EMBO reports

# SCF$^{FBXW7}$ regulates G2-M progression through control of CCNL1 ubiquitination.

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## Review Timeline:

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| Submission Date: | 14th Mar 22 |
| Editorial Decision: | 19th Apr 22 |
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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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

**1st Editorial Decision** **19th Apr 2022**

Dear Stephane,

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

As you will see, the referees acknowledge that the findings are potentially interesting. However, they also point out several technical concerns and have a number of suggestions for how the study should be strengthened, and I think that all of them should be addressed.

Please note that we also ask from the editorial side to specify the number of experiments and their nature (i.e., biological or technical repeats) in the figure legends and to include all data (screening results) in the manuscript. We also recommend supplying source data for figures and quantification (see also point 9 below).

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

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (July 19, 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

***** IMPORTANT NOTE:
We perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

1) A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.

2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision. *****

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines
()

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their

respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here:

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) Please note that a Data Availability section at the end of Materials and Methods is now mandatory. In case you have no data that requires deposition in a public database, please state so instead of refereeing to the database.
See also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

8) Figure legends and data quantification:

The following points must be specified in each figure legend:
- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)
- If the data are obtained from n {less than or equal to} 2, use scatter blots showing the individual data points.

Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

See also the guidelines for figure legend preparation:
https://www.embopress.org/page/journal/14693178/authorguide#figureformat

- Please also include scale bars in all microscopy images.

9) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available .

10) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

11) As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

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

I look forward to seeing a revised form of your manuscript when it is ready and please let me know if you have questions or comments regarding the revision.

Kind regards,

Martina

Martina Rembold, PhD

Senior Editor
EMBO reports

************************

Referee #1:

SCF-FBXW7 regulates G2-M progression through control of CCNL1 ubiquitination

Using a genetic screen, the authors identify FBXW7 as a synthetic-lethal interaction with CCLN1. They show that FBXW7 binds CCLN1 through a conserved motif, and CCLN1 degradation is dependent on FBXW7 and this binding motif. Consistent with these findings, CCLN1 is ubiquitinated in vitro by a FBXW7-dependent mechanism. Cell cycle analysis showed that the CCLN1-CDK11 complex regulates cell proliferation, and CCLN1 overexpression shortens mitosis. Finally, cells lacking FBXW7 activity are hypersensitive to treatment with a CDK11 inhibitor, suggesting that this molecular axis could be exploited to treat in FBXW7 tumors.

While the uncovered findings are interesting, several conclusions are not justified with the included experiments. Moreover, the quality of some data needs to be improved before publication. Thus, in my opinion the paper needs major revisions before publication in EMBO Reports.

Experimental points:

Figure 1:

- A better/clearer representation of the experimental outline shown in panels A, C, and E would be helpful. For example, the library used in in panel C should be labeled, as well as the time used for selection. The time should also be included in panel E, and the cells' color needs to change, as green could be mis-interpreted as GFP-expressing cells.
- A statistical analysis should be included for the experiment shown in panel F.
- Controls showing APC and Fbxw7 knock out are missing in panel G. The data included in Suppl. Figure 1 is not sufficient to properly control for this experiment, as the APC knock out was not shown.

Figure 2:

- In panel B, except for CCNL1, it seems that data showing fitness genes has been omitted. It would also be relevant to include known FBXW7 substrates and reveal whether CDK11 was identified in this screening approach. The actual screening data should be uploaded as a list.
- Panel C: a color labeling, to indicate the difference between the data points, is needed on the figure panel as well as in the figure legend.
- Panel D: a control for mCherry-AVVS1 to GFP-AVVS1 is missing. I also propose to include the deletion controls currently shown in part in Supp. Figure 1E to the main Figure. A control demonstrating proper depletion of CCNL1 in FBXW7-deleted cells is missing.
- A statistical analysis of the experiments shown in panel D and E should be included.

Figure 3:

- Panel A: Quantification of the CCNL1 bands should be included (in comparison to the three replicates). Blotting CDK11 levels in the same experiment is also recommended, together with a control for FBXW7 depletion.
- Panel C and G: t1/2 should be removed, as the longer time points were not part of the measurements shown in panels B and F. Instead, a statistical analysis comparing WT and FBXW7-deleted cells at time 8 h should be included.
- The authors should clearly state that different cell lines (HPAF II or HEK293) that were used for each panel.
- Panel H: The authors are suggested to do a similar IP experiment, but pulling on HA-CCNL1 instead, and probe not only for the presence of FBXW7, but also for the presence of SCF subunits (CUL1, SKP1), and not other cullin components such as (CUL4 or DDB1). Moreover, the authors should include CDK11 in this analysis. Finally, please state the number of replicates in the figure legends.
- Panel I: please include protein markers. Moreover, in panels I and J, show the full HA-blot to visualize the ubiquitination bands/smear. To improve these experiments, the authors should pull on ubiquitin, and then blot for the tag on their substrate. Expression of wild type CCNL1 vs mutant CCNL1 should be shown in the input.
- Panel I: controls showing proper depletion of FBXW7 are missing. Expression of wild type CCNL1 should be shown in the input.
- The authors should include the binding-deficient FBXW7(R465C) mutant in their analysis.
- It is important to include an experiment demonstrating that CCNL1 stabilization is dependent on other SCF components, such

as SKP1, in order to solidify the conclusion that degradation of CCNL1 is indeed SCF-dependent.

Figure 4:

- The cell cycle experiment shown in Figure 4 are not convincing and inconclusive. For example, the two time points at 0 and 1 h after nocodazole treatment are very variable between WT and FBXW7-depleted cells, therefore no solid conclusions can be made from the subsequent time points. Moreover, cell cycle dependent cycling of CCNL1 is unjustified with the data shown in panel B.
- Panel C: the G1 peak of the different cell types is shifted, and this makes the comparison difficult. In addition, the gating shown in Supp. Fig. 2C shows variable areas, which does not allow proper conclusions to be made. The authors need to re-analyze the data with the same gating settings in all the conditions, and also include statistical analysis in their representation.
- Panel D, E, and F: investigating the time to cytokinesis is not a proper readout for the G2-M phenotype. In addition, the authors should comment on why the time of mitosis in wild type cells is 400 mins, which is unusually long.
- Panel G: the gating and the raw data should be shown in a Supp. Fig. with a statistical analysis. All conditions should be measured with the same gating settings. Quantification of the number of cells in G2-M in FBXW7-deleted vs. wild type cells should be stated in the text.
- The authors are suggested to perform live cell imaging experiments to quantify the number of cycling cells in different conditions. This can be achieved with bright field imaging, and should also reveal the length of mitosis.
- Controls for CCNL1 and FBXW7 levels are missing in panel C.

