nature portfolio

Peer Review File

Molecular mechanisms of CAND2 in regulating SCF ubiquitin ligases

Corresponding Author: Professor Xing Liu

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The article "Molecular mechanisms of CAND2 in regulating SCF ubiquitin ligases" by Kankan Wang et al., describes how CAND2, a CAND1 homolog, regulates the dynamic assembly of SCFs and how this impacts SCF-dependent protein degradation. To do so, they use biochemical, biophysical, and structural techniques. The general approach is similar to the one by Raistma et al 2017 (https://doi.org/10.1016/j.cell.2017.10.016), using CAND1/2 DKO 293 cells and assessing F-box exchange by similar biochemical assays and SILAC proteomics/interactomics. The main finding from this study is that CAND2, like CAND1, acts as an F-box protein exchange factor. Structural studies unveiled a similar interaction architecture between CAND1-CUL1 and CAND2-CUL1. However, the authors found that CAND2 has a much lower efficiency (up to 10 times lower) in exchanging the F-box proteins. They explain this by showing how the CAND2-SCF intermediate complex is less prone to dissociation than the CAND1-SCF intermediate.

The manuscript is well-written, and the design of the experiments, the quality of the data and the interpretation seem adequate. However, I have some comments/suggestions that may need to be addressed before the publication of this study in Nature Communications, in its actual form:
- One main concern is that all the experiments have been performed using one cell line (human; 293 cells) and overexpressing proteins or after incubation with recombinant proteins. This does not always explain what happens in the cells at endogenous levels or in different cells. I would recommend expanding these findings to additional cell lines and using cell models where the expression levels of CAND1 and CAND2 are different at the endogenous level.
- Also, it would be interesting to see (or to explain, based on the structures) how mutations associated with disease of CAND1/CAND2 affect the FBP exchange, SCF-dependent ubiquitylation and subsequent degradation of substrates.
- The need to add 10x more CAND2 than CAND1 to induce FBP exchange is concerning. What are the protein concentrations and how do they compare to the actual concentration of these proteins in the cell? Would you expect these differences in concentration to be as high between different cellular compartments?
- In line with the previous comment, there is not a clear explanation of how, despite the structural analogies, the authors think CAND2 is much less efficient than CAND1 in disassembling the SCF complex.
- It would be interesting to show how CUL1 Neddylation regulates the interaction between CAND2 and CUL1. I am not sure there is any experiment in this study that shows, in the same experiment, the two conditions (+/- MLN4924).
- Similarly to what has been done in Figure 1 with TNF-a and IkBa levels and FAC and IRP2 levels, it would have been interesting to investigate what additional SCF substrates are regulated by CAND1/CAND2 by performing a total proteome analysis in CAND1 KO and CAND2 KO cells (+/- MLN4924) and the presence/absence of a proteasome inhibitor (for example MG132 or Bortezomib). This would have informed of the potential different cellular functions of both proteins. This remains an important question in my opinion.
- In the SILAC proteomics study in Figure 2, DKO cells and DKO3xFlag-CUL1 cells were labelled with heavy and light SILAC medium, respectively. Then mixed and 3xFlag-CUL1 was immunoprecipitated. MS analysis of the H/L ratios were used as an estimation of FBP changes. A concern is that the total proteome composition of the two cell lines, DKO and DKO overexpressing CUL1, may be quite different and therefore affect the outcome of the experiment. Has this been investigated/addressed somehow?
- In the same experiments, it would be nice to show if CAND1/CAND2 affect the binding of non-F-box proteins to CUL1. Perhaps as a supplementary figure/table.
- Figure 1H. It would be beneficial to quantify and plot the ratios n8CUL1/FlagIRP2 as it is not easy to appreciate (differences look quite small).

- Fig S2 B and E, when on the top of the blot image says SKP2 incubation, does it mean SKP1*SKP2?

