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Cullin neddylation and substrate-adaptors counteract SCF inhibition by S. cerevisiae Lag2, an orthologue of human Cand1

Edyta Siergiejuk, Daniel Scott, Brenda Schulman, Kay Hofmann, Thimo Kurz, Matthias Peter

Corresponding author: Matthias Peter, ETH Zurich

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Transaction Report:

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

1st Editorial Decision 24 September 2009

Thank you for submitting your manuscript for consideration by the EMBO Journal. I have now received the reports back from three referees. As we discussed, I tried to get the same reviewers as had seen the manuscript from the Kamura lab; only two of them were able to review your study (#s 1 and 2), and I therefore also involved a third, new reviewer.

As you will see, referees 1 and 2 are broadly positive, although referee 1 in particular raises a number of issues with the study in its current form. Referee 3, on the other hand, finds that your work is primarily confirmatory of what is already known about CAND1 in mammalian systems, and is therefore not supportive of publication. Given that refs 1 and 2 are those who also reviewed the Kamura lab study, and in the interests of consistency, we are prepared to overlook the more negative assessment of referee 3, and to invite you to submit a revised version of your manuscript.

All three referees do raise a number of concerns that it would be valuable to address by additional experiments. We would (as I'm sure would you) like to get the paper published in 2009. We should be able to make a final decision without sending the revised version out for re-review, and so a revision received by the end of October/beginning of November should be early enough to make it into the final 2009 issue. I suggest that the best way forward would be for you to go through the reviewers' comments with your co-workers, and then - if necessary - we could discuss how best to proceed. I would like to stress that it will be important to respond to all the comments raised, even if the suggested experiments can not be performed within this time-frame. Please just let me know if you have any questions about this.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Best wishes,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Cullin neddylation and substrate-adaptors counteract SCF inhibition by S. cerevisiae Lags, an orthologue of human Cand1

This manuscript identifies an SCF-interacting protein, Lag2 that is a CAND1 orthologue in yeast S. cerevisiae. Lag2 was initially identified as a longevity factor, directly interacts with the non neddylated form of cullin, Cdc53. This interaction requires the conserved region between Lag2 and Cand1. Lag2 over expression counteracts SCF function in vivo in a neddylation-dependent manner and prevents Cdc53 neddylation in vitro. Lag2 is also partially neddylated in vivo on a lysine residue adjacent to Cdc53 binding site. Lag2 is released from Cdc53 in the presence of substrate-specific adaptors, Skp1, which allow subsequent neddylation of Cdc53. Overall the authors identify a Cand1 orthologue in yeast and show that Lag2 is a regulator of SCF neddylation cycle. This paper answer some aspects of Lag2 and its modification in SCF neddylation cycle and is therefore of interest to general readers.

Specific comments:

The author show that Skp1 adaptor counteract Lag2 function by releasing Lag2 from Cdc53, thus allowing cullin neddylation. If that is the case, immunoprecipitation of Lag2 would pull down unmodified Cdc53 (which they see), but no (or little) Skp1 adaptor protein. In particular when Lag2 is overexpressed, Cdc53 bound to Lag2 should be free of any Skp1. The authors need to show that Lag2 and Skp1 binding to Cdc53 are mutually exclusive to propose that binding of adaptor protein triggers the release of Cand1/Lag2.

Lag2 over-expression in rub1Δ partially displays phenotype similar to cdc53-1 mutant (2D). What is the phenotype when Lag2 is overexpressed in a rub1Δ dcn1Δ double null? Since both proteins contribute towards neddylation of Cdc53, phenotype resembling cdc53-1 (in % cell with elongated buds) would further support the role of Lag2 in inhibiting SCF complex in a neddylation-dependent manner.

Even with drastic over expression of Lag2 (figure 2 and Lag2 western in 2E), the change in steady state Sic1 level is at best marginal. Also, the levels of Sic1 is not much different in Lag2 vs. Lag2GN(551) (lanes 2 vs. 3 and 5 vs. 6). Providing quantification after normalization would be useful. It might be more direct to look at Sic1 ubiquitination levels (with MG132) in WT or rub1Δ with and without Lag2 over expression.