Figure 5:

- Panel C: The protocol for generating FBWX7-deletion and CCNL1 overexpression cell lines is not stated in the Materials and Methods. Authors should give a rational why they used FBXW7 clones only for figure 5.
- Authors should state which clone number they used in panel D. The gating and the raw data should be shown in a Suppl. Fig. and analyzed with the same settings for all conditions. Moreover, a statistical analysis should be included.

Figure 6:

- Panel B: a western blot showing FBWX7 levels in the different cancer cell lines should be included.
- Panel C: can the authors explain why SiHa cells show higher sensitivity to OTS964 treatment at 25 nM as compared to Caski, although they do not over-express CCNL1?
- Panel D: referring to Suppl. Fig. 2D, the authors should include the raw data of treated and untreated cells. The same settings should be applied for treated and untreated conditions, and a statistical analysis should be added.
- Panel E is not cited in the main text.

Text issues:

- Line 90: The title of the paragraph used: "Generation of a cell line model addicted to FBXW7 loss-of-function" does not recapitulate the results presented in this section.

- Line 103: the authors should comment whether α-catenin could be a potential substrate of FBXW7, and whether it would be directed for degradation in a FBXW7-dependent manner, hence, FBXW7 would also be considered as a negative regulator of α-catenin, like APC, AXIN1 and CSNK1A1.

- Line 110: using selective pressure of LGK974 for cell line generation and afterwards using this for an assay is not optimal. The authors should comment on this.

- Line 112: The sensitivity of FBXW7-deleted cells to LGK974 in Figure 1F, does not explain the strong resistance effect noticed in Figure 1D. The authors should comment on this discrepancy. The authors should also state the fold of change, as they quantified the relative confluence, instead of writing "lower than for APC knockout". The authors should comment whether this could be due to the incomplete knock out of FBXW7 (as shown in Suppl. Fig 1B)?

- Line 135 and 137: there is no Suppl. Fig 1F, as cited in these lines.

- Line 136: can the authors state the fold change in speed, instead of stating "much slower"?

- Line 165: The conclusion is overstated. Evidence that degradation of CCNL1 is SCF dependent is lacking.

- Line 179: the authors refer to G2 phase, they analyze and present however G2/M.

- Line 183: The authors mention "Consistent with hyperactivity of CCNL1-CDK11..", however a reference/citation is lacking.

- Line 190: instead of stating "higher proportion", the authors should state the % of cells, and measure fold of change when applicable.

- Line 192: the conclusion is overstated, if we compare the number of G2 cells. Could the authors also comment whether this slight increase in FBXW7-deleted cells is sufficient to explain the striking resistance to LGK974 shown in Figure 1D? In addition, the cells were selected with LGK974 for cell line generation, therefore they need to be bypassing LGK974 cell cycle arrest.

- Line 200: would a knock down of CDK11 is expected to exhibit a similar effect as the OTS964 treatment?

- Line 201: FBXW7-/- HPAF-II cells were isolated - please describe the cell line generation in Material and Methods. The rationale on why to use different clones in Figure 5 is lacking.

- Line 213: what do the authors mean by "loss of post-translational control"?

- Line 220: The authors mention "... tumor harboring both FBXW7 mutation or CCNL1", however they need to state which type of FBXW7 mutation they analyzed: loss or gain of function mutations? Please state this for consistency.

- Line 234: the authors need to include the HPAFII wild type cell line in the same analysis to justify the statement on the comparison of cells in G2-M phase.

- Line 337: do the authors use 5% antibiotic in their cell culture media?

- Line 421: in which experiment was cytoplasmic fractionation used?

- Line 429: please describe the generation of HPAF-II cell lines overexpressing CCLN1 and GFP-tubulin cells.

Referee #2:

In this manuscript, O'Brien and colleagues reported that CCNL1 is a new substrate of FBXW7, and FBXW7 regulates CCNL1 stability during cell cycle progression. They also demonstrated that CCNL1 is synthetic lethal with FBXW7. FBXW7 loss-of-function and CCNL1 overexpression sensitize cells to a CDK11 inhibitor, OTS964. Their findings implicate that CCNL1 levels could represent a biomarker for CDK11 inhibitor in cancer treatment. Overall, the experiments are well executed, and the manuscript are professionally written. To strengthen this study, the following concerns should be addressed.

1. In Fig 3E, the concentrations of FBXW7-Skp1 in this assay are extremely high, while the binding signal was saturated at much lower concentrations. To obtain a reliable Kd, it's recommended that the authors perform this experiment with full dose response curves that span four log units centered around the Kd value.

2. In Fig 3H, mutant CCNL1 still weakly binds to FBXW7. It's necessary to investigate if this is a non-specific binding or CCNL1 has another binding site besides the TPALS motif. Some FBXW7 substrates have more than 1 CPD motif. In the over-expressed setting, each motif is sufficient to bind to FBXW7. Expressing FBXW7 that are mutated at the substrate-binding sites (Arg465, Arg479, Arg505) would help to answer this question.

Referee #3:

In this study, O'Brien and colleagues have carried out a genome-wide CRISPR screen and identified a synthetic lethal genetic interaction between the SCF substrate receptor FBXW7, a well-known tumor suppressor gene, and the non-canonical cyclin CCNL1. The authors reported that CCNL1 is a substrate of the SCF-FBXW7 ubiquitin ligase complex and that FBXW7-mediated degradation of CCNL1 controls progression from the G2 phase of the cell cycle into mitosis. They also found that FBXW7 loss of function and CCNL1 overexpression sensitize cells to CDK11 inhibition and proposed that the FBXW7-CCNL1-CDK11 molecular axis may be therapeutically relevant in cancer cells.