Reviewer #2

(Remarks to the Author)
The manuscript by Wang et al. attempts to dissect a potential role for the exchange factor Cand2 in SCF dynamics. The homolog Cand1 has been extensively studied and is generally considered the main exchange factor for cullin-1 ligases (SCF). Cand1 is necessary for a dynamic cycle of Skp1/Fbox protein interaction with the core ligase so that a complete repertoire of the up to 69 F-box proteins are represented as SCF complexes. This system also integrates substrate abundance to adjust SCF subtype representation to substrate demands. The authors explore a potential similar role for the Cand1 homolog Cand2 in SCF regulation. The manuscript reports a thorough biochemical/biophysical characterization with the conclusion that Cand2 can catalyze Skp1/Fbox exchange of SCF complexes in vitro, albeit with different kinetics. The in vitro experiments are excellent and of high quality. Where the study falls short is to clearly demonstrate that Cand2 has a physiological role in regulating SCF ligases. There is one set of experiments that tries to address this question by analyzing degradation of IkBa or Irp2. These experiments are performed in an artificial system where CAND1 and CAND2 are knocked out and complementation by ectopic expression of CAND1 or CAND2 was performed. Both proteins can partially complement the degradation defect of the double knockout cells. These experiments imply that Cand2 CAN perform similar functions as Cand1 but does not address whether Cand2 DOES perform these functions under physiological conditions. In fact, extensive and well-designed MS-based experiments would suggest otherwise, because observed effects are mainly driven by Cand1 and not by Cand2 at equal concentrations. It is also noteworthy that protein expression levels of Cand2 are much lower than that of Cand1 in most cell systems. Based on the protein atlas 293T cells (used in the reported experiments) have the highest level of Cand2, and even in these cells it reaches only about 30% of Cand1 levels. In general, most of the cell-based experiments are performed in artificial configurations with overexpression of components. Because of the lack of any physiological context of an otherwise excellent biochemical/biophysical study of Cand2 function I think this manuscript is better suited for a more specialized journal and I cannot recommend the study for publication in Nature Communications.

Major points:
1) the most important concern is biological relevance of Cand2 in the context of SCF as outlined above. The authors may not have found the right tissue system, right stress conditions, etc. were Cand2 plays an important physiological role in SCF regulation. Alternatively, Cand2 may play a very local role in subcellular compartments as implied by the authors. It is however also possible that Cand2 is more important and effective in the context of other cullin-type ligases. It is critical to address this question and clearly demonstrate a role for Cand2 in SCF regulation in a physiological context (with endogenous protein levels) to support publication in Nat. Comm.
2) Fig. 1c would benefit from showing degradation rates for IkBa in WT cells
3) Fig. 1f should show the degradation kinetics of Irp2.
4) Fig. 1H: This is an important experiment and the authors should show quantitation of replicates with appropriate statistical analyses.

Other points:
1) The manuscript would benefit from some discussion or introduction of Cand2 function in other Cullin-ligases.
2) Fig. 1A may benefit from showing other cullins as well to see side-by-side comparison between of Cand1 and Cand2 binding preference in this cell system.
3) Fig. 1H: Can the authors comment on why Irp2 binds to un-neddylated Cul1? I would have expected specific binding to neddylated SCF-Fbxl5
4) Page 5 line 169: S3D? Shouldn't that be S3C?
5) Figure S2E: should be labelled Skp1/Skp2 not Skp2. It is a bit confusing because looking at the figure it seems that Skp2 displaces Cand2.
6) Page 6, line 191: Fig 2F? Shouldn't that be 2G?

Reviewer #3

(Remarks to the Author)
This manuscript by Wang et al. presents a detailed study of molecular, structural, kinetic, and cellular biology, comparing the functions of CAND2 and CAND1. The authors provide biochemical, proteomic, and kinetic evidence supporting the role of CAND2 as a substrate receptor exchange factor, capable of sustaining an adaptive cellular response akin to that of CAND1. It is noteworthy, however, that CAND2 exhibits markedly lower efficiency in this role, indicating that further investigations will be important to elucidate the evolutionary benefits of maintaining two such diverse exchange factors. The quality of the presented data is high and the research is timely. The primary limitation of this manuscript is the narrow scope of structural analyses, which precludes a direct comparison with the structural mechanism described for CAND1 last year. While a comprehensive conformational analysis of the CAND2-SCF complexes would be beyond the scope of this study, specific suggestions to enhance the presentation and discussion of the structural biology findings are given below.

Major points
- The structural work reported in this manuscript is of a binary complex between CUL1(/RBX1)-CAND2 and the obtained

structure has a single dominant conformation which closely resembles that previously reported for CUL1(/RBX1)-CAND1 both by x-ray crystallography and cryo-EM. However, very little could be learnt about the structural basis of the molecular mechanism of the exchange of FBPs from these structures. The complex dynamic conformations of the rock and rolling CAND1 including the clash with SKP1 and the importance of the pincers were only visualized by two groups when ternary complexes were reconstituted and studied by cryo-EM. It is thus very important to re-word the relevant sections of the main text of this work to make clear that the docking comparisons presented in Fig 4E and F are just a guess and the interpretation should be treated with the respective caution.