Does Lag2 stoiciometrically bind unmodified Cdc53-Hrt1 when they are over expressed in E coli and purified? It will be informative to see the coomassie gel of the purified trimeric complex.

How are the neddylation reactions in Figures 3 and 6 normalized for loading?

On page 7, there is an error that says G551A, N551A instead of N552A.

Referee #2 (Remarks to the Author):

Through sequence analysis and a series of biochemical studies, Siergiejuk et al. have identified in this manuscript the yeast Lag2 protein as the long missing functional orthologue of human Cand1, a regulator of the cullin-RING E3 ubiquitin ligase complexes. Despite significant sequence diversity, the authors were able to pin down Lag2 as the yeast Cand1 orthologue by focusing on the functionally important regions of Cand1 sequence. This effort is remarkable and can be a great lesson for a general audience. For the rest of the paper, most of the conclusions derived from these studies, including their final model, are simply consistent with the current understanding of the cullin assembly cycle. The story would be more exciting if it helps solve several remaining key issues. For example, what role does Dcn1Δ plays in the cycle presented in Figure 7? How does the neddylation system cooperate with Cop9 to ensure the right timing of the assembly steps in Figure 7?

Referee #3 (Remarks to the Author):

The paper by Siergiejuk et al. describes the identification of Lag2 as an orthologue of CAND1 in budding yeast. The authors have shown that Lag2 is conjugated with Nedd8/Rub1Δ though the biological significance of this modification remains to be elucidated. Moreover, the authors provided in vivo and in vitro data supporting the previous claim by Hershko in 2006 that interaction between Skp1/F-box protein and Cullin 1 (Cdc53)/Rbx1 (Hrt1) appears to displace Lag2/CAND1, thereby promoting neddylation.

While this work is solid providing strong evidence for Lag2 acting as CAND1, it, however, lacks novelty at levels required for publication at EMBO. It should be noted that Lag2 neddylation is very low and its significance remains elusive. On the mechanistic level, this work is in strong support of the Hershko model that binding of the substrate-specific adaptors triggers CAND1/Lag2 disassociation. However, it does not provide additional insights into this displacement reaction.

One comment on Fig. 6: Western analysis should be shown to monitor the concomitant association of Skp1/Cdc4 and displacement of Lag2.

1st Revision - authors' response 01 November 2009

We were happy to learn that you and reviewers 1 and 2 support publication of a revised version of our manuscript EMBOJ-2009-72423R entitled " Cullin neddylation and substrate-adaptors counteract SCF inhibition by the CAND1-like protein Lag2 in S. cerevisiae" in EMBO Journal.

In this paper, we not only describe the identification and characterization of a CAND1-orthologue in S. cerevisiae, but also suggest an order of events leading to the activation of cullin-based E3-ligases. Specifically, the presence of substrate-specific adaptors initiate the release of Cand1/Lag2 from the cullin, while subsequent neddylation of the cullin, and perhaps Lag2 itself, facilitates the removal and prevents re-association of Lag2/Cand1. In contrast to reviewer 3, we are convinced that these results strongly advance our understanding of how Cullin/Cand1 complexes are regulated in vivo. We are also confident that the identification of Lag2 as budding yeast Cand1 will lead to further advances in the field by allowing the use of S. cerevisiae as simple model organism to study this process. Finally, as Lag2 was originally identified as a regulator of longevity in yeast, it is possible that Lag2, and perhaps Cand1-like activities, may also regulate targets other than cullin-based E3-ligases, as no cullin ligase has so far been implied in yeast ageing.