In my opinion, the evidence demonstrating that CCNL1 is a substrate of the SCF-FBXW7 ubiquitin ligase is weak. The interaction of CCNL1 with FBXW7 is shown only with exogenously expressed proteins. The authors should test the ability of ectopically expressed FLAG-tagged FBXW7 to coimmunoprecipitate with endogenous CCNL1. Conversely, does exogenous HA-tagged CCNL1 coimmunoprecipitate with endogenous FBXW7? A complex with endogenous CCNL1 and FBXW7 should also be assessed. Have the authors tested the specificity of the CCNL1-FBXW7 interaction by comparing CCNL1

coimmunoprecipitation with other F-box proteins?

The polyubiquitylation of CCNL1 in FBXW7-/- cells should be tested. Does ectopic expression of FBXW7 in these cells stimulate CCNL1 polyubiquitylation?

The biological role of FBXW7-dependent degradation of CCNL1 in normal cells is not clear. What is the effect of expressing a non-degradable CCNL1 mutant in cells when compared with cells expressing wild type CCNL1?

Minor:
Which FBXW7 isoform targets CCNL1 for degradation? Have the authors analyzed CCNL1 colocalization with FBXW7 isoforms?

Martina Rembold, PhD
Senior Editor
EMBO reports

Re: EMBOR-2022-55044-T

Dear Dr. Rembold,

First and foremost, I would like to thank you for handling our manuscript and the reviewers for providing excellent constructive criticisms that guided new experiments to strengthen our study. As you will see below in the point-by-point response to the reviewers, we feel that we have now addressed the vast majority of the questions and comments of the reviewers. We are grateful for the extension you provided as the new results we are now including made the manuscript stronger. We are therefore hopeful that our revised manuscript will now meet the reviewers and your expectation for publication in Embo Reports.

Please do not hesitate to contact me if you need more information,

Stephane Angers

**Referee #1:**

SCF-FBXW7 regulates G2-M progression through control of CCNL1 ubiquitination

Using a genetic screen, the authors identify FBXW7 as a synthetic-lethal interaction with CCLN1. They show that FBXW7 binds CCLN1 through a conserved motif, and CCLN1 degradation is dependent on FBXW7 and this binding motif. Consistent with these findings, CCLN1 is ubiquitinated in vitro by a FBXW7-dependent mechanism. Cell cycle analysis showed that the CCLN1-CDK11 complex regulates cell proliferation, and CCLN1 overexpression shortens mitosis. Finally, cells lacking FBXW7 activity are hypersensitive to treatment with a CDK11 inhibitor, suggesting that this molecular axis could be exploited to treat in FBXW7 tumors.

While the uncovered findings are interesting, several conclusions are not justified with the included experiments. Moreover, the quality of some data needs to be improved before publication. Thus, in my opinion the paper needs major revisions before publication in EMBO

Reports.

We would like to thank this reviewer for the in-depth review of our manuscript and the suggestions that have helped strengthened the findings.

Experimental points:

**Figure 1:**

- A better/clearer representation of the experimental outline shown in panels A, C, and E would be helpful. For example, the library used in in panel C should be labeled, as well as the time used for selection. The time should also be included in panel E, and the cells' color needs to change, as green could be mis-interpreted as GFP-expressing cells.

The suggested modifications have been incorporated.

- A statistical analysis should be included for the experiment shown in panel F.

The statistical analysis is now added to panel F.

- Controls showing APC and Fbxw7 knock out are missing in panel G. The data included in Suppl. Figure 1 is not sufficient to properly control for this experiment, as the APC knock out was not shown.

We now include TIDE analysis results that show robust gene editing at the indicated locus (Figure EV1A).

**Figure 2:**

- In panel B, except for CCNL1, it seems that data showing fitness genes has been omitted. It would also be relevant to include known FBXW7 substrates and reveal whether CDK11 was identified in this screening approach. The actual screening data should be uploaded as a list.

We have now provided the raw data for all the screens included in the manuscript as EV File 1. CDK11a and CDK11b are pan-essential genes (according to the Broad Institute Cancer Dependency Map: DepMAP) that are targeted by the CDK11 gRNA in the library. CDK11a/b are therefore essential in both the wild-type cells and in the FBXW7-knockout context, so these genes would not be identified as part of this analysis that was performed to identify differentially essential genes between wild-type cells and FBXW7 knockout cells.

- Panel C: a color labeling, to indicate the difference between the data points, is needed on the figure panel as well as in the figure legend.

We have updated the figure as suggested to improve clarity.

.

- Panel D: a control for mCherry-AVVS1 to GFP-AVVS1 is missing. I also propose to include the deletion controls currently shown in part in Supp. Figure 1E to the main Figure.

A control demonstrating proper depletion of CCNL1 in FBXW7-deleted cells is missing.

The mCherry-AAVS1/GFP-AAVS1 ratio was used to normalize each data points, and as such would be plotted as a straight line on the graph which is not informative. We have updated the figure legend for clarity. The western blot presented in Figure EV1E was performed in FBXW7-depleted cells and show effective CCNL1 knockout.

- A statistical analysis of the experiments shown in panel D and E should be included.

Statistical analysis are now included for both panels D and E.

**Figure 3:**

- Panel A: Quantification of the CCNL1 bands should be included (in comparison to the three replicates). Blotting CDK11 levels in the same experiment is also recommended, together with a control for FBXW7 depletion.

Quantification of CCNL1 expression is now included in Figure EV2A. We also have provided a western blot controlling for FBXW7 expression as suggested. We do not anticipate CDK11 levels to change since CCNL1 regulates its activity.

- Panel C and G: t1/2 should be removed, as the longer time points were not part of the measurements shown in panels B and F. Instead, a statistical analysis comparing WT and FBXW7-deleted cells at time 8 h should be included.

We have updated these panels as suggested: we removed the half-life calculations and present a statistical analysis at T8.

- The authors should clearly state that different cell lines (HPAF II or HEK293) that were used for each panel.

As suggested, we have updated all figures to clearly indicate the cell lines used in each experiment.

- Panel H: The authors are suggested to do a similar IP experiment, but pulling on HA-CCNL1 instead, and probe not only for the presence of FBXW7, but also for the presence of SCF subunits (CUL1, SKP1), and not other cullin components such as (CUL4 or DDB1). Moreover, the authors should include CDK11 in this analysis. Finally, please state the number of replicates in the figure legends.