Minor points
- It would be helpful to explain why class 1 as opposed to class 3 (or both) were chosen for the initial refinement in the data processing illustrated in Fig S4A
- The complex used for the structural work is referred to as CUL1-CAND2. It would be sensible to explicitly indicate that RBX1 was present in the relevant section of the main text as well as Table 1
- It is technically wrong to refer to a molecular model built into a cryo-EM map at about 3.5A resolution as atomic.
- Relevant panels in Fig S4 need scale bars
- The discussion section starting in the last paragraph of p 9 seems to cite only one of the two studies describing the structural basis of the mechanism of CAND1. The two papers are fairly similar in this respect and it would be more balanced to also cite both.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I would like to thank the authors for their efforts to respond to the concerns raised during the first revision. In general, their responses are sound and satisfactory and address my initial concerns. However, there are a couple of comments that are in line with the general lack of biological/physiological context and relevance, also highlighted by reviewer #2:

1) I appreciate the addition of data using a second cell line (JMSU-1), it solidifies the findings on HEK293 cells. However, it seems like a wasted opportunity that the authors tested CAND1/2 expression in other two cell lines (TOV-112D and JOHS-4) and did not do any additional confirmation by, for example, trying to knockdown or knockout the CAND2 gene and looking at IRP2 level changes by FAC (as in Fig. 2B). Can the authors explain why they did not attempt that? Or if they did, was there a technical issue?

2) In Figure 2, using the new cell line JMSU-1, to identify additional substrates whose turnover rates are regulated by CAND2, the authors performed a dynamic SILAC proteome analysis. This is also a great addition but I also have a couple of comments regarding this experiment: A) The number of replicates, at least I have not seen it, seems to be missing and should be included. B) It would be interesting to know if the proteins whose halflives are affected by CAND2 are involved in certain biological/cellular processes. There are several bioinformatic tools to perform such types of analysis such as string.db, Panther GO, and others.

I believe that addressing these points would provide a more comprehensive context for the study's findings and enhance its impact. Thank you again for your efforts in revising this manuscript.

Reviewer #2

(Remarks to the Author)
The authors did a good job addressing my concerns with additional experiments. I support publication of the study but suggest that for figures 1E, 2C, and 2G some statistical evaluation is included, such as providing standard deviations for the calculated half-life and p-values when T1/2s are compared.

Reviewer #3

(Remarks to the Author)
All my comments have been addressed sufficiently well and the manuscript is of high enough quality and sufficiently broad interest to the ubiquitin and TPD fields to merit publication.

Point-by-point response to the reviewers' comments for Wang et al. "Molecular mechanisms of CAND2 in regulating SCF ubiquitin ligases".

We sincerely thank the editor and reviewers for their thoughtful evaluation of our manuscript. We appreciate the critical and constructive feedback from diverse perspectives, which has helped strengthen the study and improve the quality of the manuscript. To address the suggestions and concerns, we have incorporated new data (primarily in the new Figure 2, Figure S2, Table S1 and S2) and revised text (colored in blue in the manuscript). Please find our specific responses to the reviewers' comments below. All source data are provided in separate files.

Reviewer #1 (Remarks to the Author):

The article "Molecular mechanisms of CAND2 in regulating SCF ubiquitin ligases" by Kankan Wang et al., describes how CAND2, a CAND1 homolog, regulates the dynamic assembly of SCFs and how this impacts SCF-dependent protein degradation. To do so, they use biochemical, biophysical, and structural techniques. The general approach is similar to the one by Raistma et al 2017 (https://doi.org/10.1016/j.cell.2017.10.016), using CAND1/2 DKO 293 cells and assessing F-box exchange by similar biochemical assays and SILAC proteomics/interactomics. The main finding from this study is that CAND2, like CAND1, acts as an F-box protein exchange factor. Structural studies unveiled a similar interaction architecture between CAND1-CUL1 and CAND2-CUL1. However, the authors found that CAND2 has a much lower efficiency (up to 10 times lower) in exchanging the F-box proteins. They explain this by showing how the CAND2-SCF intermediate complex is less prone to dissociation than the CAND1-SCF intermediate.

The manuscript is well-written, and the design of the experiments, the quality of the data and the interpretation seem adequate. However, I have some comments/suggestions that may need to be addressed before the publication of this study in Nature Communications, in its actual form.

Response: We thank the reviewer for the overall positive feedback and helpful suggestions on how to improve this study. Our responses to the reviewer's points are detailed below.