In the revised version, we have now addressed all criticism raised by the reviewers, and included several new experiments in the revised Figures, and changed the text according to the suggestions. In particular, we now show that Lag2 co-immunoprecipitates with un-neddylated Cdc53, but not with Skp1 (new Fig. 1B). Conversely, Cdc53 but not Lag2 is present in Skp1 immunoprecipitates (new Suppl. Fig. 2B). Moreover, we have further analyzed a potential role of Dcn1Δ in the release of Lag2. First, we do not find an interaction of Dcn1Δ and Lag2 in vivo or in vitro (new Fig. 1B, and Suppl. Fig. 6A), and addition of Dcn1Δ does not alter the activity of Skp1/Cdc4 to remove Lag2

from Cdc53 (Suppl. Fig. 6A). These results are complemented by genetic data suggesting that in contrast to skp1-12 cells, deletion of Lag2 does not restore Cdc53 neddylation in dcn1Δ cells (Suppl. Fig. 6B). Finally, as requested by reviewer 1, we now analyzed the rub1Δ dcn1Δ double mutant, and as expected the results demonstrate that Dcn1Δ and Rub1Δ counteract Lag2 function in vivo by promoting Cdc53-neddylation (new Fig. 2A and 2D). Together, these results strongly suggest that the Skp1/Cdc4 complex and Cdc53 neddylation cooperate to remove bound Lag2 and thus promote activation of the SCF E3 ligase. A detailed point-by-point reply to all reviewer's comments is attached below.

We would like to thank you and the reviewers for their valuable input and are confident that the final manuscript is now ready for publication in EMBO Journal. Please do not hesitate to contact us if you have any additional concerns or questions.

Point-by-point response to the reviewers' comments:

Referee 1:

1. The author show that Skp1 adaptor counteracts Lag2 function by releasing Lag2 from Cdc53, thus allowing cullin neddylation. If that is the case, immunoprecipitation of Lag2 would pull down unmodified Cdc53 (which they see), but no (or little) Skp1 adaptor protein. In particular when Lag2 is overexpressed, Cdc53 bound to Lag2 should be free of any Skp1. The authors need to show that Lag2 and Skp1 binding to Cdc53 are mutually exclusive to propose that binding of adaptor protein triggers the release of Cand1/Lag2.

As requested by the reviewer, we have now included co-immunoprecipitation experiments to analyze Lag2 complexes in vivo. While Lag2 co-immunoprecipitated un-neddylated Cdc53, we did not detect co-immunoprecipitation of endogenous Skp1 or Dcn1Δ with Lag2 (Figure 1B). Conversely, we were unable to detect Lag2 in Skp1 immunoprecipitates, while as expected Cdc53 is readily bound (Suppl. Fig. 2B). These new results suggest that endogenous Skp1 is not found in Cdc53/Lag2 complexes in vivo. However, we can detect a complex between Cdc53, Skp1 and Lag2 in vitro (Figure 1B for reviewers), implying that Lag2 can remain bound to Cdc53 even in the presence of Skp1. As depicted in the Figure 7, we thus favor a model in which Skp1/F-box and Cdc53 neddylation cooperate to fully remove Lag2. We have revised the text accordingly to better discuss this step.

2. Lag2 over-expression in rub1Δ ; partially displays phenotype similar to cdc53-1 mutant (2D). What is the phenotype when Lag2 is overexpressed in a rub1Δ ; dcn1Δ ; double null? Since both proteins contribute towards neddylation of Cdc53, phenotype resembling cdc53-1 (in % cell with elongated buds) would further support the role of Lag2 in inhibiting SCF complex in a neddylation-dependent manner.

As suggested by the reviewer, we have now compared overexpression of Lag2 in rub1Δ , dcn1Δ and rub1Δ dcn1Δ double mutants, both by serial dilution assays (new Figure 2A) and counting the number of cells with elongated buds (revised Figure 2D). As expected, deletion of Rub1Δ and Dcn1Δ are not additive, implying that they function in the same pathway. We have added these results and implications in the revised text.