We thank all the reviewers for helpful suggestions. Legends have been updated accordingly. We performed co-immunoprecipitation experiments and identified an interaction between CCNL1 with both Cul1 and Cul4A (Figure EV2E). This was unexpected but is also seen with the well-studied FBXW7 substrate Jun (Cang 2017 PNAS) The biological implications for a potential regulation of CCNL1 by a Cul4 ligase remain unclear. In addition, as suggested by other reviewers, we have performed experiments showing that endogenous FBXW7 is immunoprecipitated with FLAG-CCNL1 (Figure 4B).

We also provide further evidence that CCNL1 is regulated by a SCF complex, we showed that overexpression of a dominant-negative Cul1 (dN-Cul1) leads to stabilization of CCNL1 (Figure 3D, quantification, and statistics in EV2B).

The interaction between CCNL1 and CDK11 is well described (Renshaw JBC 2019, Loyer JBC 2008, Loyer & Tremblay Seminars in Cell & Developmental Biology 2020) and was not the subject of the experiments showed in Fig.3.

- Panel I : please include protein markers. Moreover, in panels I and J, show the full HA-blot to visualize the ubiquitination bands/smear. To improve these experiments, the authors should pull on ubiquitin, and then blot for the tag on their substrate. Expression of wild type CCNL1 vs mutant CCNL1 should be shown in the input.

This is now related to Figure 4. We have attempted various versions of this assay, including pulling down Myc-Ub as suggested by the reviewer. We however had lower sensitivity and poor reproducibility using this format. The most robust results were obtained using pull-down of HA-CCNL1, and blotting for Myc-Ub. As suggested, we present the full blots and the inputs.

- Panel I: controls showing proper depletion of FBXW7 are missing. Expression of wild type CCNL1 should be shown in the input.

As suggested, we modified the figures to include input lanes. Depletion of FBXW7 in these cells is now included in Figure EV2C.

- The authors should include the binding-deficient FBXW7(R465C) mutant in their analysis.

We thank the reviewer for this excellent suggestion. We now included cycloheximide chase experiments carried in cells expressing the R465C mutant of FBXW7 and demonstrate that CCNL1 levels are stabilized (Figure EV2G).

- It is important to include an experiment demonstrating that CCNL1 stabilization is dependent on other SCF components, such as SKP1, in order to solidify the conclusion that degradation of CCNL1 is indeed SCF-dependent.

This was another excellent suggestion. To provide further evidence that CCNL1 is regulated by a SCF complex, we showed that overexpression of a dominant-negative Cul1 (dN-Cul1) leads to stabilization of CCNL1 (Figure 3D, quantification, and statistics in EV2B).

**Figure 4: (new Figure 5)**

- The cell cycle experiment shown in Figure 4 are not convincing and inconclusive. For example, the two time points at 0 and 1 h after nocodazole treatment are very variable between WT and FBXW7-depleted cells, therefore no solid conclusions can be made from the subsequent time points. Moreover, cell cycle dependent cycling of CCNL1 is unjustified with the data shown in panel B.

We are unsure what the reviewers is referring to here. The cell cycle variation of CCNL1 levels in Figure 5B exhibited a similar trend than CyclinB1 (admittedly more moderate). Importantly the cycling of CCNL1 levels is always inhibited in FBXW7-/- cells.

- Panel C: the G1 peak of the different cell types is shifted, and this makes the comparison difficult. In addition, the gating shown in Supp. Fig. 2C shows variable areas, which does not allow proper conclusions to be made. The authors need to re-analyze the data with the same gating settings in all the conditions, and also include statistical analysis in their representation.

Cell cycle distributions by propidium iodide staining are very sensitive to the number of cells being stained through the process. We control for this by cell counting at the start of the experiment, but variability still does occur. We are measuring G2/M accumulation within each cell line and comparing the G2/M counts between cell lines in an effort to mediate this variability. pH3S10 is used to confirm the G2/mitotic fraction for gating as well to mediate variability between cell lines. Authors have added a statistical analysis to the figure and updated the figures to represent 2n and 4n cells for ease of comparison.

- Panel D, E, and F: investigating the time to cytokinesis is not a proper readout for the G2-M phenotype. In addition, the authors should comment on why the time of mitosis in wild type cells is 400 mins, which is unusually long.

HPAF-II cells are a slower-growing cell line, which may be the reason for a longer than usual time of mitosis. In addition, cells are exiting chemical-induced arrest, which may lead to an apparent slower mitosis than an unperturbed cells. We performed these assays as they were previously similarly employed to characterize the activity of CDK11 in this context (Renshaw 2019 JCB).

We nevertheless understand the point of the reviewer that these experiments may not be a proper readout of the G2-M phenotype when considered in isolation. To supplement this data, we performed experiments using the PIP-FUCCI reporter to measure cell cycle timing. Using nocodazole, we quantified the reduction in mCherry expression (as cells exit mitosis) and measured this at both the population level (Figure EV3B,C) and in individual cells (EV3B,D). We also measured mitotic timing in unperturbed cells using the PIP-FUCCI reporter, and report similar results as when mitotic exit is quantified following nocodazole arrest (Figure EV3D). The PIP-FUCCI results in both unperturbed and nocodazole arrested cells revealed a shortened mitotic timeline than cells treated with monastrol, suggesting that release from monastrol-arrest is contributing to the longer than anticipated length of mitosis observed.

- Panel G: the gating and the raw data should be shown in a Supp. Fig. with a statistical analysis. All conditions should be measured with the same gating settings. Quantification of the number of cells in G2-M in FBXW7-deleted vs. wild type cells should be stated in the text.

Gating strategy for the new panel 5G has been added as Figure EV4A. A statistical analysis has been provided in the figure, and the text has been updated to describe the change in cell counts in the G2-M phase.

- The authors are suggested to perform live cell imaging experiments to quantify the number of

cycling cells in different conditions. This can be achieved with bright field imaging, and should also reveal the length of mitosis.