1) One main concern is that all the experiments have been performed using one cell line (human; 293 cells) and overexpressing proteins or after incubation with recombinant proteins. This does not always explain what happens in the cells at endogenous levels or in different cells. I would recommend expanding these findings to additional cell lines and using cell models where the expression levels of CAND1 and CAND2 are different at the endogenous level.

Response: We appreciate this important concern and have conducted new experiments to address it (described in Line 146-154). We found that the CAND2 to CAND1 protein ratio is significantly higher in JMSU-1 cells compared to HEK293 cells (Fig. S2H). Therefore, we knocked out *CAND2* in JMSU-1 cells using CRISPR and repeated the iron-induced IRP2 degradation assay. The results are consistent with those from HEK293 cells: knocking out *CAND2* (*CAND2*-KO) increased the $t_{1/2}$ of IRP2 by 1.7-fold in JMSU-1 cells (Fig. 2B-C) and by 1.8-fold in HEK293 cells (Fig. 1D-E).

2) Also, it would be interesting to see (or to explain, based on the structures) how mutations associated with disease of CAND1/CAND2 affect the FBP exchange, SCF-dependent ubiquitylation and subsequent degradation of substrates.

Response: This is an interesting point that we have explored to some extent. In multiple studies reporting genetic variations/mutations of *CAND2* associated with diseases, altered levels of *CAND2* expression were observed. Three missense mutations have been found: V77A, Q408R, and H709R. V77 is near but not directly located on the CUL1-CAND2 interface; Q408 is distant from the interface; and H709 is located on the CUL1-CAND2 interface. All three residues are positioned at the ends of alpha-helices. Since it was reported that point mutations changing secondary structures are primarily found at the ends of secondary structure units (PMID: 27935949), it is possible that these mutations alter the CAND2 secondary structure and thereby affect the CUL1-CAND2 interaction. Alternatively, the H709R mutation may directly disrupt the CUL1-CAND2 interaction, while the V77A and Q408R mutations may affect the CAND2•SCF intermediate complex where CAND2 undergoes significant conformational changes.

3) The need to add 10x more CAND2 than CAND1 to induce FBP exchange is concerning. What are the protein concentrations and how do they compare to the actual concentration of these proteins in the cell? Would you expect these differences in concentration to be as high between different cellular compartments?

4) In line with the previous comment, there is not a clear explanation of how, despite the structural analogies, the authors think CAND2 is much less efficient than CAND1 in disassembling the SCF complex.

Response: These two comments are related, so we will discuss them together here.

First, to clarify the "1x" CAND concentration, we revised the text in Line 208: CAND1 was added to the DKO cell lysate "*at a ratio to ^{3xFLAG}CUL1 equivalent to the endogenous ratio (1x)*". In other words, "1x" equals the amount of CAND1 that would be present if WT HEK293 cells were used, estimated based on previous measurements (ref. 9). This mimics a condition where DKO is "rescued" by CAND1. Note that the [CAND1] (absolute concentration) in the cell lysate for the IP experiment is much lower than endogenous [CAND1], due to dilution by the lysis buffer—an intrinsic limitation of IP experiments. The "10x" concentration of CAND2 was chosen arbitrarily for experimental purposes. Essentially, we treated this IP experiment as a biochemical assay, and we expected to observe concentration-dependent effects. The conclusion from this experiment is that CAND2 can mediate multiple F-box protein exchange, but with lower efficiency than CAND1. This is a qualitative observation.

Quantitative insights into the lower exchange efficiency of CAND2 come from the kinetic measurements (summarized in Fig. 8E), particularly the K_M for CAND-catalyzed SCF dissociation (Fig. 8D). K_M represents the [CAND] at which half the k_{off} is achieved for CAND-accelerated SCF disassembly. While the k_{off} values for CAND1 and CAND2 are similar (0.35 s^{-1} vs. 0.29 s^{-1}), the K_M for CAND2 is much higher (~14x) than for CAND1. This explains why higher [CAND2] is required for F-box protein exchange in the IP assay mentioned above. These results and interpretations are discussed in Line 328-343.

This K_M also reflects the binding affinity of the intermediate CAND•SCF complex (CAND+SCF ↔ CAND•SCF → CAND•CUL1+FBP), where CUL1 is simultaneously bound by an

FBP and a CAND. Recent structural studies have captured the CAND1•SCF complex and characterized an ensemble of its conformations (ref. 11, 12). The higher K_M for CAND2 suggests a lower binding affinity of the CAND2•SCF complex, further indicating that the conformations of CAND2•SCF differ from those of CAND1•SCF in a way that makes FBP dissociation less favorable. This likely provides a molecular explanation for the lower exchange efficiency of CAND2. Note that the K_M discussed here is different from the K_D measured for the CAND•CUL1 complex, where CAND and CUL1 form a stable complex (shown in Fig. 5A-D) with much higher affinity. These discussions are elaborated in Line 361-379.

