. Fig. 2B and others: The relative amounts of the P and N forms of Sre1 in the wild-type and mutant backgrounds are somewhat puzzling. First, even under normoxic conditions, there appears to be less precursor present in the dsc mutants than in the wild type - why? Second, the processing of P into N in wild type appears not to be at the expense of the precursor. Quite the contrary, P also increases over time. Because this complicates the interpretation of the processing kinetics, these experiments should be performed as pulse chase or cycloheximide shut-off experiments.

4. Fig. 3CDE: Why is there a double band of the P form? The size distribution of the CN fragments produced by Rbd2 appears to differ between the experiments - why? It would be interesting to include human RHBDL4 in the analysis.

5. Fig. 2EF and others: Many Sre1/2 blots lack control blots demonstrating equal loading of all lanes.

6. The authors stress the "structural" similarity between the ERAD E3 ligase gp78 and the Dsc complex. However, there is no evidence for such a similarity, as the structure of neither E3 has been

solved yet. Moreover, Zhang et al (MBoC 26, 4439-4450; 2015) recently showed that gp78 has a role downstream of Hrd1 in mammalian ERAD, also putting into question a potential similarity with the Dsc complex.

1st Revision - authors' response 15 July 2016

Response to Reviewers

We thank the reviewers for their supportive comments and helpful suggestions that improved the manuscript. Major changes to the manuscript include the addition of new Figures 5 and 9 as well as additional supporting figures. We address both general and specific comments below.

In the original manuscript, we reported that Rbd2 is an active Golgi rhomboid protease that is required for fission yeast SREBP cleavage. Using a new proximity biotinylation assay, we found that Rbd2 binds Cdc48 and that this binding is required for SREBP cleavage. In the absence of Rbd2, ubiquitinylated SREBP is degraded, demonstrating that regulation of Rbd2 activity may serve as a new control point for the fungal SREBP pathway. In this revised manuscript, we additionally report the following:

1. Rbd2 interacts with Sre2, suggesting that SREBPs are direct substrates for Rbd2.
2. Overexpression of Rbd2 bypasses the requirement for Cdc48, indicating that Cdc48 functions in substrate recruitment.
3. A comprehensive mutant screen identified residues in Sre2 required for interaction with the Dsc E3 ligase and a single residue K743 in the luminal loop required for Rbd2 function. These studies indicate that SREBP is a unique, multi-span rhomboid substrate and highlight the need for future studies of this proteolytic reaction.
4. Two studies in *Aspergillus fumigatus* published since our submission (Dhingra et al. *mSphere* 2016; Vaknin et al. *Infect Immun* 2016) demonstrate that the Rbd2 homolog is required for SREBP activation and virulence in this human pathogen. We demonstrate that Cdc48 binds *in vitro* to this *A. fumigatus* RbdB protease, suggesting that the Cdc48-dependent mechanism described underlies fungal pathogenesis in *Aspergillus*.
5. It was reported in *Aspergillus nidulans* that the signal peptide peptidase SppA is required for cleavage of what is likely the product of the Rbd2 reaction. We show that neither fission yeast signal peptide peptidase Ypf1 nor two other aspartyl proteases are required for SREBP activation, indicating that a second protease remains to be discovered in this pathway.
6. Regarding proteasomal degradation of SREBP in the absence of Rbd2 activity, we show that this process does not require the ERAD E3 ligases Hrd1 or Doa10. Given that Hrd1 is the candidate dislocase (Baldridge & Rapoport, 2016), these data indicate that a novel mechanism exists in the secretory pathway for the extraction and degradation of membrane proteins.

We were delighted that each reviewer found this study significant and that Reviewer #2 recommended "*publication with minimal or no changes*". While it is certainly intriguing that Rbd2 displayed unique sequence preference in the TatA cleavage assays, we agree that conclusions about whether Rbd2 defines a new rhomboid class require detailed studies. We modified our conclusions in the revised manuscript and will address these questions in future studies. Reviewers 1 and 3 raised questions about the novelty of our findings given an existing paper (Kim et al, 2015) showing that Rbd2 is required for SREBP activation in fission yeast. In addition, the reviewers noted similarities between Rbd2 and the ER-localized RHBDL4 in that both proteases bind to Cdc48/p97.

There are now 3 published reports (one in *S. pombe* and two in *A. fumigatus*) implicating Rbd2 in SREBP activation (Dhingra et al, 2016; Kim et al, 2015; Vaknin et al, 2016). However, each of these papers provides only a cursory examination of Rbd2 function, a point highlighted by the title from Kim et al. "Identification of Rbd2 as a candidate protease". Indeed, we confirm

all of the results reported by Kim et al. in a single figure, Figure 2. Using this as a starting point, we demonstrate that Rbd2 is a Golgi-localized SREBP protease that employs Cdc48 in a novel mechanism to recruit substrate. While the interaction of Rbd2 with Cdc48 is reminiscent of RHBDL4, a fundamentally different mechanism is at work here and the protein-protein interactions involve different structural motifs. RHBDL4 is an ER-resident protease that recruits substrate using a ubiquitin-interacting motif (UIM) and binds p97 through a VBM motif (Fleig et al, 2012). p97 functions to extract cleaved RHBDL4 products for subsequent degradation. In contrast, Rbd2 resides in the Golgi, replacing the function of the mammalian Site-1 protease and functions in a signaling pathway, not in protein quality control. Cdc48 binds to Rbd2 through a SHP box, and Cdc48 functions in substrate recruitment, not in protein degradation. Rather than diminish the novelty of our findings, the parallels to RHBDL4 make the current story more interesting and show that rhomboids have evolved multiple ways to recruit substrates and interact with Cdc48/p97. Our findings add to the growing list of non-catalytic functions in rhomboids and again highlight how little we understand about the many functions of p97/Cdc48.

Specific reviewer comments shown in italics are addressed below.

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To address the reviewer's comments, we performed experiments using the HEK293 cell heterologous cleavage assay to test whether Rbd2 cleaves yeast SREBPs. Despite exhaustive

efforts, we failed to detect SREBP cleavage, possibly due to the absence of the Dsc E3 ligase and ubiquitinylated substrate.

As an alternative, we tested whether the unique substrate sequence preference was also observed for SREBP cleavage in yeast using Sre2MS mutagenesis. We assayed cleavage of alanine-substitution mutants in the N-terminus of the second transmembrane segment of Sre2MS to test the requirement of large hydrophobic residues. Processing of each mutant was normal, indicating that Sre2MS cleavage does not require any individual large hydrophobic residues. Given that we have not mapped the cleavage site(s), we cannot rule out that the cleavage site shifts in these mutants. These results are included in Figure 5A and 5B in the revised manuscript. This difference in sequence requirements may explain why SREBP processing requires Dsc E3 ubiquitination machinery unlike in the case of TatA cleavage. Based on our new findings on SREBP sequence requirements, we agree that defining Rbd2 as a member of a new rhomboid class requires additional mutagenesis studies. We will pursue these experiments in future studies and have removed this conclusion from the manuscript.

2) Identification of the cleavage sites for the physiological Sre1 or Sre2 substrates would also be important to support the interpretation that Rbd2 acts as analog of S1P and not as S2P as speculated by the authors in the Discussion. Intuitively, one might think that as an intramembrane protease Rbd2 would be acting as S2P in fission yeast and not as S1P. Sre1 or Sre2 cleavage site identification will also show whether there is indeed intramembrane cleavage in TM2 of these substrates as speculated in the Discussion. Actually, I think it would be important to lay out the relevant arguments for the above interpretation at least in part a bit earlier in the text, probably already in the introduction.

The final processed products of both Sre1 and Sre2 (Sre1N and Sre2N) terminate at a cytosolic position, ~10 amino acids prior to TM1 (Cheung & Espenshade, 2013; Stewart et al, 2011) (Figure R1A). Thus, we do not think that Rbd2 cleaves to produce the active transcription factor. Rather, we hypothesized that Rbd2, like other rhomboid proteases, cleaves within TM2 toward the luminal N-terminus because the rhomboid active site lies near the lipid bilayer/luminal interface. If the Rbd2-mediated N-terminal cleaved form ('i + ii', Figure R1B) is processed to a final product ('i') very quickly, it may be difficult to capture. Thus following the reviewer's suggestion to identify the Sre2 cleavage site, we aimed to capture the C-terminal cleaved form of Sre2MS ('iii') that generated by Rbd2 (Figure R1B). To this end, we tagged the N-terminus and C-terminus of Sre2MS with Flag and GFP, respectively. In wild-type cells (WT), a Flag-tagged N-terminal fragment was observed with expected molecular weight (Figure R1C, lane 4). However, the predicted ~32 kDa C-terminal GFP fragment did not accumulate in WT cells although the full-length precursor was detected by anti-GFP antibody (lane 1). Proteasome inhibition with Bortezomib did not lead to accumulation of the C-terminal fragment in WT cells (Figure R1D, lanes 1-2), and attempts to inhibit vacuolar proteolysis with Bafilomycin also had no effect (Figure R1E, lanes 1, 7 and 10). Because we were not able to capture the C-terminal fragment or intermediate forms, we were unable to identify the Rbd2-mediated cleavage site. We observed ~35 kDa and 25 kDa GFP signals that accumulated in *dsc1⊿* background, possibly due to off pathway vacuolar degradation of the Sre2MS, but we did not investigate this further.