We agree with the reviewer. To supplement our initial data we performed experiments using the PIP-FUCCI reporter to measure cell cycle timing. Using nocodazole, we measured the reduction in mCherry expression (as cells exit mitosis) and measured this at both the population level (Figure EV3B,C) and in individual cells (EV3B,D). We also quantified mitotic timing in unperturbed cells using the PIP-FUCCI reporter, and report similar results as mitotic exit following nocodazole arrest (Figure EV3D).

- Controls for CCNL1 and FBXW7 levels are missing in panel C.

The same cell lines are used in all experiments, and a western blot of CCNL1 expression is included in Figure 5F.

**Figure 5 (new figure 6):**
- Panel C: The protocol for generating FBWX7-deletion and CCNL1 overexpression cell lines is not stated in the Materials and Methods. Authors should give a rational why they used FBXW7 clones only for figure 5.

We have updated the materials and methods. We opted for clones for this experiment as the pooled FBXW7ko cells used throughout the paper may have fraction of cells with unedited alleles that could confound the experiments when performing clonogenic assays. We however understand the point of the reviewers and have repeated the experiment using the polyclonal lines to be consistent with the other Figures.

- Authors should state which clone number they used in panel D. The gating and the raw data should be shown in a Suppl. Fig. and analyzed with the same settings for all conditions. Moreover, a statistical analysis should be included.

Clones were not used in panel 4D; the pooled parental cells were used in this assay, and we have indicated that in the legend. Authors have added raw gating data to Figure EV4B.

**Figure 6:**
- Panel B: a western blot showing FBWX7 levels in the different cancer cell lines should be included.

As suggested, we now provide, in Figure 7B, western blot showing FBXW7 levels in the studied cell lines.

- Panel C: can the authors explain why SiHa cells show higher sensitivity to OTS964 treatment at 25 nM as compared to Caski, although they do not over-express CCNL1?

We anticipate some variability in response to any small molecule between cell lines, for example due to expression of drug resistance transporters or other genomic or epigenomic differences.

- Panel D: referring to Suppl. Fig. 2D, the authors should include the raw data of treated and

untreated cells. The same settings should be applied for treated and untreated conditions, and a statistical analysis should be added.

As suggested, we now provide gating strategies in Figure EV4C, and a statistical analysis has been performed and added to Figure 7D.

- Panel E is not cited in the main text.

Panel E is now cited in the text.

**Text issues:**

- Line 90: The title of the paragraph used: "Generation of a cell line model addicted to FBXW7 loss-of-function" does not recapitulate the results presented in this section.

The title has been updated to more accurately reflect the results.

- Line 103: the authors should comment whether α-catenin could be a potential substrate of FBXW7, and whether it would be directed for degradation in a FBXW7-dependent manner, hence, FBXW7 would also be considered as a negative regulator of α-catenin, like APC, AXIN1 and CSNK1A1.

There is one publication implicating FBXW7 as a regulator of BCatenin, but no direct ubiquitination of Bcatenin by FBXW7 is observed (Jiang et al, Tumour Biology, 2016). We observe no stabilization of BCatenin in the cytoplasmic fraction following FBXW7-knockout (Figure 1G).

- Line 110: using selective pressure of LGK974 for cell line generation and afterwards using this for an assay is not optimal. The authors should comment on this.

We may not have been clear enough in the text, but we believe that this was one of the most important part of the study. The selective pressure exerted with LGK974 on FBXW7-edited cells promoted the selection of cells that are exquisitely dependent on loss of FBXW7 function for growth. We believe that this is one of the first in vitro models for FBXW7 loss of function that enabled us to identify new mechanisms for this important tumor suppressor gene.

- Line 112: The sensitivity of FBXW7-deleted cells to LGK974 in Figure 1F, does not explain the strong resistance effect noticed in Figure 1D. The authors should comment on this discrepancy. The authors should also state the fold of change, as they quantified the relative confluence, instead of writing "lower than for APC knockout". The authors should comment whether this could be due to the incomplete knock out of FBXW7 (as shown in Suppl. Fig 1B)?

The text has been updated with the suggested comments.
Screening conditions were much longer (35 days) and at a lower concentration of LGK974 (20nM), which is likely the cause of the discrepancy between the strong hit from the screen versus the clonogenic data.

- Line 135 and 137: there is no Suppl. Fig 1F, as cited in these lines.

The text has been updated to fix this error.

- Line 136: can the authors state the fold change in speed, instead of stating "much slower"?

The text has been updated with suggested comments.

- Line 165: The conclusion is overstated. Evidence that degradation of CCNL1 is SCF dependent is lacking.

We have performed further experiments as suggested by the reviewer to further demonstrate the role of SCF E3 ligases in regulation of CCNL1 stability.

- Line 179: the authors refer to G2 phase, they analyze and present however G2/M.

The text has been updated with the suggested comment.

- Line 183: The authors mention "Consistent with hyperactivity of CCNL1-CDK11..", however a reference/citation is lacking.

The text has been updated with the suggestion.

- Line 190: instead of stating "higher proportion", the authors should state the % of cells, and measure fold of change when applicable.

The text has been updated to reflect these suggestions.

- Line 192: the conclusion is overstated, if we compare the number of G2 cells. Could the authors also comment whether this slight increase in FBXW7-deleted cells is sufficient to explain the striking resistance to LGK974 shown in Figure 1D? In addition, the cells were selected with LGK974 for cell line generation, therefore they need to be bypassing LGK974 cell cycle arrest.

The text has been updated to rephrase the conclusion.

The LGK974 resistance screen was performed over 35 days, with a much lower concentration of LGK974 (20nM) than what was used for selection of our FBXW7ko cell line, and what was used in our follow-up experiments including the cell cycle arrest assay (Figure 5G). The cell cycle arrest assay was performed at much higher concentration of LGK974, and over a much shorter time period (2 days) to assess acute responses to loss of Wnt signalling. This assay was performed to understand how FBXW7ko cells are bypassing LGK974-induced cell cycle arrest, following our findings that the FBXW7-CCNL1 axis is involved in progression through mitosis. The results of this assay suggest that FBXW7ko cells are partially resistant to LGK974-induced cell cycle arrest and enter the cell cycle.

- Line 200: would a knock down of CDK11 is expected to exhibit a similar effect as the OTS964 treatment?

CDK11 is an essential gene in most cell lines according to DepMAP. If the timing of knockdown experiments could be correctly monitored, we expect that similar increased sensitivity could be detected.