When considering the cellular environment where CAND2 functions as an exchange factor for SCF, the K_M (231 nM) serves as an important reference. In a cell or a cellular compartment, [CAND2] does not need to be 10x [CAND1] to promote FBP exchange. For example, when [CAND2] = K_M, CAND2 can disassemble SCF at k_{off} = 0.15 s^{-1} (half of 0.29 s^{-1}) and effectively promote FBP exchange. Even when [CAND2] < K_M, such as at 20 nM, SCF disassembly is still accelerated (k_{off} = 0.024 s^{-1}, increased by 28,235-fold; Fig. 8D, S10B), suggesting that CAND2 can still be effective in promoting FBP exchange under these conditions. In this scenario, cellular compartmentation could further enhance CAND2 activity by excluding CAND1 from the same compartment. To better reflect these considerations, we revised the text in Line 399-403 of the Discussion section.

5) It would be interesting to show how CUL1 Neddylation regulates the interaction between CAND2 and CUL1. I am not sure there is any experiment in this study that shows, in the same experiment, the two conditions (+/- MLN4924).

Response: We have now added Fig. S2B, which shows that CAND2 preferentially binds to unneddylated CUL1. This is the same as CAND1. Because CUL1•CAND2 is structurally similar to CUL1•CAND1, we expect that neddylation regulates CAND2 in a manner similar to that of CAND1. Discussions on how CAND2 cooperates with neddylation to control SCF assembly are included in the last paragraph on Page 11 (starting at Line 380).

6) Similarly to what has been done in Figure 1 with TNF-a and IkBa levels and FAC and IRP2 levels, it would have been interesting to investigate what additional SCF substrates are regulated by CAND1/CAND2 by performing a total proteome analysis in CAND1 KO and CAND2 KO cells (+/- MLN4924) and the presence/absence of a proteasome inhibitor (for example MG132 or Bortezomib). This would have informed of the potential different cellular functions of both proteins. This remains an important question in my opinion.

Response: We agree with the reviewer that understanding which CRL target proteins are differentially affected by CAND1 vs. CAND2 is important. Indeed, we are establishing physiologically relevant cell models to address this question in an independent project. The focus of this study is twofold: 1) to establish that *CAND2*-KO has an impact on the assembly and activity of cellular SCFs, and 2) to characterize the biochemical properties of CAND2 in the context of SCF regulation. We chose SCFs as the primary focus in this study due to their advanced conceptual understanding and the availability of research tools. We understand that the data in our initial manuscript were not strong enough to achieve the first goal, so we added two sets of experiments for improvement, summarized as follows:

First, to identify additional SCF substrates regulated by CAND2, as the reviewer suggested, we measured global protein turnover rates in JMSU-1 cells +/- *CAND2*-KO using a dynamic SILAC approach (ref. 44). The results are presented in Fig. 2D-G and described in Line 155-170. Briefly, among the proteins detected by mass spectrometry whose half-lives were affected by *CAND2*-KO, some were previously reported as SCF substrates. We followed up with BCL10 and conducted a cycloheximide chase experiment, confirming that its half-life was increased by 1.4-fold in *CAND2*-KO cells.

Second, in response to Reviewer 2's comment, we compared levels of endogenous SCFs assembled in WT vs. *CAND2*-KO HEK293 cells, using previously established methods (ref. 9). The results are presented in Fig. 2A and described in Line 136-145, showing that *CAND2*-KO led to changes in the assembly of 13 SCFs out of 35 detected.

7) In the SILAC proteomics study in Figure 2, DKO cells and DKO3xFlag-CUL1 cells were labelled with heavy and light SILAC medium, respectively. Then mixed and 3xFlag-CUL1 was immunoprecipitated. MS analysis of the H/L ratios were used as an estimation of FBP changes. A concern is that the total proteome composition of the two cell lines, DKO and DKO overexpressing CUL1, may be quite different and therefore affect the outcome of the experiment. Has this been investigated/addressed somehow?

Response: The reviewer has raised an important concern. Potential differences in proteome composition and stoichiometry between different cell lines were also key considerations in our experimental design. Therefore, ^{3xFLAG}CUL1 was not overexpressed. Instead, the 3xFLAG tag was introduced at the N-terminus of endogenous CUL1 via CRISPR knock-in in DKO cells. We have emphasized this design by specifying "endogenous ^{3xFLAG}CUL1" in the text and figures (Lines 97, 138, 428; Figs. 2A, 3A, 3C, 4A, S2B, S2D).