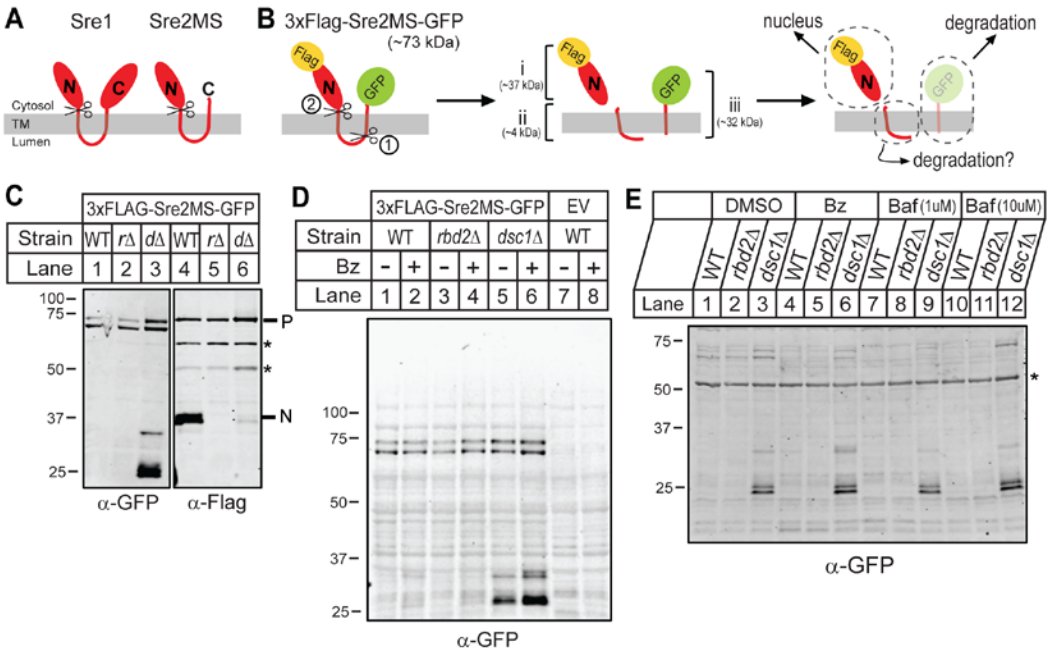

Figure R1. Sre2MS intermediates do not accumulate. (A) Diagram of Sre1 and Sre2 with predicted final cleavage site. (B) Model of Sre2MS cleavage activation. Rbd2 initiates cleavage ①, which generates intermediate forms, (i+ii) and (iii). The (i+ii) form is further processed ② to create the final product (i). The final product form (i) travels to the nucleus to act as a transcription factor while the C-terminal intermediate form (iii) disappears. (C) Whole cell lysates from wild-type (WT), *rbd2Δ* (*rΔ*) or *dsc1Δ* (*dΔ*) cells carrying a plasmid expressing 3xFlag-Sre2MS-GFP were analyzed by western blot with anti-GFP or anti-Flag antibody. Asterisks indicate nonspecific bands. P and N denote Sre2MS precursor and cleaved forms.(D) WT, *rbd2Δ* or *dsc1Δ* cells carrying either a plasmid expressing 3xFlag-Sre2MS-GFP or empty vector (EV) were treated with bortezomib (Bz,1 mM) for 3 hr. Whole cell lysates were analyzed by western blot with anti-GFP antibody. (E) WT, *rbd2Δ* or *dsc1Δ* cells carrying a plasmid expressing 3xFlag-Sre2MS-GFP were treated with either DMSO (vehicle), bortezomib (Bz), or bafilomycin (Baf) at indicated concentrations for 3 hr. Whole cell lysates were analyzed by western blot with anti-GFP antibody.

In order to identify residues important for proteolytic cleavage, we performed further mutagenesis on Sre2MS (Figures 5, S1 and S2 in the revised manuscript). Through this mutagenesis study, we identified lysine 743 in the luminal loop as the only single residue required for Rbd2-mediated Sre2MS cleavage. This luminal lysine residue may serve as part of a substrate recognition motif located outside the membrane that may access the hydrophilic cavity surrounding Rbd2 active site, as suggested in other models of rhomboid-substrate recognition (Strisovsky et al, 2009). Alternatively, the arginine may separately block cleavage. Should Rbd2 cleave in the luminal loop of Sre2, it would functionally replace S1P by initiating Sre2 cleavage activation and yield a type II intermediate requiring additional processing.

During the preparation of our revised manuscript, two papers reported a requirement for a rhomboid in *Aspergillus fumigatus* SREBP cleavage activation (Dhingra et al, 2016; Vaknin et al, 2016). In *Aspergillus nidulans*, cleavage of a type II intermediate requires SppA, an aspartyl signal peptide peptidase homolog (Bat-Ochir et al, 2016). Collectively, these studies support a model for *Aspergillus* SREBP cleavage in which Rbd2 initiates SREBP cleavage activation followed by SppA, which releases SREBP-N from the membrane. Combined with our Sre2MS mutagenesis analysis, we believe that Rbd2 likely acts as an S1P analog in which Rbd2 initiates SREBP cleavage. The intermediate cleavage product is subsequently processed by a second unknown protease to generate the soluble active transcription factor.

3) Related to 2), an obvious and easily testable guess would then be that the fission yeast SPP homologue that is expected to cleave substrates in type II membrane orientation could act as S2P. SPP knock out and overexpression experiments would immediately tell. If positive, adding such data would make the story stronger by more clearly providing an advance over the previous studies mentioned above.

We appreciate the reviewer's excellent suggestion. Because Rbd2 likely yields a type II intermediate, and because *A. nidulans* SREBP cleavage requires signal peptide peptidase SppA (Bat-Ochir et al, 2016), we tested whether fission yeast also utilizes signal peptide peptidase for Sre1 cleavage activation. To address the reviewer's question, we examined Sre1 cleavage under hypoxia in deletion strains of candidate proteases including *ypf1* (SppA homolog, intramembrane aspartyl protease) and two other aspartyl proteases *yps1* and *sxa1* (Figure EV4 in the revised manuscript). However, we did not observe any Sre1 cleavage defect under hypoxia in the deletion strains, leaving unknown the identity of the second protease in fission yeast. Our previous effort to screen for genes required for Sre1 cleavage under hypoxia using *S. pombe* non-essential haploid deletion collection did not identify any obvious candidate proteases (Burr et al, 2016; Stewart et al, 2011), indicating that the second protease for *S. pombe* SREBP may exist redundantly (compensating for each other's loss of function) or it may be essential gene that has escaped our screening. Together, our efforts and parallel studies in *Aspergillus* indicate that a second protease (presumably a S2P analog) exists in *S. pombe*, but further work is required to determine its identity.

4) The authors suggest that due to the involvement of the Dsc E3 ubiquitin ligase, Rbd2 likely cleaves Sre1 and Sre2 substrates in ubiqitinylated form. Biochemical experiments directly demonstrating ubiquitinylation of Sre1 or Sre2 are not provided so far. To demonstrate that these are indeed ubiquitinylated and to clarify the important question in which form, mono- or poly-ubiquitinylated, Sre1 or Sre2 will become substrates of Rbd2, such experiments should be shown. The model in Fig. 8 is somewhat confusing as it shows that polyubiquitinated Sre can be substrate for both proteases, i.e. Rbd2 and proteasome would compete for substrate raising the question how the access to Rbd2 versus proteasome is regulated, if not by different substrate ubiquitination states.

As the reviewer notes, no direct biochemical evidence exists supporting SREBP (Sre1 or Sre2) ubiquitinylation despite continuous efforts. To identify the candidate ubiquitinylation site(s) on Sre2MS we tested cleavage of Sre2MS in which the 5 cytosolic lysines were mutated to arginine. However, a cytosolic 5K->R Sre2MS mutant was cleaved normally (Figure R2, lanes 1-4). For these experiments, we used an HA-tagged Sre2MS because the Flag tag contains two lysine residues.

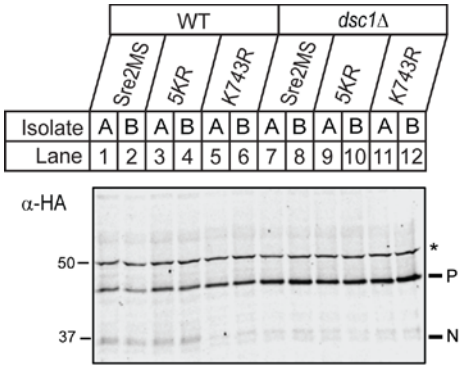

Figure R2. Cleavage analysis of Lysine-deficient Sre2MS mutant. Wild-type (WT) or *dsc1Δ* yeast containing a plasmid expressing HA-tagged Sre2MS WT, 5KR (all five cytosolic-facing lysine residues changed to arginine) or K743R (a single luminal lysine residue changed to arginine) were analyzed for Sre2MS cleavage. Western blot was performed using anti-HA IgG of whole cell lysates. Two independent isolates (A and B) are shown for each strain. P and N denote Sre2MS precursor and cleaved forms. The asterisk denotes nonspecific band.