3. Even with drastic over expression of Lag2 (figure 2 and Lag2 western in 2E), the change in steady state Sic1 level is at best marginal. Also, the levels of Sic1 is not much different in Lag2 vs. Lag2GN(551) (lanes 2 vs. 3 and 5 vs. 6). Providing quantification after normalization would be useful. It might be more direct to look at Sic1 ubiquitination levels (with MG132) in WT or rub1Δ ; with and without Lag2 over expression.

As requested, we have replaced the previous experiment with a new experiment, which more clearly shows the accumulation of Sic1 in cells overexpressing Lag2 (revised Figure 2E). Moreover, we have quantified and normalized Sic1 levels in all Lag2 overexpressing cells, and the analysis shows that Sic1 levels are 2.5 - 3 fold increased in rub1Δ cells overexpressing wild-type Lag2 but not the $Lag2^{GN(551)}$ mutant (Figure 2 for reviewers, result cited in revised text). As shown in Figure 2D, only about 40% of the cells arrest with a characteristic cdc53-phenotype, suggesting that the toxicity of Lag2 overexpression in neddylation-deficient cells may not solely be caused by inhibition of

Cdc53. We have now included these results and revised the text accordingly.

4. Does Lag2 stoiciometrically bind unmodified Cdc53-Hrt1 when they are over expressed in E coli and purified? It will be informative to see the coomassie gel of the purified trimeric complex.

A coomassie blue stained gel of the purified components expressed in Sf9 cells is shown in Suppl. Figure 3. Importantly, we now also included a titration experiment followed by detection of Cdc53/Hrt1 complexes using a non-denaturing gel. As expected, this analysis shows that purified Cdc53/Hrt1 forms a nearly 1 : 1 complex with Lag2 (Suppl. Figure 4). Finally, we attached to this letter a coomassie blue stained gel of the complex expressed in E. coli (Figure 1A for reviewers). It is clear that the preparations contain an additional protein, probably a chaperone that remains associated with the Cdc53/Hrt1 complex under all conditions.

5. How are the neddylation reactions in Figures 3 and 6 normalized for loading?

We have included a detailed protocol for these experiments in the revised Material and Methods section. Briefly, these experiments were performed with fully purified proteins (see Suppl. Figure 3), which were quantified spectroscopically and diluted from a master mix to which Skp1, Skp1-Cdc4, Lag2 etc. were added. Several independent experiments were performed and quantified with identical results.

6. On page 7, there is an error that says G551A, N551A instead of N552A.

Thanks - we have corrected this mistake.

Referee 2:

1) The story would be more exciting if it helps solve several remaining key issues. For example, what role does Dcn1Δ plays in the cycle presented in Figure 7? How does the neddylation system cooperate with Cop9 to ensure the right timing of the assembly steps in Figure 7?

As requested by the reviewer, we have carefully analyzed a potential role for Dcn1Δ in the cycle shown in Figure 7. Genetic evidence shown in Suppl. Figure 6B excludes a role of Dcn1Δ in the removal of Lag2 from Cdc53 in vivo. Supporting this finding, we added a new experiment demonstrating that addition of Dcn1Δ did not alter the ability of Lag2 to prevent neddylation of Cdc53 (Suppl. Figure 6B). Finally, we have also included additional data showing that Dcn1Δ fails to co-immunoprecipitate with Lag2 in vivo (revised Figure 1B), but may weakly bind to Cdc53/Hrt1/Lag2 complexes in vitro (Suppl. Figure 6A). Based on these results we conclude that Dcn1Δ primarily promotes neddylation of Cdc53 after removal of Lag2 by Skp1/Cdc4 (Figure 7). We have included a possible role of the COP9/signalosome in the inactivation of SCF complexes by deneddylating Cdc53 (Figure 7). As discussed in the revised text, this step is predicted to trigger a conformational change that may weaken the interaction with the Skp1/F-box complex and promote rebinding of Lag2.