- Line 201: FBXW7-/- HPAF-II cells were isolated - please describe the cell line generation in Material and Methods. The rationale on why to use different clones in Figure 5 is lacking.

We have updated the text with the suggested comments. The Materials and methods section was also updated. We used clones in this assay to control for the fact that the parental polyclonal line may contain unedited FBXW7 cells, but we have added the parental cell line to this analysis to keep it more consistent with the remainder of the manuscript.

- Line 213: what do the authors mean by "loss of post-translational control"?

Post-translational control in this context is referring to the ubiquitination and subsequent degradation of CCNL1.

- Line 220: The authors mention "... tumor harboring both FBXW7 mutation or CCNL1", however they need to state which type of FBXW7 mutation they analyzed: loss or gain of function mutations? Please state this for consistency.

The text has been updated as suggested.

- Line 234: the authors need to include the HPAFII wild type cell line in the same analysis to justify the statement on the comparison of cells in G2-M phase.

We have updated the text to more accurately reflect the experiment presented.

- Line 337: do the authors use 5% antibiotic in their cell culture media?

Text has been updated; we use 1% antibiotic-antimycotic.

- Line 421: in which experiment was cytoplasmic fractionation used?

Cytoplasmic fractionation was used in the Bcatenin accumulation assay in Figure 1G. HPAF-II cells, and most other cell lines, have a high level of membrane-associated Bcatenin that is not subjected to Wnt signaling regulation. We have clarified this in the figure legend and text.

- Line 429: please describe the generation of HPAF-II cell lines overexpressing CCLN1 and GFP-tubulin cells.

We have updated the materials and methods.

**Referee #2:**
In this manuscript, O'Brien and colleagues reported that CCNL1 is a new substrate of FBXW7, and FBXW7 regulates CCNL1 stability during cell cycle progression. They also demonstrated that

CCNL1 is synthetic lethal with FBXW7. FBXW7 loss-of-function and CCNL1 overexpression sensitize cells to a CDK11 inhibitor, OTS964. Their findings implicate that CCNL1 levels could represent a biomarker for CDK11 inhibitor in cancer treatment. Overall, the experiments are well executed, and the manuscript are professionally written. To strengthen this study, the following concerns should be addressed.

We appreciate the positive comments of the reviewer and for the suggested experiments to strengthen our manuscript.

1. In Fig 3E, the concentrations of FBXW7-Skp1 in this assay are extremely high, while the binding signal was saturated at much lower concentrations. To obtain a reliable Kd, it's recommended that the authors perform this experiment with full dose response curves that span four log units centered around the Kd value.

As suggested by the reviewer, we repeated this experiment with the full dose-response curves (Figure 3F).

2. In Fig 3H, mutant CCNL1 still weakly binds to FBXW7. It's necessary to investigate if this is a non-specific binding or CCNL1 has another binding site besides the TPALS motif. Some FBXW7 substrates have more than 1 CPD motif. In the over-expressed setting, each motif is sufficient to bind to FBXW7. Expressing FBXW7 that are mutated at the substrate-binding sites (Arg465, Arg479, Arg505) would help to answer this question.

We are thankful to the reviewer for this important observation that we have attempted to address in multiple ways. As a result, we identified a limitation of the experiment (as suspected by the reviewer) and have designed alternative experiments.

We first performed repeats of this binding assay with the addition of additional controls. When lysates from cells expressing HA-CCNL1 alone were subjected to immunoprecipitation with anti-FLAG beads, we observed background binding. While conducting the study, we have dedicated hours of work to try to purify CCNL1 from bacteria and insect cells and tried several shorter constructs but invariably failed in purifying soluble proteins. The co-IP experiments are therefore consistent with our recombinant protein purification experience indicating that CCNL1 is an intrinsically difficult protein to keep in solution, which we believe explain the residual non-specific binding. We therefore decided to remove the binding data of the mutant CCNL1 from the manuscript and to rather study the impact of the mutations on the stability of the protein.

To further substantiate the role of FBXW7 in degradation of CCNL1, we now include an additional experiment showing that expression of the FBXW7-R465C mutant, which is known to prevent substrate binding, leads to stabilization of CCNL1 whereas over-expression of wild-type FBXW7, accelerates CCNL1 degradation (Figure EV2G,H).

**Referee #3:**

In this study, O'Brien and colleagues have carried out a genome-wide CRISPR screen and identified a synthetic lethal genetic interaction between the SCF substrate receptor FBXW7, a well-known tumor suppressor gene, and the non-canonical cyclin CCNL1. The authors reported

that CCNL1 is a substrate of the SCF-FBXW7 ubiquitin ligase complex and that FBXW7-mediated degradation of CCNL1 controls progression from the G2 phase of the cell cycle into mitosis. They also found that FBXW7 loss of function and CCNL1 overexpression sensitize cells to CDK11 inhibition and proposed that the FBXW7-CCNL1-CDK11 molecular axis may be therapeutically relevant in cancer cells.

Authors would first like to thank the reviewer for their helpful suggestions to strengthen our manuscript. All comments have been addressed below.

In my opinion, the evidence demonstrating that CCNL1 is a substrate of the SCF-FBXW7 ubiquitin ligase is weak. The interaction of CCNL1 with FBXW7 is shown only with exogenously expressed proteins. The authors should test the ability of ectopically expressed FLAG-tagged FBXW7 to coimmunoprecipitate with endogenous CCNL1. Conversely, does exogenous HA-tagged CCNL1 coimmunoprecipitate with endogenous FBXW7?

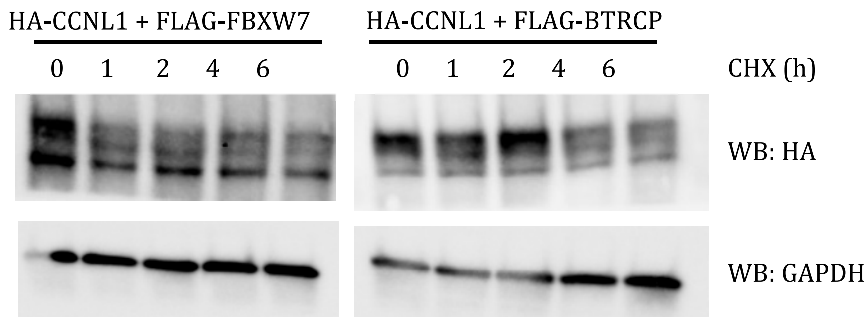
We have performed the suggested experiments and now show that endogenous CCNL1 is co-immunoprecipitated with FLAG-FBXW7 and reciprocally that endogenous FBXW7 is co-immunoprecipitated with FLAG-CCNL1 (Figure 4A,B).