Since cleavage of this mutant still required the Dsc E3 ligase, we suspect that ubiquitinylation may occur on non-lysine residues when no lysine acceptors are available as previously reported in other cases (Boban et al, 2015; Kravtsova-Ivantsiv & Ciechanover, 2012). Because of the difficulty of identifying ubiquitylation sites, it was also hard to address which form (mono- or poly-ubiquitinylated) of SREBP is a substrate for Rbd2. However, given that Ubc4 is required for SREBP cleavage, we speculate that a mono-ubiquitinylated form of SREBP is the likely a substrate for Rbd2. Ubc4 has previously shown to modify the substrate at multiple lysines to generate mono-ubiquitinylated products (Rodrigo-Brenni & Morgan, 2007; Stoll et al, 2011). In contrast in the absence of functional Rbd2, uncleaved SREBP is likely poly-ubiquitinylated because most proteasomal substrates, if not all, are poly-ubiquitinylated.

We apologize that our model in the original manuscript was confusing. To clarify our model, we separated one condition from the other: one with functional Rbd2 being present and the other when Rbd2 is not available. The new model can be found in Figure 10 in the revised manuscript.

Minor points:
- The authors frequently switch between Sre1 and Sre2 analyses. May this could be better structured.

We recognize that using multiple substrate constructs can be confusing, but we employ both Sre1 and Sre2 to show that these functions are independent of oxygen and lipid regulation. In revision experiments, we focused on Sre2 and Sre2MS to minimize this.

- p. 28, the composition of the IP wash buffer should be given.

The revised manuscript provides the detailed procedure as the reviewer requested.

- in Figure 5B, C and 6A, the labeling of bound fraction is not fully clear (1x, 5x, 25x: in relation to what?)

We clarified this point as requested. Old figures are now Figure 6B, 6C, and 7A in the revised manuscript.

- in Figure 5B, lower panel, the bands of the GST-fusion proteins should be labeled.

We thank the reviewer for noting this. The labeling has been added in Figure 6B in the revised manuscript.

- in Figure 5C, a description of the results and a corresponding interpretation for the ∆200-240 construct that shows no binding is missing.

These results are described on page 16, line 15 of the revised manuscript along with the ∆200-225 construct. Both failed to bind Cdc48.

- in Figure 7B and C, the protein bands should be labeled.

These protein bands have now been properly labeled in Figure 8B and C in the revised manuscript.

- In Figure EV2, the labeling indicating the presence or absence of oxygen is reverted (there is cleavage in the presence of oxygen).

The reviewer is correct. We fixed our error.

- In Figure EV2, what is the band in lane 7? Shouldn´t this lane be empty?

The reviewer is correct that the lane 7 should be blank or show little Cdc48 signal. While lane 7 does show reduced Cdc48, this experiment was performed during the early optimization of the APEX labeling assay. We have repeated this experiment using G246R and S130A more than three times, and the revised manuscript contains an image representative of these optimized results (Figure EV2B).

Referee #2:

This study by Hwang et al. describes the identification and functional analysis of Rhomboid2 (Rbd2) in the activation pathway of fission yeast SREBPs. They first establish a requirement for Rbd2 in Sre1/2 activation (consistent with a recent report from Kim et al., 2015), then do an admirably thorough job of establishing exactly when and how it acts in the pathway. These experiments indicate that Rbd2 acts after Dsc complex mediated Sre1/2 ubiquitination, and requires both its catalytic activity and Cdc48 recruitment to initiate Sre1/2 processing. The results were convincing, the topic is of wide importance, and the manuscript is very well written. Overall, I have little to criticize here, and can support publication with minimal or no changes. The below comments are provided for the authors' consideration out of my own curiosity, but they need not be addressed for the paper to be accepted.

1) The evidence is most consistent with Rbd2 directly cleaving Sre1 and Sre2, but it formally remains possible that it works indirectly. While the most rigorous demonstration would be in vitro reconstitution of the cleavage in vitro (well beyond the scope of this study), a step in that direction might be to show a physical interaction between Rbd2 (presumably a catalytically inactive mutant) and Sre1 or Sre2. Has this been done?

We agree that an *in vitro* cleavage assay would demonstrate that Sre1 and Sre2 are direct substrates of Rbd2, and we will work toward this in the future. As an intermediate step, we employed the heterologous HEK293 cell cleavage system to test Rbd2 cleavage of multiple SREBP substrate variants. Unfortunately, these experiments failed to yield a positive result, possibly due to the lack of Dsc E3 ubiquitin ligase machinery. In addition following the reviewer's suggestion, we tested for a physical interaction between Rbd2 and Sre2MS using APEX technique. We used a Rbd2-S130A catalytically inactive mutant to capture substrate-enzyme binding. The APEX labeling experiment showed that Rbd2-S130A interacts with GFP-tagged Sre2MS, supporting that Sre2MS is a direct substrate for Rbd2. The revised manuscript includes these new data in Figure 9D.

2) The authors seem to demur on whether Cdc48 recruitment might involve ubiquitinated client. The most sensible model seems to be one where a combination of the SHP domain on Rbd2 and ubiquitin on the client collaborate to mediate optimal Cdc48 binding (i.e., Cdc48 would be a coincidence detector that is only recruited at the right place/time). A comment about this might be worthwhile in the discussion. It is also potentially testable using their APEX assay if they observe that less Cdc48 is recruited to Rbd2 in Dsc mutant cells.

At the reviewer's suggestion, we performed the APEX assay to test whether Rbd2, Cdc48 and ubiquitin on SREBP might cooperatively contribute to complex formation. As the reviewer predicted, Cdc48 biotin labeling in *dsc1⊿* was marginally, but consistently, reduced compared to that in WT (~20% reduction) (Figure R3E). Given the magnitude of this effect and the fact that we cannot ascribe reduced binding specifically to a lack of SREBP substrate (only to loss of the Dsc1 E3 ligase), we chose not to include this in the revised manuscript.

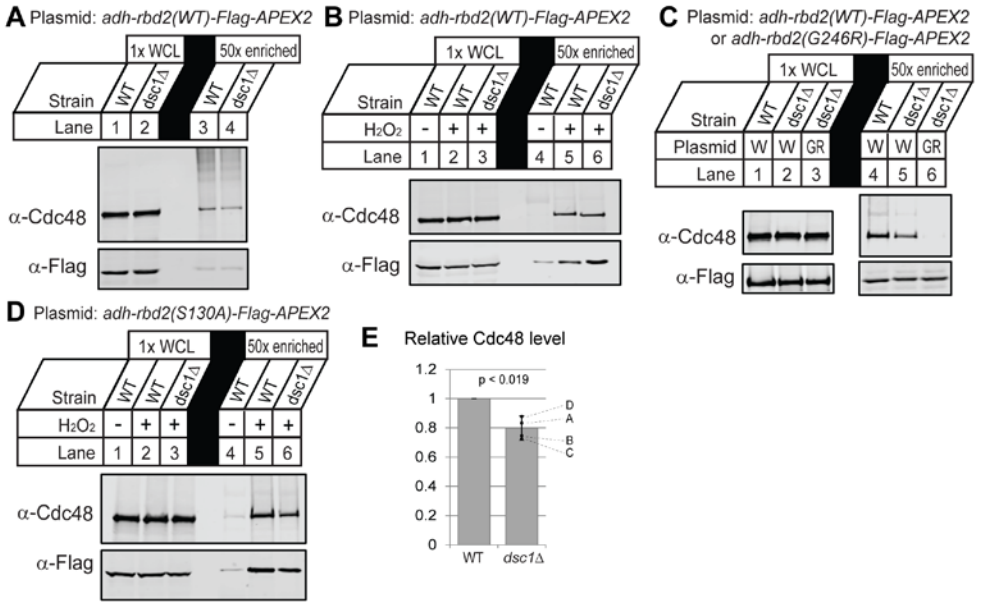

Figure R3. Dsc1 promotes optimal Cdc48 binding to Rbd2. (A) WT or *dsc1Δ* cells carrying *rbd2-Flag-APEX2* were lysed after biotin-labeling. Biotinylated proteins were then enriched using streptavidin magnetic beads. Whole cell lysates (WCL) or 50x enriched eluates were analyzed by western blot anti-Cdc48 serum, or anti-Flag IgG. (B) APEX/western assay was performed as in (A). APEX assay without H_2O_2 (lanes 1 and 4) served as a negative control. (C) *dsc1Δ* cells carrying *rbd2 (G246R)-Flag-APEX2* (lanes 3 and 6, GR) served as a negative control. (D) WT or *dsc1Δ* cells carrying *rbd2 (S130A)-Flag-APEX2* were lysed after biotin-labeling. APEX assay without H_2O_2 (lanes 1 and 4) served as a negative control. (E) The intensity of Cdc48 band in *dsc1Δ* sample from blots in (A)-(D) was quantified using Li-COR software and intensity relative to corresponding Flag level was normalized to that in WT sample. The data are presented as bar graph of relative intensity of Cdc48 in each subfigures (A) through (D).