8) In the same experiments, it would be nice to show if CAND1/CAND2 affect the binding of non-F-box proteins to CUL1. Perhaps as a supplementary figure/table.

Response: We have now included the full dataset in Table S3 and mentioned it in Line 201.

9) Figure 1H. It would be beneficial to quantify and plot the ratios n8CUL1/FlagIRP2 as it is not easy to appreciate (differences look quite small).

Response: We have now added a bar graph displaying the ratios with two replicates (Fig. 1H). The WT group shows the largest change, which is easier to perceive visually.

10) Fig S2 B and E, when on the top of the blot image says SKP2 incubation, does it mean SKP1*SKP2?

Response: We have changed the label from SKP2 to SKP1•SKP2 wherever applicable.

Reviewer #2 (Remarks to the Author):

The manuscript by Wang et al. attempts to dissect a potential role for the exchange factor Cand2 in SCF dynamics. The homolog Cand1 has been extensively studied and is generally considered the main exchange factor for cullin-1 ligases (SCF). Cand1 is necessary for a dynamic cycle of Skp1/Fbox protein interaction with the core ligase so that a complete repertoire of the up to 69 F-box proteins are represented as SCF complexes. This system also integrates substrate abundance to adjust SCF subtype representation to substrate demands. The authors explore a potential similar role for the Cand1 homolog Cand2 in SCF regulation. The manuscript reports a thorough biochemical/biophysical characterization with the conclusion that Cand2 can catalyze Skp1/Fbox exchange of SCF complexes in vitro, albeit with different kinetics. The in vitro experiments are excellent and of high quality. Where the study falls short is to clearly demonstrate that Cand2 has a physiological role in regulating SCF ligases. There is one set of experiments that tries to address this question by analyzing degradation of IkBa or Irp2. These experiments are performed in an artificial system where CAND1 and CAND2 are knocked out and complementation by ectopic expression of CAND1 or CAND2 was performed. Both proteins can partially complement the degradation defect of the double knockout cells. These experiments imply that Cand2 CAN perform similar functions as Cand1 but does not address whether Cand2 DOES perform these functions under physiological conditions. In fact, extensive and well-designed MS-based experiments would suggest otherwise, because observed effects are mainly driven by Cand1 and not by Cand2 at equal concentrations. It is also noteworthy that protein expression levels of Cand2 are much lower than that of Cand1 in most cell systems. Based on the protein atlas 293T cells (used in the reported experiments) have the highest level of Cand2, and even in these cells it reaches only about 30% of Cand1 levels. In general, most of the cell-based experiments are performed in artificial configurations with overexpression of components. Because of the lack of any physiological context of an otherwise excellent biochemical/biophysical study of Cand2 function I think this manuscript is better suited for a more specialized journal and I cannot recommend the study for publication in Nature Communications.

Response: We thank the reviewer for the insightful summary and thoughtful discussion of our study. Based on the reviewer's comments, we realized that we did not make it sufficiently clear that the IRP2 degradation work was conducted in HEK293 cells with *CAND2* knocked out by CRISPR. To clarify this, we have revised the text in Line 117 and 119-120, to improve the presentation. We fully agree with the reviewer on the importance of investigating the role of CAND2 in a physiologically relevant context, along with studying its biochemical properties. More responses to the reviewer's comments are detailed below.

Major points:

1) the most important concern is biological relevance of Cand2 in the context of SCF as outlined above. The authors may not have found the right tissue system, right stress conditions, etc. were Cand2 plays an important physiological role in SCF regulation. Alternatively, Cand2 may play a very local role in subcellular compartments as implied by the authors. It is however also possible that Cand2 is more important and effective in the context of other cullin-type ligases. It is critical to address this question and clearly demonstrate a role for Cand2 in SCF regulation in a physiological context (with endogenous protein levels) to support publication in Nat. Comm.

Response: We appreciate the reviewer's critical point regarding the need to establish a physiological role for CAND2 in SCF regulation. In addition to the evaluation of iron-induced IRP2 degradation in HEK293 cells with *CAND2* knockout (*CAND2*-KO) as mentioned above, we followed Reviewer 1's suggestion and repeated the experiment using JMSU-1 cells +/- *CAND2*-KO. The results were consistent: *CAND2*-KO increased the $t_{1/2}$ of IRP2 by 1.8-fold in HEK293 cells (Fig. 1D-E) and by 1.7-fold in JMSU-1 cells (Fig. 2B-C). Additionally, the $t_{1/2}$ of BCL10, a target of $SCF^{\beta\text{-}TRCP}$, was increased by 1.4-fold in *CAND2*-KO JMSU-1 cells (Fig. 2F-G, Line 164-169).