A complex with endogenous CCNL1 and FBXW7 should also be assessed.

We have performed several experiments to detect the interaction using endogenous proteins but have not been able to reliably detect it. There are many possible limitations to explain this, the main one being the very transient interaction between E3 ligases and their substrates, which are being ubiquitinated and degraded. The levels of expression of the proteins and the quality of the antibodies may also be limiting. Nevertheless, we surmise that, collectively, the results we are presenting strongly support CCNL1 being a new substrate of the SCF-FBXW7 E3 ligase.

Have the authors tested the specificity of the CCNL1-FBXW7 interaction by comparing CCNL1 coimmunoprecipitation with other F-box proteins?

Following this suggestion by the reviewer, we attempted some experiments with the widely studied F-Box protein BTRCP. Due to high expression levels and high levels of BTRCP auto-ubiquitination, the co-IP experiments were difficult to interpret with a high level of non-specific binding. Although the data was suggestive of an interaction, we are not confident to include these experiments. Nevertheless, to satisfy the reviewer we performed experiments to test whether BTRCP overexpression leads to accelerated CCNL1 degradation using a cycloheximide chase assay and demonstrated that BTRCP overexpression if not affecting CCNL1 degradation rates. We could in principle include these data but we feel that an interaction with another F-box protein was not supported by our genetic experiments, which we feel are compelling.



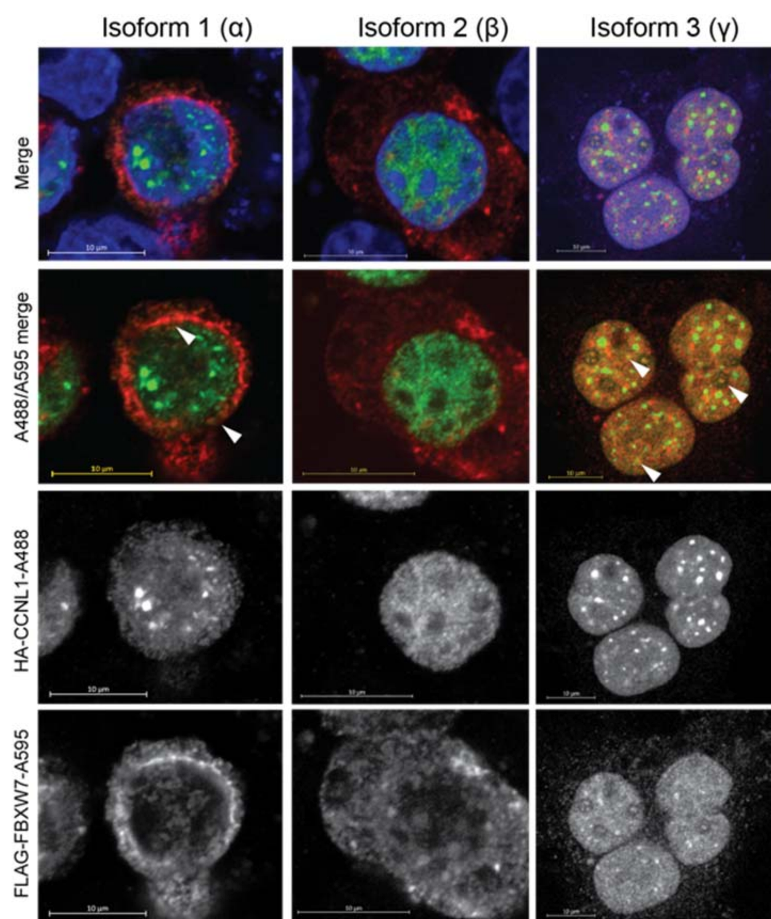

The polyubiquitylation of CCNL1 in FBXW7-/- cells should be tested. Does ectopic expression of FBXW7 in these cells stimulate CCNL1 polyubiquitylation?

Those are excellent recommendations by the reviewer. We performed ubiquitination experiments in FBXW7ko cells and showed that CCNL1 ubiquitination was reduced (Figure 4E). To address whether addition of ectopic FBXW7 stimulates ubiquitination, we demonstrated that expression of a gRNA resistant FBXW7 cDNA led to increased CCNL1 ubiquitination (Figure 4F).

The biological role of FBXW7-dependent degradation of CCNL1 in normal cells is not clear. What is the effect of expressing a non-degradable CCNL1 mutant in cells when compared with cells expressing wild type CCNL1?

We thank the reviewer for this important recommendation. We overexpressed CCNL1 in parental cells to assess whether CCNL1 abundance is impacting the mitotic phenotype described. We show that mitotic time is directly proportional to CCNL1 levels (figure 5E and 5F).

Minor:
Which FBXW7 isoform targets CCNL1 for degradation? Have the authors analyzed CCNL1 colocalization with FBXW7 isoforms?
We performed immunofluorescence experiments using the three isoforms of FBXW7 and HA-CCNL1 in HEK293T cells. We confirmed the previous localization of FBXW7 isoform 1 (alpha) to the nucleoplasm, isoform 2 (beta) to the cytoplasm and FBXW7 isoform 3 (gamma) to the nucleolus. The co-localization experiments suggest a stronger localization between nuclear CCNL1 and FBXW7 isoform 3. We attached the results here, but we don’t necessarily feel that they should be included in the manuscript. The specific role of FBXW7 isoforms could be the subject of future work.



Isoform 1 (α)
Isoform 2 (β)
Isoform 3 (γ)
Merge
A488/A595 merge
HA-CCNL1-A488
FLAG-FBXW7-A595

**1st Revision - Editorial Decision** **27th Sep 2022**

Dear Stephane,

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

As you will see, all referees are positive about the study and request only minor changes to clarify text and figures. Please also discuss potential limitations regarding the interaction between FBXW7 and CCNL1 and its specificity.