3) Do the authors think that Dsc-dependent precursor degradation occurs directly from the Golgi, or involves trafficking back to the ER where putative dislocation machinery exists? The model seems to indicate the former, but it wasn't clear whether this was demonstrated anywhere.

In Figure EV1 in the revised manuscript, we addressed whether the two known ERAD E3 ligases in *S. pombe*, Hrd1 (a candidate dislocase (Baldridge & Rapoport, 2016)) and Doa10, were involved in degradation of SREBP in the absence of functional Rbd2. Asc homologs are not present in *S. pombe*. Neither Hrd1 nor Doa10 was required for degradation. Thus, it appears unlikely that SREBP is degraded from the ER in the absence of Rbd2.

4) I didn't understand the basis for stating that Rbd2 is a founding member of a new class of rhomboid protease. What makes it different? It's substrate specificity, the role in SREBP activation, or something else? To my eye, these differences didn't warrant its designation as a 'new class' since such fine distinctions might well lead to each rhomboid being in its own class!

To our knowledge, all known rhomboid proteases cleave after small hydrophobic residues. Our finding that Rbd2, unlike other rhomboid proteases, preferentially cleaves TatA model substrate after large hydrophobic amino acid residue led us to conclude that Rbd2 is a new class of rhomboid protease with unique substrate specificity. However, we agree that additional mutagenesis studies are required to support this conclusion and thus we removed this conclusion.

Referee #3:

Hwang et al. describe a role of the fission yeast rhomboid protease Rbd2 in SREBP activation. The authors identified Rbd2 in a genetic screen and demonstrate by bioinformatic analyses and cell-based functional assays that it is a novel type of rhomboid protease. They show that Rbd2 is required for the processing of the fission yeast SREBP homologs Sre1 and Sre2 into their N-terminal, active fragments. Furthermore, they show that the Sre1/2 precursor proteins are rapidly degraded in the absence of Rbd2, and that this degradation requires the E3 ubiquitin ligase activity of the Dsc complex and the 26S proteasome. Finally, the authors show that Rbd2 contains a C-terminal SHP box motif that mediates binding to Cdc48, and that Cdc48 binding to Rbd2 is required for Sre1/2 processing. The authors propose a model where Rbd2 cleaves TM2 of Sre1/2 in a Dsc complex- and Cdc48-dependent manner to generate a Golgi membrane-anchored, N-terminal type-II intermediate. This intermediate is proposed to be subsequently released into the cytosol upon cleavage by a further, unknown protease.

This is an interesting paper on an important topic. The experiments are technically sound and well presented, and the conclusions are plausible. However, some key findings of this study are not entirely novel. The processing of Sre1 by Rbd2 and the phenotypic analysis of rbd2 mutants were recently published by Kim et al (BBRC 468, 606-610; 2015). The concept of Cdc48 recruitment to a rhomboid protease is reminiscent of the Rbd2-related human RHBDL4 protease involved in ERAD of membrane proteins (Fleig et al, Mol Cell 47, 558-569; 2012). Given this, the present story would benefit from additional mechanistic insights into the role of Cdc48: Does

Cdc48 interact with the (ubiquitinylated?) precursor, with the postulated intermediate, with Sre1/2N, and/or with the C-terminal Sre1/2 cleavage products? Is Cdc48 required for the release of Sre1/2N from the Golgi membrane, in analogy to its role in ERAD and Spt23 processing in budding yeast?

In response to these general questions, we now provide evidence in Figure 9 that Cdc48 functions as an adaptor to recruit SREBP substrate to Rbd2. Our inability to observe an intermediate or the SREBP C-terminal fragment prevented some of the recommended experiments. However, overexpression of Rbd2-G246R that does not bind to Cdc48 bypasses the cleavage defect, indicating that Cdc48 is not required for membrane extraction of an intermediate or the final cleavage product.

Further points:

1. Fig. 1E: The colocalization of Rbd2 with Golgi markers could be driven by unspecific oligomerization/aggregation of the huge tandem FP tags. Is there any other evidence that Rbd2 localizes to the Golgi and not, for instance, to the ER?

We did not observe ER localization of Rbd2-6xmCherry in our experiments, and it would be unusual for a dysfunctional ER-resident protein to localize to the Golgi (normally it is the reverse). To explore this further, we conducted additional experiments to test the function of tagged Rbd2. First, we compared growth on $CoCl_2$ of two *rbd2⊿* yeast strains: one expressing Rbd2-6xmCherry and the other with Rbd2-1xGFP. While tagging Rbd2 at its C-terminus partially reduced function, there was no difference between 6xmCherry and 1xGFP (Figure R4). Thus, Golgi-localized Rbd2-6xmCherry is active.

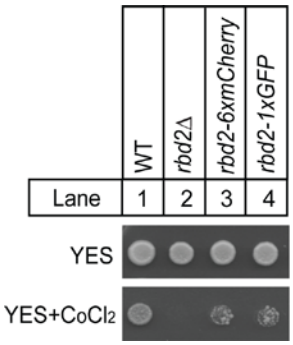

Figure R4. *rbd2-6xmCherry* rescues *rbd2*Δ growth. Wild-type (WT) and *rbd2Δ* yeast containing a plasmid expressing either *rbd2-6xmCherry* or *rbd2-1xGFP* were grown on rich medium (YES)in the presence of absence of cobalt chloride ($CoCl_2$)

Second, we tested subcellular localization of Rbd2 using APEX2 technology (Figure R5). Rbd2-APEX fusion protein labeled Golgi-localized Dsc5 in WT yeast while the labeling of the same protein decreased 5-fold in *dsc1⊿*, a condition in which the Dsc E3 ligase is trapped in the ER (Raychaudhuri & Espenshade, 2015). These data indicate that tagged Rbd2-APEX fusion protein (which is about the same size as Rbd2-1xGFP) colocalizes in the Golgi with the Dsc E3 ligase.

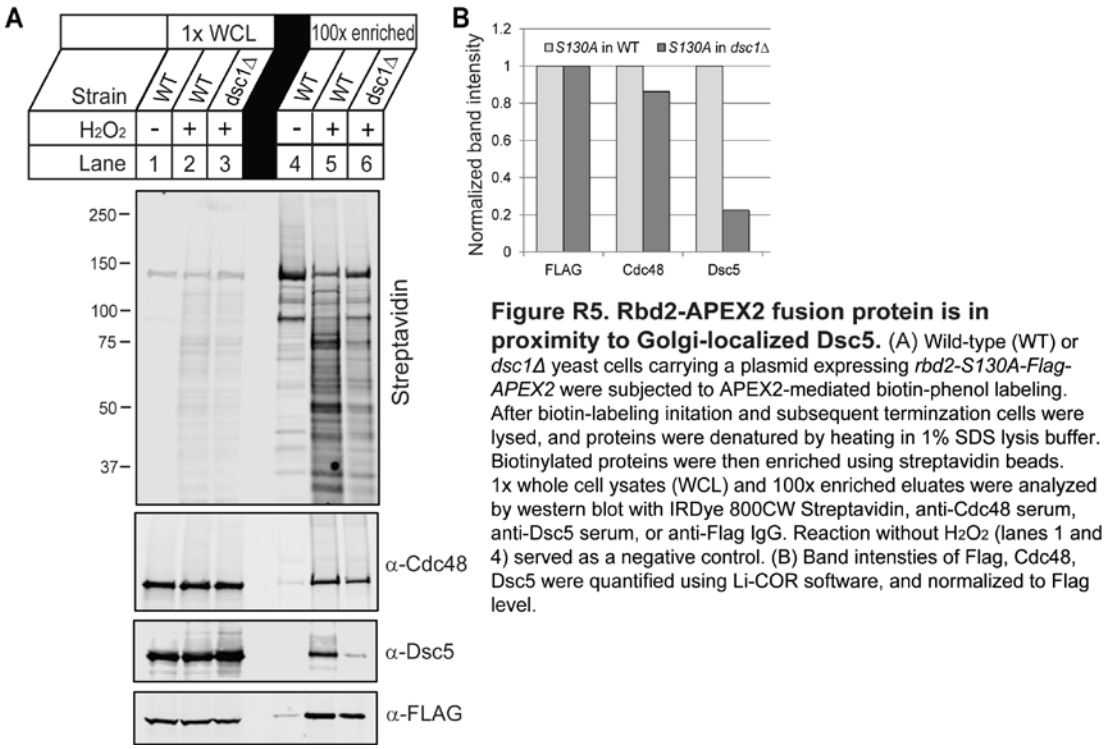

Figure R5. Rbd2-APEX2 fusion protein is in proximity to Golgi-localized Dsc5. (A) Wild-type (WT) or *dsc1Δ* yeast cells carrying a plasmid expressing *rbd2-S130A-Flag-APEX2* were subjected to APEX2-mediated biotin-phenol labeling. After biotin-labeling initation and subsequent terminzation cells were lysed, and proteins were denatured by heating in 1% SDS lysis buffer. Biotinylated proteins were then enriched using streptavidin beads. 1x whole cell ysates (WCL) and 100x enriched eluates were analyzed by western blot with IRDye 800CW Streptavidin, anti-Cdc48 serum, anti-Dsc5 serum, or anti-Flag IgG. Reaction without H_2O_2 (lanes 1 and 4) served as a negative control. (B) Band intensties of Flag, Cdc48, Dsc5 were quantified using Li-COR software, and normalized to Flag level.