Referee 3:

The reviewer confirms that the data presented in this paper are solid and provide strong evidence for Lag2 acting as CAND1, but feels that the findings lacks novelty at levels required for publication at EMBO. We strongly disagree with the latter statement, as we not only describe the identification and thorough characterization of a CAND1-orthologue in S. cerevisiae, but also suggest an order of events leading to the activation of cullin-based E3-ligases. Specifically, the presence of substrate-specific adaptors initiate the release of Cand1/Lag2 from the cullin, while subsequent neddylation of the cullin, and perhaps Lag2 itself, facilitates the removal and prevents re-association of Lag2/Cand1. While these conclusions are consistent with the cycle presented in the paper by Bornstein et al. (2006), our data provide for the first time strong in vivo evidence for this mechanism. We agree that the physiological role of Lag2/CAND1 remains to be investigated. However, we are confident that the identification of Lag2 as budding yeast Cand1 will now open the possibility to study this process using S. cerevisiae as simple model organism. For example, Lag2 was originally identified as a regulator of longevity in yeast, and our discoveries now suggest that

cullin-based E3-ligases or perhaps other targets may be involved in ageing.

1) One comment on Fig. 6: Western analysis should be shown to monitor the concomitant association of Skp1/Cdc4 and displacement of Lag2.

See also response to point 1 by reviewer 1. As requested, we have now included co-immunoprecipitation experiments to analyze Lag2 complexes in vivo. In contrast to un-neddylated Cdc53, we did not detect specific co-immunoprecipitation of endogenous Skp1 or Dcn1Δ with Lag2 (Figure 1B). Conversely, we are unable to detect Lag2 in Skp1 immunoprecipitates, while as expected Cdc53 is readily bound. These new results suggest that endogenous Skp1 is not found in Cdc53/Lag2 complexes in vivo. However, we can detect a complex between Cdc53, Skp1 and Lag2 in vitro (Figure 1B for reviewers), implying that Lag2 can remain bound to Cdc53 even in the presence of Skp1. As depicted in Figure 7, we thus favor a model in which Skp1/F-box and Cdc53 neddylation cooperate to fully remove Lag2. We have revised the text accordingly to better discuss this step.

Figure 1.