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

- Reference format: The abbreviation 'et al' should be used if more than 10 authors (i.e., list the first 10 authors only followed by et al). The year needs to be in brackets.
You can download the respective EndNote file from our Guide to Authors
https://endnote.com/style_download/embo-reports/

- Dataset EV1: Please add the legend to a separate tab of the xls file and please change the callout in the Data availability section to Dataset EV1 instead of EV file 1.

- Movies: please provide the legend as simple README.txt file and ZIP each movie with its legend. The .zip file is then uploaded. The movie legends need to be removed from the manuscript file.

- Please add a callout in the text to Figure 6C.

- We now use AC/CRediT to specify author contributions. The information you entered in our online submission system will be automatically incorporated in the manuscript text. Therefore, please remove the current Author Contributions paragraph from the manuscript text. If you want to add free text, please use the corresponding free text form in the submission system.

- Declaration of interests: Please update the heading to 'Disclosure and competing interests statement'. For more information see https://www.embopress.org/page/journal/14693178/authorguide#conflictsofinterest

- The list of Antibodies and oligos in the Materials and Methods section should be called Table 1 and moved to the end of the file.

- I attach to this email a related manuscript file with comments by our data editors. Please address all comments and upload a revised file with tracked changes with your final manuscript submission.

- Finally, please provide a draft for the following: A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results. This information will be displayed next to the synopsis image you supplied.

We look forward to seeing a final version of your manuscript as soon as possible.

With kind regards,

Martina

Martina Rembold, PhD
Senior Editor
EMBO reports

***************************

Referee #1:

The authors have properly addressed almost all my comments and the manuscript has greatly improved. This manuscript is now suitable for publication.

Referee #2:

The manuscript has been strengthened by the extra evidence provided by the authors. I have some minor points regarding the text.

1) The binding mode of cyclin E degron, which is described in Line 185-186, is not accurate. The reference cited in Line 186, shows that phosphorylation at both T380 and S384 of cyclin E is required for high affinity binding, which argues against the author's statement that "phosphorylation at the threonine residue is sufficient for maximal binding".

2) When cyclin E S384 is not phosphorylated, it needs cooperative binding from a second degron centered at phosphorylated threonine 62. Since the CCNL1 degron identified in this manuscript, has comparable binding affinity to mono phosphorylated cyclin E degron (CCNE1 pT), the author should discuss if under physiological conditions, this CCNL1 degron is sufficient to mediate CCNL1-FBXW7 binding, and lead to CCNL1 degradation.

3) In Line 306-308, the authors again misunderstood the binding of cyclin E and c-Myc degrons. For endogenous c-Myc, it has two di-phosphorylated degrons: one is centered at phosphorylated threonine 58; the other is centered at phosphorylated threonine 244.

Referee #3:

The authors have strengthened the manuscript although the data attempting to demonstrate a specific interaction of FBXW7 with CCLN1 still remain a weak point of this study.

The authors have addressed all minor editorial requests.

## 2nd Revision - Editorial Decision 28th Sep 2022

Prof. Stephane Angers
University of Toronto
Department of Pharmaceutical Sciences
144 College Street
Toronto, ON M5S 3M2
Canada

Dear Stephane,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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

Kind regards,

Martina

Martina Rembold, PhD
Senior Editor
EMBO reports

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| Corresponding Author Name: Stephane Angers | |
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This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.
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**Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots 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. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship 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 χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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**Select "Not Applicable" only when the requested information is not relevant for your study.**

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|---|---|---|
| New materials and reagents need to be available; do any restrictions apply? | Yes | Materials and Methods |

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|---|---|---|
| For **antibodies** provide the following information:<br>- Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number<br>- Non-commercial: RRID or citation | Yes | Materials and Methods |

| DNA and RNA sequences | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| **Short novel DNA or RNA including primers, probes**: provide the sequences. | Yes | Materials and Methods |

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|---|---|---|
| **Cell lines:** Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID. | Yes | Materials and Methods |
| **Primary cultures:** Provide species, strain, sex of origin, genetic modification status. | Not Applicable | |
| Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination. | Yes | Materials and Methods |

| Experimental animals | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| **Laboratory animals or Model organisms:** Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID. | Not Applicable | |
| **Animal observed in or captured from the field:** Provide species, sex, and age where possible. | Not Applicable | |
| Please detail **housing and husbandry conditions**. | Not Applicable | |

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|---|---|---|
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| Human research participants | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants. | Not Applicable | |

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|---|---|---|
| If your work benefited from core facilities, was their service mentioned in the acknowledgments section? | Yes | Acknowledgements |

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| --- | --- | --- |
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| Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable. | Not Applicable | |

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| --- | --- | --- |
| Provide DOI OR other citation details if **external detailed step-by-step protocols** are available. | Not Applicable | |

| Experimental study design and statistics | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
| --- | --- | --- |
| Include a statement about **sample size** estimate even if no statistical methods were used. | Yes | Figure legends |
| Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described? | Not Applicable | |
| Include a statement about **blinding** even if no blinding was done. | Not Applicable | |
| Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established?<br><br>If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification. | Not Applicable | |
| For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared? | Yes | Materials and Methods |

| Sample definition and in-laboratory replication | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
| --- | --- | --- |
| In the figure legends: state number of times the experiment was **replicated** in laboratory. | Yes | Figure legends |
| In the figure legends: define whether data describe **technical or biological replicates**. | Yes | Figure legends |

## Ethics

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| --- | --- | --- |
| Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. | Not Applicable | |
| Studies involving **human participants**: 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. | Not Applicable | |
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| Dual Use Research of Concern (DURC) | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
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| Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm | Not Applicable | |
| If you used a select agent, is the security level of the lab appropriate and reported in the manuscript? | Not Applicable | |
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## Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

| Adherence to community standards | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
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| State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided. | Not Applicable | |
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| 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. | Not Applicable | |

## Data Availability

| Data availability | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
| --- | --- | --- |
| Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section? | Not Applicable | |
| Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement? | Not Applicable | |
| Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided? | Yes | Materials and Methods; citations |
| If publicly available data were reused, provide the respective **data citations in the reference list**. | Yes | Citations list |