2. Fig. 2B and others: Sre1N runs as multiple bands. What is the identity of these bands? Do they represent distinct cleavage products or posttranslational modifications (ubiquitinylation?)? Are they all soluble forms of Sre1N, or are some of them membrane-associated?

Sre1N is heavily phosphorylated, so we treat cell lysates with alkaline phosphatase (AP) for 1 hour at 37°C. Multiple bands may represent AP-resistant phosphorylated forms or may result from incomplete AP treatment. Since the size difference between multiple bands is within less than 10 kDa, it is unlikely that the modification is polyubiquitinylation. Analysis of microsomes indicates that these species are extracted by high pH buffer and therefore not integral to the membrane (Figure R6, lane 2 vs. 3).

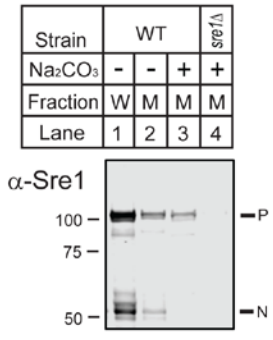

Figure R6. Cleaved Sre1N is not integral to the membrane. Wild-type (WT) or *sre1Δ* cells were grown for 2 hr in the absence of oxygen. Microsomes were prepared by a modification of the method described in Sakai *et al.*, Cell 1996). Briefly, cells were lysed mechanically with glass beads in B88 buffer (20 mM Hepes, 150 mM potassium acetate, 250 mM sorbitol, 5 mM magnesium acetate pH 7.4). After removal nuclei and unbroken cells, the sup was spun at 47,000 rpm for 10 min at 4°C. The pellet was washed twice with B88 buffer. To remove any peripherally associated proteins, the pellet was incubated wtih 0.1M sodium carbonate (pH 11) for 30 min at 4°C, and spun at 47,000 rpm. This step was repeated twice. The obtained pellet was dissolved in gel loading buffer. Whole cell lysates were independently prepared from the same yeast culture (lane 1, W). Phosphatase-treated microsome fraction before and after sodium carbonate washes (lanes 2 and 3) along with phosphatase-treated whole cell lysates (lane 1) were analyzed by western blot with anti-Sre1 IgG. P and N denotes Sre1 precursor and nuclear form, respectively. It should be noted that multiple bands observed in whole cell lysates likely represent different phosphorylated forms.

3. Fig. 2B and others: The relative amounts of the P and N forms of Sre1 in the wild-type and mutant backgrounds are somewhat puzzling. First, even under normoxic conditions, there appears to be less precursor present in the dsc mutants than in the wild type - why? Second, the processing of P into N in wild type appears not to be at the expense of the precursor. Quite the contrary, P also increases over time. Because this complicates the interpretation of the processing kinetics, these experiments should be performed as pulse chase or cycloheximide shut-off experiments.

As the reviewer pointed out, Sre1 precursor level increases over time. This is due to increased transcription of the *sre1* gene through a positive feedback loop, and this serves as another indication of Sre1 activity (Hughes et al, 2005). Sre1 precursor is reduced in *dsc* mutants due to the absence of basal Sre1 activity and to reduced levels of the Sre1 binding partner Scp1 in *dsc* mutants (Shao & Espenshade, 2014). Scp1 is required for Sre1P stabilization (Hughes et al, 2009). We agree that pulse-chase analysis would be a better assay, but this is not feasible given the low abundance of these transcription factors. In addition, a fraction of Sre1 precursor is degraded through a Hrd1-dependent pathway in wild-type cells in the presence of oxygen (Hughes et al, 2009), so a cycloheximide chase experiment monitoring the disappearance of Sre1P would not be specific for the cleavage reaction. Given that our parallel experiments with Sre2 and Sre2MS align with those of Sre1, we do not feel as though these limitations affect our conclusions.

4. Fig. 3CDE: Why is there a double band of the P form? The size distribution of the CN fragments produced by Rbd2 appears to differ between the experiments - why? It would be interesting to include human RHBDL4 in the analysis.

The GFP-TatA-Flag substrate contains an N-linked glycosylation site in its luminal N-terminal domain. PNGaseF treatment, which removes core N-linked oligosaccharides, increased the mobility of the upper species (Figure R7), and this species now comigrated with the lower band. Thus, the substrate doublet results from incomplete N-linked glycosylation of the substrate. Likewise, the cleaved N-terminal product is N-glycosylated. The referenced figures are now Figures 4CDE in the revised manuscript.

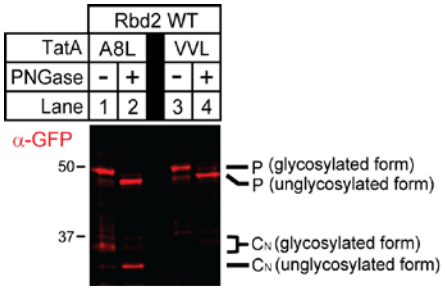

Figure R7. GFP-TatA-Flag protein is incompletely glycosylated.
Human HEK293 cells were transfected with plasmids expressing the indicated GFP-TatA-Flag substrate (either A8L or VVL) and Rbd2. 48 hours post-transfection, whole cell lysates were treated with or without PNGaseF for 1 hr at 37°C, then analyzed by western blot with anti-GFP IgG. P and C_N denote precursor and C-terminal cleaved products, respectively.

Given that Cdc48 functions differently with Rbd2 than RHBDL4, we chose not to include RHBDL4 experiments in the revised manuscript. However, this comparison will be critical in future studies analyzing the substrate sequence specificity for Rbd2.

5. Fig. 2EF and others: Many Sre1/2 blots lack control blots demonstrating equal loading of all lanes.

Given the large number of westerns in this study, it would be impractical to repeat all of these experiments. To demonstrate equal loading on our western blots, we included loading controls either by showing a larger area of the original Li-COR images that contained nonspecific bands (Figure 2F, 3C) or probing the blots for additional proteins (Dsc5 for Figure 2E and Flag for Figure 7H).

6. The authors stress the "structural" similarity between the ERAD E3 ligase gp78 and the Dsc complex. However, there is no evidence for such a similarity, as the structure of neither E3 has been solved yet. Moreover, Zhang et al (MBoC 26, 4439-4450; 2015) recently showed that gp78 has a role downstream of Hrd1 in mammalian ERAD, also putting into question a potential similarity with the Dsc complex.

We apologize for any confusion. We intended to convey that there is conservation in the overall architecture between the Dsc E3 complex and gp78 complex (Lloyd et al, 2013). Dsc1(E3 ligase)-Dsc2(UBA)-Dsc5(UBX) form a complex resembling gp78(E3)-UBAC2(UBA)-UBXD8(UBX). We clarified this in the revised manuscript.

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2nd Editorial Decision 02 August 2016

Thank you for submitting your revised manuscript for our consideration. It has now been assessed once more by the three original referees, whose comments are copied below. As you will see, they all consider the study significantly improved and now in principle suitable for publication in The EMBO Journal. Still, referees 1 and 3 retain several concerns based in part on the outcome and interpretation of the revision experiment, which I would kindly ask you to address through an additional round of minor revision. These additional modifications to the text and some of the figures should not require further experiments, but it will be important to carefully respond to the reviewer's points and to more carefully state/qualify various conclusions and assertions throughout the manuscript.

In addition, I would also like to ask you to carefully revisit all figure panels showing blots/gels/autoradiographs. We noted that they generally suffer from contrast/brightness over-adjustments that makes the background appear almost flat, and in order to allow their proper inspection by readers, it will be important to reassemble these figures with panels that more closely retain a relation to the original scans/blots. Alternatively, you may want to upload the original scans as 'figure source data' files with the revision; we would ask for a single PDF/JPG/GIF file per figure comprising the original, uncropped and unprocessed scans of the gel/blot panels used in the respective figures. These should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files would be linked as 'source data' to the respective figures in the online publication of your article.

Finally, when revising the text, please make sure to reference each of the two Appendix Tables S1 and S2 at least once in the main manuscript (probably in the methods section).