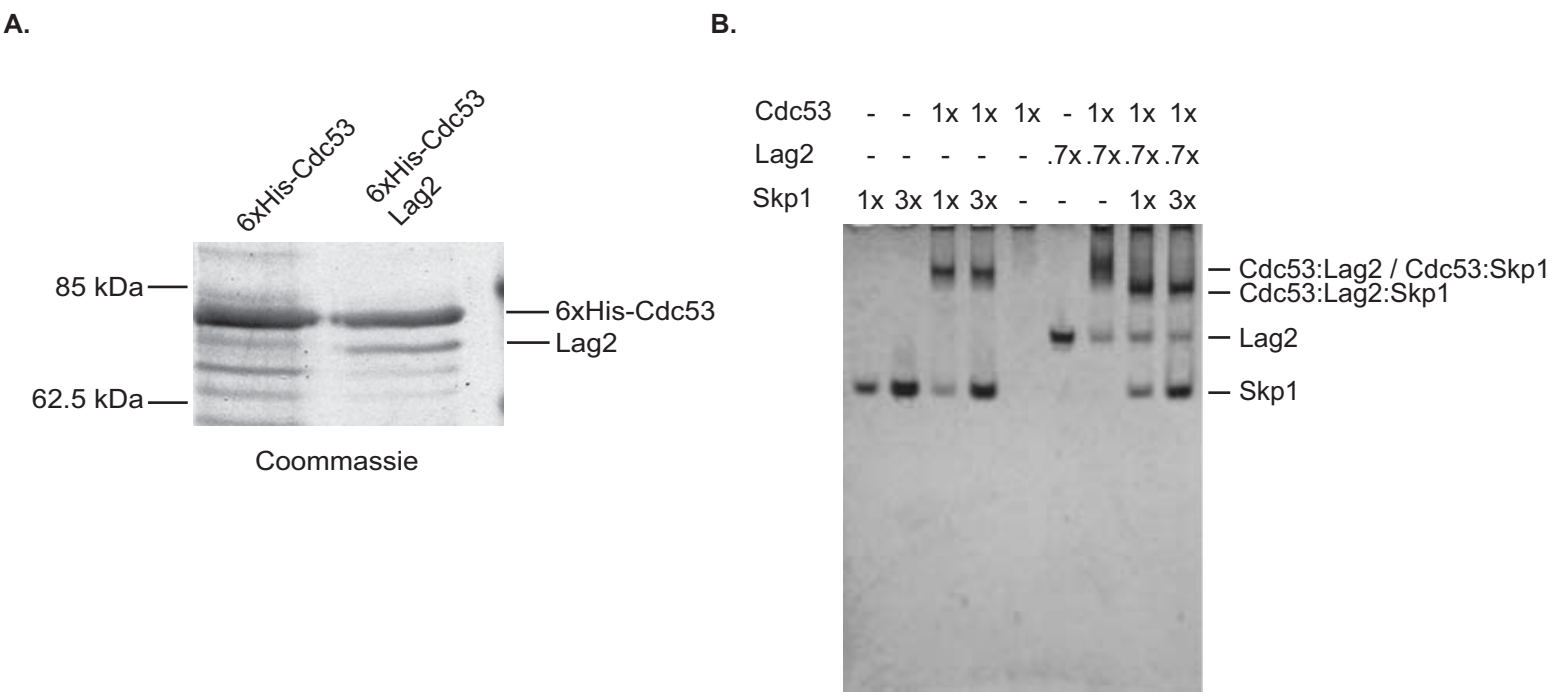

Figure 1: Purification and characterization of Cdc53/Hrt1/Skp1 and Cdc53/Hrt1/Lag2 complexes from E.coli and Sf9 cells

A: Cdc53/Hrt1 and Skp1 (lane 1) or Lag2 (lane 2) were co-expressed from poly-cistronic vectors in E.coli Rosetta cells, and purified over Ni-NTA and GSH resin as described in Material and Methods. A fraction of the complexes was analyzed by SDS-PAGE followed by coomassie blue staining. The positions of 6xHis-Cdc53 and Lag2 are indicated.

B: Skp1 associates with the Cdc53/Hrt1/Lag2 complex in vitro. Purified proteins from Sf9 cells were mixed in the indicated molar ratios and incubated for 30 minutes on ice in 25mM Tris pH=7.6 50mM NaCl 8% Glycerol 1mM DTT. Samples were ran at 130V on native gels for 30 minutes, and visualized by coomassie blue staining.

Figure 2.

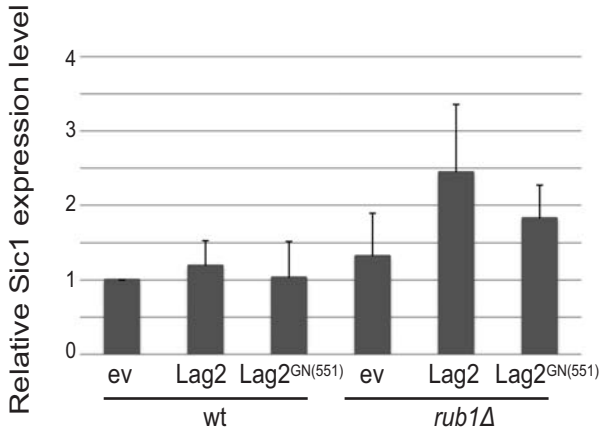

Figure 2. Quantification of Sic1 levels upon Lag or $Lag2^{GN(551)}$ overexpression in wild type and *rub1Δ* cells.

Wild type (wt) or *rub1*Δ cells transformed with an empty control plasmid (ev) or plasmids overexpressing Lag2 or Lag2GN(551) from the inducable *GAL1,10*-promoter were grown in raffinose medium, and analyzed three hours after addition of 2% galactose. Sic1 levels were quantified by immunoblotting and normalized to the actin reference. The average from three independent experiments was plotted as fold increase relative to overexpression of empty vector (ev) controls in wild type cells.