Furthermore, we assessed the effect of *CAND2*-KO on the levels of endogenous SCFs assembled in HEK293 cells expressing endogenous ^{3xFLAG}CUL1, using an IP procedure in the presence of a "sponge" protein to suppress post-lysis FBP exchange, as reported previously (ref. 9). The results are presented in Fig. 2A and described in Line 136-145. Briefly, 13 of 35 detected SCFs showed significant changes in response to *CAND2*-KO. This result directly illustrates an impact of CAND2 on SCF regulation.

We have also added data showing the binding of $CAND1^{HA}$ and $CAND2^{HA}$ to cullins other than CUL1 (Fig. S2C) and added related discussion (see also responses below). [Information redacted due to its confidentiality]

2) Fig. 1c would benefit from showing degradation rates for IkBa in WT cells

Response: We have now added data for IκBα degradation in WT vs. DKO cells in Fig. S2E-F. In summary, defective IκBα degradation in DKO cells, as well as its rescue by reintroducing $CAND1^{HA}$, were reported previously (ref. 8). The new finding reported here is that introducing $CAND2^{HA}$ also rescues the defective IκBα degradation in DKO cells (Fig. 1B-C).

3) Fig. 1f should show the degradation kinetics of Irp2.

Response: We have now shown the degradation kinetics of IRP2 in WT and *CAND* knockout cells in Figure 1E.

4) Fig. 1H: This is an important experiment and the authors should show quantitation of replicates with appropriate statistical analyses.

Response: We have now added a bar graph displaying the ratios of $CUL1^{N8}/^{FLAG}$IRP2 with two replicates (Fig. 1H).

Other points:

1) The manuscript would benefit from some discussion or introduction of Cand2 function in other Cullin-ligases.

Response: In the discussion section, Line 412-415, we added "*Additionally, both CAND1 and CAND2 bind multiple cullins (Figure S2C), and CAND1 is known to promote CRL3- and CRL4-mediated protein ubiquitination. Thus, CAND2 is expected to affect protein ubiquitination through regulating CRLs beyond SCF*".

2) Fig. 1A may benefit from showing other cullins as well to see side-by-side comparison between of Cand1 and Cand2 binding preference in this cell system.

Response: We have added Fig. S2C, which shows the binding of $CAND1^{HA}$ and $CAND2^{HA}$ to three other cullins in the HEK293 cell lysate.

3) Fig. 1H: Can the authors comment on why Irp2 binds to un-neddylated Cul1? I would have expected specific binding to neddylated SCF-Fbxl5

Response: We agree with the reviewer, and we also anticipated that only or primarily the neddylated CUL1 would be co-IP'd with IRP2. We repeated this experiment a few times with the WT and DKO cells using different buffers and anti-FLAG beads, and we consistently observed the band representing un-neddylated CUL1. Importantly, the level of un-neddylated CUL1 did not change before and after FAC treatment; only the neddylated CUL1 did. This leads us to believe that un-neddylated CUL1 stays in the IP samples in an iron (or FBXL5)-independent manner.

5) Figure S2E: should be labelled Skp1/Skp2 not Skp2. It is a bit confusing because looking at the figure it seems that Skp2 displaces Cand2.

Response: We have changed the label from SKP2 to SKP1•SKP2 wherever applicable.

4) Page 5 line 169: S3D? Shouldn't that be S3C?
6) Page 6, line 191: Fig 2F? Shouldn't that be 2G?

Response: Thanks for pointing them out. When revising the manuscript, we have checked all figure and table numbers to ensure they are correctly referenced.

Reviewer #3 (Remarks to the Author):

This manuscript by Wang et al. presents a detailed study of molecular, structural, kinetic, and cellular biology, comparing the functions of CAND2 and CAND1. The authors provide biochemical, proteomic, and kinetic evidence supporting the role of CAND2 as a substrate receptor exchange factor, capable of sustaining an adaptive cellular response akin to that of CAND1. It is noteworthy,

however, that CAND2 exhibits markedly lower efficiency in this role, indicating that further investigations will be important to elucidate the evolutionary benefits of maintaining two such diverse exchange factors. The quality of the presented data is high and the research is timely. The primary limitation of this manuscript is the narrow scope of structural analyses, which precludes a direct comparison with the structural mechanism described for CAND1 last year. While a comprehensive conformational analysis of the CAND2-SCF complexes would be beyond the scope of this study, specific suggestions to enhance the presentation and discussion of the structural biology findings are given below.