In order to prepare and upload this final modifications, I am returning the manuscript to you once more for minor revisions. Once we will have received the final version and files, we should be able to swiftly proceed with formal acceptance and publication of the study in The EMBO Journal!

REFEREE COMMENTS

Referee #1:

In their revised version, Hwang et al. have amended their original manuscript and provide important additional data. These include a classical co-immunoprecipitation-based substrate-trapping assay using catalytically inactive Rbd2, which now provides evidence that Rbd2 directly interacts with SREBP substrate. The authors have added new data showing that overexpression of a Cdc48-binding defective mutant of Rbd2 bypasses the requirement of Cdc48 for SREBP cleavage by Rbd2. The authors also show that the second step of SREBP activation is mediated by a protease different from SPP. Unfortunately, an additional mutational analysis of target residues for SREBP cleavage that were predicted from the cleavage site analysis of TatA in the original manuscript did, however, not fit to their initial hypothesis. Likewise, direct evidence for SREBP ubiquitinylation could also not be achieved yet.

The new data are logically incorporated into the flow of the story and make the manuscript stronger now also providing a clearer advance over the previously published studies. I appreciate that the authors now also better explain the different functional roles of Cdc48 in ERAD by RHBDL4 (cleavage product extraction) and SREBP activation by Rbd2 (substrate recruitment) in the revised version.

As detailed in the following, I have a few questions and comments related to the author´s response and the new data coming in with this revision:

1) Addressing my previous first and second points, the authors failed to demonstrate that the unique and unusual feature of Rbd2 for a rhomboid protease of cleaving after large amino acids observed toward its non-physiological TatA substrate is transferable to its fission yeast SREBP substrates.

Unfortunately, determination of cleavage sites of SREBPs was not possible and the deeper mutational analysis presented in Figure 5 now showed that none of the large amino acids in the target region within and N-terminal to TM2 are required for SREBP cleavage by Rbd2. Only one mutant, and extremely intriguing, a conservative lysine to arginine substitution, blocked substrate cleavage by Rbd2.

Although the authors have thus removed their previous conclusion from the abstract, I would still recommend that they also reword a few sentences, which still read like quite definite general statements. Since cleavage after large residues is only demonstrated for TatA substrate cleavage, sentences such as "Rbd2 is an active rhomboid protease with unique sequence specificity" (Heading of Figure 4 legend) or "As such, Rbd2 is an active rhomboid intramembrane protease that cleaves preferentially after large hydrophobic residues" (In the Results) are, at the current stage of research, overstatements.

2) Addressing my previous third point, the authors show that the fission yeast SPP homolog is not involved in SREBP processing demonstrating that the liberation of the N-terminal transcription factor domain is mediated by a yet to be discovered protease acting as a mammalian S2P. This new finding is compared to recent findings obtained by others for Aspergillus, where a SPP homolog mediates the second cleavage. I appreciate that the authors have done this experiment as it brings more clarity regarding the identity of the players involved and not involved in fission yeast SREBP processing.

3) Addressing my previous fourth point, the authors state in their response letter that they fail to show by various approaches that SREBP is indeed ubiquitinylated. Although the genetic evidence strongly suggests SREBP ubiquitinylation, direct evidence is thus still missing. I therefore suggest slight rewording of sentences such as "DSC E3 ligase ubiquitinylates SREBP" in the abstract and other places. It would also be good to mention in the manuscript that based on the authors´ mutational analysis outlined in their response letter, ubiquitinylation of SREBP might involve non-classical sites.

4) In the abstract and other places in the manuscript, it should be more precisely stated that it is the Cdc48-binding defective mutant of Rbd2 (and not wt Rbd2), which bypasses the requirement of Cdc48 for SREBP cleavage. In this regard, is it really clear that this finding "demonstrates" that Cdc48 is involved in the substrate-recruitment pathway? Could this finding on the contrary not also be taken as an argument that Cdc48 is not required for substrate recognition (Overexpressed mutant Rbd2 that cannot bind Cdc48 can still cleave SREBP)? (See also point 10).

5) In the new figure 5B, it is stated that all mutants are cleaved normally, however, cleavage of L754A is apparently enhanced. It may be also worthwhile to write a few words about the behavior of S751L mutant, as the small serine residue fits to the typical sequence requirement for rhomboid cleavage.

6) In the new Fig. 5C, a longer exposure should be shown to clearly see processing in lanes 1 and 2 for the R753L mutant of Sre2MS.

7) The description of the new data presented in Fig. 5 in the Results regarding the categorization of the 19 mutants is difficult to understand. In particular the explanations for the expected results regarding precursor accumulation of mutant substrate in the rbd2Δ and, more importantly, in the dsc1Δ background are not easy to understand. I recommend including an accompanying graphics here illustrating the expected processing and degradation pathway of wt and mutant SREBP including ubiquitination-state and fate of cleavage product and precursor in the rbd2Δ and dsc1Δ background. For an easy overview, prior to the more complicated Figure 5F, it might also be good to include a simple figure showing the cleavage efficiency of all mutants tested compared to wt (N/P ratios). Finally, on page 16, at the end of the chapter describing the mutational analysis presented in Fig. 5, an overall conclusion of the mutational analysis should be given.

8) In Fig. 6A, the asterisk is supposed to mark identical residues in the SHP domain of the proteins shown. Note that there is an error for the second to last position. The glycine residues for p47, Dfm1 and Rbd2 are not identical in all four proteins aligned. It is replaced by an aspartate in UFD1.

9) On page 19, the statement that the failure to detect a SREBP cleavage product for rbd2-G246R mutant might potentially also be due to improper degradation of a cleaved product is confusing. If there was an improper degradation of the cleavage product, it should be detectable.

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12) On page 12, is stated that codon-optimized is expressed 35-fold higher than normal 3xHA-rbd2. How is this quantified if there is no detectable expression of 3xHA-rbd2 as the authors state?

Minor points:

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- In Fig. 10, to avoid confusion it would be good to indicate that "U" can be monoubiquitin (likely in case of SREBP as Rbd2 substrate) or polyubiquitin in case of SREBP as proteasome substrate.

- In Appendix Fig. S2, the quantitations for WT Sre2MS are missing.

In conclusion, this work provides important novel information of how fission yeast SREBPs are recruited to Rbd2. Addressing the few remaining points coming with this revision should be straightforward and finally make the manuscript suitable for publication in EMBO J.

Referee #2:

I was positive about this manuscript in the first round, and my favorable opinion has only increased with revision. The authors have gone to considerable lengths to improve the manuscript and address all of the referee comments. I am fully supportive of its publication in EMBO.

Referee #3:

In their revised manuscript, Hwang et al. satisfactorily addressed all questions/concerns that had been listed under "further points". However, a remaining weakness of their study is the lack of mechanistic insights into the role of Cdc48. The model shown in new Fig. 10 contains several speculative elements that are not backed up by the data:
First, despite circumstantial evidence from the analysis of Dsc mutants, there is so far no proof that the Sre1/2 precursor is in fact ubiquitinylated. The authors´ experiments employing proteasome inhibitor (this study) or Cdc48 mutants (Stewart et al, JBC 2012) do not support this point, as no accumulation of ubiquitinylated Sre1/2 precursor was observed under either condition.
Second, the existence and nature of a Sre1/2 cleavage intermediate have not been tested and hence are highly speculative at this point.
Third, and most importantly, the authors fail to provide a convincing explanation for the Cdc48 requirement in this pathway. They claim that Cdc48 functions as an "adaptor" to "recruit" Sre1/2, mainly based on the overexpression experiments shown in new Fig. 9. However, they should note that their conclusion (high levels of Rbd2-G246R bypass the need for the adaptor Cdc48) is just one possible interpretation of their results. The data are equally consistent with the possibility that overexpression drives Rbd2-G246R, by the law of mass action, into a weak/transient interaction

with Cdc48 that is sufficient for cleavage to occur. The weak but detectable residual binding of Rbd2-G246R to Cdc48 seen in Figs. 7AC and 9D actually supports this latter possibility. In order to formally prove that Cdc48 activity is not required for Sre1/2 cleavage upon overexpression of Rbd2, the Rbd2-G246R variant would have to be overexpressed in a Cdc48 mutant background.
The function of the ATPase Cdc48 typically goes beyond mere recruitment and has been described as "segregase" activity by the Jentsch lab. Could the authors come up with some related functions of Cdc48 in Sre1/2 cleavage? E.g., could Cdc48 free Sre1/2 from binding partners preventing cleavage? Or (partially) dislocate Sre1/2 in order to expose the cleavage site(s) to Rbd2? At any rate, a more meaningful model and an in-depth discussion of its mechanistic aspects are required. (Minor point: Cdc48 has been shown by the Espenshade lab to interact with Dsc5 - why not show this in the model?)

2nd Revision - authors' response 31 August 2016

Response to Reviewers

We thank the reviewers for their time, supportive comments, and helpful suggestions that further improved the manuscript. We address both general and specific comments below.

Significant changes to the manuscript include:

1. To improve the description of the results in Fig 5 as recommended by Reviewer 1, we added a model (new Fig 5C), and now present the data in old Fig 5F as two histogram figures (new Fig 5G and 5H). In addition, we reordered the Fig 5 panels to improve clarity.

2. In response to the Reviewers and Editor, we edited the images in the figures listed below to display more background. Please note that these digital images were acquired using a LI-COR Odyssey CLx imager. Since signals for these experiments were acquired in the linear range of this instrument, the "exposure" does not affect quantification.

Figure 2B, C, D, E, G
Figure 3B, D
Figure 8B, C, F, H
Figure 9A
Figure EV1A, B
Figure EV3
Figure EV4

3. In response to a comment from Reviewer 1, we include a new Fig S1A that directly compares cleavage of Sre2MS mutants in the second transmembrane segment.

Reviewer Comments

Referee #1:

In their revised version, Hwang et al. have amended their original manuscript and provide important additional data. These include a classical co-immunoprecipitation-based substrate-trapping assay using catalytically inactive Rbd2, which now provides evidence that Rbd2 directly interacts with SREBP substrate. The authors have added new data showing that overexpression of a Cdc48-binding defective mutant of Rbd2 bypasses the requirement of Cdc48 for SREBP cleavage by Rbd2. The authors also show that the second step of SREBP activation is mediated by a protease different from SPP. Unfortunately, an additional mutational analysis of target residues for SREBP cleavage that were predicted from the cleavage site analysis of TatA in the original manuscript did, however, not fit to their initial hypothesis. Likewise, direct evidence for SREBP ubiquitinylation could also not be achieved yet.

The new data are logically incorporated into the flow of the story and make the manuscript stronger now also providing a clearer advance over the previously published

studies. I appreciate that the authors now also better explain the different functional roles of Cdc48 in ERAD by RHBDL4 (cleavage product extraction) and SREBP activation by Rbd2 (substrate recruitment) in the revised version.

As detailed in the following, I have a few questions and comments related to the author´s response and the new data coming in with this revision:

1) Addressing my previous first and second points, the authors failed to demonstrate that the unique and unusual feature of Rbd2 for a rhomboid protease of cleaving after large amino acids observed toward its non-physiological TatA substrate is transferable to its fission yeast SREBP substrates. Unfortunately, determination of cleavage sites of SREBPs was not possible and the deeper mutational analysis presented in Figure 5 now showed that none of the large amino acids in the target region within and N-terminal to TM2 are required for SREBP cleavage by Rbd2. Only one mutant, and extremely intriguing, a conservative lysine to arginine substitution, blocked substrate cleavage by Rbd2.

Although the authors have thus removed their previous conclusion from the abstract, I would still recommend that they also reword a few sentences, which still read like quite definite general statements. Since cleavage after large residues is only demonstrated for TatA substrate cleavage, sentences such as "Rbd2 is an active rhomboid protease with unique sequence specificity" (Heading of Figure 4 legend) or "As such, Rbd2 is an active rhomboid intramembrane protease that cleaves preferentially after large hydrophobic residues" (In the Results) are, at the current stage of research, overstatements.

We thank the Reviewer for noting these two statements that we overlooked in the revised version of the manuscript. The legend to Figure 4 (p. 38) now reads, “Rbd2 is an active rhomboid protease.”

In addition, we edited the following sentences on p. 14 in the Results section:
“As such, Rbd2 is an active rhomboid intramembrane protease that cleaves preferentially after large hydrophobic residues. This unique substrate sequence preference has never been observed for any rhomboid protease.”

Now reads:
“As such, Rbd2 is an active rhomboid intramembrane protease that can cleave after large hydrophobic residues. This unique property has not been observed for any rhomboid protease.”

Finally, we edited the following sentence on p. 21 in the Discussion:
“Although Rbd2 cleavage of TatA required substrate helix-destabilizing residues, Rbd2 preferentially cleaved TatA substrates after large hydrophobic side chains (Leu and Phe) (Fig 4F), revealing a unique substrate sequence preference not observed for any other rhomboid protease.

Now reads:
“Although Rbd2 cleavage of TatA required substrate helix-destabilizing residues, Rbd2 preferentially cleaved TatA substrates after large hydrophobic side chains (Leu and Phe) (Fig 4F), revealing a unique property not observed for any other rhomboid protease.”

2) Addressing my previous third point, the authors show that the fission yeast SPP homolog is not involved in SREBP processing demonstrating that the liberation of the N-terminal transcription factor domain is mediated by a yet to be discovered protease acting as a mammalian S2P. This new finding is compared to recent findings obtained by others for Aspergillus, where a SPP homolog mediates the second cleavage. I appreciate that the authors have done this experiment as it brings more clarity regarding the identity of the players involved and not involved in fission yeast SREBP processing.

3) Addressing my previous fourth point, the authors state in their response letter that they fail to show by various approaches that SREBP is indeed ubiquitinylated. Although the genetic evidence strongly suggests SREBP ubiquitinylation, direct evidence is thus still missing. I therefore suggest slight rewording of sentences such as "DSC E3 ligase ubiquitinylates SREBP" in the abstract and other places. It would also be good to mention in the manuscript that based on the authors´ mutational analysis outlined in their response letter, ubiquitinylation of SREBP might involve non-classical sites.

Thank you for the suggestions. We edited the Abstract as suggested and qualified our statements throughout the manuscript. In the absence of *rbd2*, SREBPs are degraded by the proteasome in a Dsc-dependent manner, and the most likely mechanism is through Dsc ubiquitinylation of SREBP. Given this fact, the results in Figure 5 are most easily described by presuming Dsc-dependent ubiquitinylation. A major open question is where SREBP is ubiquitinylated. So as not to mislead the reader and to note that Dsc-dependent ubiquitinylation may involve non-classical sites, we edited the Discussion on p. 25 as follows:

“Although SREBP ubiquitinylation remains to be shown, these data provide additional support for SREBPs as Dsc E3 ligase substrates. Interestingly, preliminary studies indicate that cytosolic lysines in Sre2MS are not required for cleavage, suggesting that SREBP ubiquitinylation may involve non-classical sites.”

4) In the abstract and other places in the manuscript, it should be more precisely stated that it is the Cdc48-binding defective mutant of Rbd2 (and not wt Rbd2), which bypasses the requirement of Cdc48 for SREBP cleavage. In this regard, is it really clear that this finding "demonstrates" that Cdc48 is involved in the substrate-recruitment pathway? Could this finding on the contrary not also be taken as an argument that Cdc48 is not required for substrate recognition (Overexpressed mutant Rbd2 that cannot bind Cdc48 can still cleave SREBP)? (See also point 10).

As suggested, changes were made to the Abstract (p. 2) and Results (p. 19). We understand the Reviewer's point that these data could support an argument that Cdc48 is not required for substrate recognition. However, this is only seen under conditions in which Rbd2 is overexpressed.

5) In the new figure 5B, it is stated that all mutants are cleaved normally, however, cleavage of L754A is apparently enhanced. It may be also worthwhile to write a few words about the behavior of S751L mutant, as the small serine residue fits to the typical sequence requirement for rhomboid cleavage.

Cleavage of Sre2MS L574A is not elevated compared to wild-type Sre2MS. We added a new Fig S1A that directly compares two isolates for each mutant.

6) In the new Fig. 5C, a longer exposure should be shown to clearly see processing in lanes 1 and 2 for the R753L mutant of Sre2MS.

As noted in the introduction to this Response, we adjusted the contrast of several images to the same degree. There is simply very low cleavage for the R753L mutant (now Fig 5E).

7) The description of the new data presented in Fig. 5 in the Results regarding the categorization of the 19 mutants is difficult to understand. In particular the explanations for the expected results regarding precursor accumulation of mutant substrate in the rbd2Δ and, more importantly, in the dsc1Δ background are not easy to understand. I recommend including an accompanying graphics here illustrating the expected processing and degradation pathway of wt and mutant SREBP including ubiquitination-state and fate of cleavage product and precursor in the rbd2Δ and dsc1Δ background. For an easy overview, prior to the more complicated Figure 5F, it might also be good to include a simple figure showing the cleavage efficiency of all mutants tested compared to wt (N/P ratios). Finally, on page 16, at the end of the chapter describing the mutational analysis presented in Fig. 5, an overall conclusion of the mutational analysis should be given.

We thank the Reviewer for this helpful suggestion that improved the explanation of these results. We now include a new Fig 5C, split the old Fig 5F into new Figs 5G and 5H histograms, and reordered other panels.

Finally, we added a summary sentence to this section of results on p. 16:
"This extended mutagenesis study demonstrates that Sre2MS cleavage does not require large hydrophobic residues in the second transmembrane segment, but identifies a luminal lysine residue required for cleavage."

8) In Fig. 6A, the asterisk is supposed to mark identical residues in the SHP domain of the proteins shown. Note that there is an error for the second to last position. The glycine residues for p47, Dfm1 and Rbd2 are not identical in all four proteins aligned. It is replaced by an aspartate in UFD1.

We thank the Reviewer for catching this error. The two symbols had shifted to the right. This has been corrected.

9) On page 19, the statement that the failure to detect a SREBP cleavage product for rbd2-G246R mutant might potentially also be due to improper degradation of a cleaved product is confusing. If there was an improper degradation of the cleavage product, it should be detectable.

We edited this to improve clarity. The sentence on p. 19 now reads:
“The inability to detect a cleavage product could be due to a failure to cleave SREBP, or alternatively a cleaved product may be degraded when Rbd2 fails to recruit Cdc48.”

10) Further on page 19, the rationale given for the overexpression experiment is difficult to understand: "Rhomboid proteases exhibit weak binding affinity for substrates (Dickey et al, 2013). Given that Cdc48 cofactors bind ubiquitin (Stolz et al, 2011), we hypothesized that Cdc48 plays a role in SREBP recognition. If this were true, we reasoned that increasing Rbd2 enzyme concentration would bypass the requirement for Cdc48." This should be better explained. The logic of these connections are not fully clear.

Thank you for the suggestion. The sentence on p. 19 now reads:
“Rhomboid proteases exhibit weak binding affinity for substrates (Dickey et al, 2013). Given that Cdc48 cofactors bind ubiquitin (Stolz et al, 2011), we hypothesized that Cdc48 plays a role in SREBP recognition by recruiting SREBP substrate to Rbd2 through cofactor binding. If this were true, we reasoned that increasing the concentration of Cdc48-binding defective Rbd2 enzyme would bypass the requirement for Cdc48.”

11) In the description of the results presented in Fig. 9 on page 20, the authors state Rbd2-APEX2 failed to detect an interaction with Cdc48 under endogenous conditions. This is confusing as in Fig. 7 binding under such conditions is shown. Further on page 20, for Fig. 9D it is also important to mention that Rbd2-S130A/G246R-APEX2 shows reduced levels of CDC48.

We edited the text to emphasize that while we detect an interaction by coIP (Fig 7A) we failed to see labeling using the proximity biotinylation assay (experiments not shown). The sentence now reads:
“Proximity biotinylation experiments using endogenous expression of Rbd2-APEX2

failed to detect an interaction with the known binding partner Cdc48, so we overexpressed Rbd2 fusion proteins using the *adh1*$^{+}$ promoter."

On p. 17, we also edited the following sentence to emphasize that the initial APEX experiment also used overexpressed Rbd2:
"To label proximal and interacting proteins of Rbd2 using APEX2 technology, we fused Flag-APEX2 to the C-terminus of Rbd2 and overexpressed the fusion protein from a plasmid in *rbd2*Δ cells."

In addition, we added a sentence describing the Cdc48 binding result:
"As expected, Rbd2-S130A/G246R-APEX2 showed reduced Cdc48 labeling (Fig 9D, lane 6)."

12) On page 12, is stated that codon-optimized is expressed 35-fold higher than normal 3xHA-rbd2. How is this quantified if there is no detectable expression of 3xHA-rbd2 as the authors state?

Thank you for noting this. Expression is detectable and the sentence now reads:
"Transfecting cells with *3xHA-rbd2* resulted in low levels of protein expression (Fig 4A, lane 2)."

Minor points:

- On page 11, eighth line from the bottom shouldn´t that read "Fig. 3 D, compare lanes 3 and 4"?

Yes, this has been corrected.

- In Fig. 10, to avoid confusion it would be good to indicate that "U" can be monoubiquitin (likely in case of SREBP as Rbd2 substrate) or polyubiquitin in case of SREBP as proteasome substrate.

The legend to Fig 10 now reads, "U, either mono-ubiquitin or poly-ubiquitin."

- In Appendix Fig. S2, the quantitations for WT Sre2MS are missing.

The results for wild-type Sre2MS are located in the top left corner of Fig. S2, labeled Sre2MS. For clarity, we changed the heading to WT.

In conclusion, this work provides important novel information of how fission yeast SREBPs are recruited to Rbd2. Addressing the few remaining points coming with this

revision should be straightforward and finally make the manuscript suitable for publication in EMBO J.

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Second, the existence and nature of a Sre1/2 cleavage intermediate have not been tested and hence are highly speculative at this point.

We agree with the Reviewer that gaps in our knowledge of this pathway exist. We edited the manuscript where possible so as not to imply that SREBP is known to ubiquitinylated. In addition, we now state explicitly in the Discussion that this is not known (p. 25):
“Although SREBP ubiquitinylation remains to be shown, these data provide additional support for SREBPs as Dsc E3 ligase substrates.”

Regarding Fig 10, we feel that it is useful to include a model for future testing that summarizes our data and hypotheses. We now introduce this model as follows (p. 26):
“Combined with our past work, these new findings lead us to speculate on a new model for activation of Sre1 (Fig 10).”

Third, and most importantly, the authors fail to provide a convincing explanation for the Cdc48 requirement in this pathway. They claim that Cdc48 functions as an "adaptor" to "recruit" Sre1/2, mainly based on the overexpression experiments shown in new Fig. 9. However, they should note that their conclusion (high levels of Rbd2-G246R bypass the need for the adaptor Cdc48) is just one possible interpretation of their results. The data are equally consistent with the possibility that overexpression drives Rbd2-G246R, by the law of mass action, into a weak/transient interaction with Cdc48 that is sufficient for

cleavage to occur. The weak but detectable residual binding of Rbd2-G246R to Cdc48 seen in Figs. 7AC and 9D actually supports this latter possibility. In order to formally prove that Cdc48 activity is not required for Sre1/2 cleavage upon overexpression of Rbd2, the Rbd2-G246R variant would have to be overexpressed in a Cdc48 mutant background.

We thank the Reviewer for noting this alternative explanation of the experiments in Fig 9 that employ a single amino acid mutant. Unfortunately, we are unable to perform the proposed experiment because Cdc48 function is required for efficient ER exit of the Dsc E3 ligase complex (unpublished data), and this will complicate the interpretation of the results.

As an alternative, we tested whether overexpression of *rbd2*Δ*SHP* that lacks the entire conserved SHP box sequence (aa 242-251) can bypass the Cdc48 requirement for SREBP cleavage. Indeed, this overexpressed Rbd2 mutant was expressed at wild-type levels and rescued Sre1 cleavage in *rbd2*Δ cells (Fig R1 below).

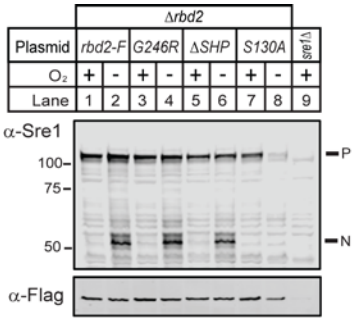

Figure R1 – Overexpression of Rbd2 bypassess requirement for Cdc48 binding to Rbd2.
Western blot of phosphatase-treated whole cell lysates from yeast strains carrying *rbd2-Flag-APEX2* (*rbd2-F*), *rbd2-G246R-Flag-APEX2* (*G246R*), *rbd2-ΔSHP-Flag-APEX2* (*ΔSHP*) or *rbd2-S130A-Flag-APEX2* (*S130A*) plasmid in *rbd2Δ* background and *sre1Δ* yeast was probed with anti-Sre1 or anti-Flag IgG. P and N denote Sre1 precursor and cleaved nuclear forms, respectively.

The function of the ATPase Cdc48 typically goes beyond mere recruitment and has been described as "segregase" activity by the Jentsch lab. Could the authors come up with some related functions of Cdc48 in Sre1/2 cleavage? E.g., could Cdc48 free Sre1/2 from binding partners preventing cleavage? Or (partially) dislocate Sre1/2 in order to expose the cleavage site(s) to Rbd2? At any rate, a more meaningful model and an in-depth discussion of its mechanistic aspects are required. (Minor point: Cdc48 has been shown by the Espenshade lab to interact with Dsc5 - why not show this in the model?)

As noted by the Reviewer, aspects of the model presented in Fig 10 are speculative. Although there are potential additional roles for Cdc48, we hesitate to comment in the absence of experimental data. Finally, we do not show Cdc48 binding to Dsc5 UBX domain because this binding is not required for SREBP cleavage and so as not to confuse the reader.

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Corresponding Author Name: Peter Espenahde
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Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- 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.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ 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?
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  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	We chose to perform at least three biological replicates for each western blot experiment, binding analysis, APEX analysis.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	N/A
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	N/A
For animal studies, include a statement about randomization even if no randomization was used.	N/A
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.	N/A
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	Yes. (Statistical test was only performed in Figure 9)
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Normal distribution. We used student's t-test for statistical analysis.
Is there an estimate of variation within each group of data?	Yes.
Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

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http://www.selectagents.gov/

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).	Yes. We provide the information for the reagents.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	N/A

* for all hyperlinks, please see the table at the top right of the document

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.	N/A
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N/A
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.	N/A

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
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.	N/A

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under '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	N/A
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).	N/A
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).	N/A
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	N/A
22. 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.	N/A

G- Dual use research of concern

23. 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.	N/A