We thank the reviewer for the helpful and actionable feedback. We have addressed all the comments as explained below.

Major points

- The structural work reported in this manuscript is of a binary complex between CUL1(/RBX1)-CAND2 and the obtained structure has a single dominant conformation which closely resembles that previously reported for CUL1(/RBX1)-CAND1 both by x-ray crystallography and cryo-EM. However, very little could be learnt about the structural basis of the molecular mechanism of the exchange of FBPs from these structures. The complex dynamic conformations of the rock and rolling CAND1 including the clash with SKP1 and the importance of the pincers were only visualized by two groups when ternary complexes were reconstituted and studied by cryo-EM. It is thus very important to re-word the relevant sections of the main text of this work to make clear that the docking comparisons presented in Fig 4E and F are just a guess and the interpretation should be treated with the respective caution.

Response: Thanks for raising this critical point. Following the reviewer's suggestion, we have added in Line 262-266: "*Nevertheless, it is important to note that the observations based on docking comparisons (Figure 5E-F) are limited by the static nature of known complex structures. Elucidating the structural basis of the CAND2-mediated SCF disassembly process will require further studies that capture the CAND2•SCF intermediate complexes in action.*"

Minor points

- It would be helpful to explain why class 1 as opposed to class 3 (or both) were chosen for the initial refinement in the data processing illustrated in Fig S4A

Response: We have added an explanation in the Method section (Line 544-547): "*Only one class with the highest number of particles was selected for the subsequent processing. This stringent selection was performed to avoid slight differences in structural conformation, which we find affects the resolution of the overall complex.*".

- The complex used for the structural work is referred to as CUL1-CAND2. It would be sensible to explicitly indicate that RBX1 was present in the relevant section of the main text as well as Table1

Response: We have updated the text on Line 246-247 to state: "*Single-particle cryo-EM imaging of purified CAND2•CUL1•RBX1 yielded a final map*…". On the bottom of Table S4, we have added:

"*Note: RBX1 is present in the complex but not modeled.*". In the Method section (Line 531-532), we now specify, "RBX1 was co-purified but not modeled in the structure."

- It is technically wrong to refer to a molecular model built into a cryo-EM map at about 3.5A resolution as atomic.

Response: Thanks for pointing out this error. We have corrected the manuscript by removing the term "atomic" (Line 248, Line 550, and the figure legend for Fig. S5C).

- Relevant panels in Fig S4 need scale bars

Response: We have included a scale bar in the updated Figure S5C.

- The discussion section starting in the last paragraph of p 9 seems to cite only one of the two studies describing the structural basis of the mechanism of CAND1. The two papers are fairly similar in this respect and it would be more balanced to also cite both.

Response: We have carefully reviewed all the references in the Discussion section and ensured that both structural studies are now cited together.

Point-by-point response to reviewer comments (2nd round) for Wang et al. "Molecular mechanisms of CAND2 in regulating SCF ubiquitin ligases".

We thank the editor and reviewers for assessing our manuscript again. We are pleased to receive positive feedback on our revised manuscript. To address the remaining suggestions and concerns, we have included additional information (in Figure 2, Figure S2, and new Table S3) and revised text (highlighted in blue in the manuscript). Please find our specific responses to the reviewers' comments below. We have also updated and provided the source data in separate files.

Reviewer #1 (Remarks to the Author):

I would like to thank the authors for their efforts to respond to the concerns raised during the first revision. In general, their responses are sound and satisfactory and address my initial concerns. However, there are a couple of comments that are in line with the general lack of biological/physiological context and relevance, also highlighted by reviewer #2:

1) I appreciate the addition of data using a second cell line (JMSU-1), it solidifies the findings on HEK293 cells. However, it seems like a wasted opportunity that the authors tested CAND1/2 expression in other two cell lines (TOV-112D and JOHS-4) and did not do any additional confirmation by, for example, trying to knockdown or knockout the CAND2 gene and looking at IRP2 level changes by FAC (as in Fig. 2B). Can the authors explain why they did not attempt that? Or if they did, was there a technical issue?

Response: While we did not anticipate technical issues in generating *CAND2* single knockout (KO) cells (CAND1/2 double knockout cells are more likely to be lethal), we chose not to generate *CAND2*-KO cells using TOV-112D or JOHS-4. We considered several factors when planning these experiments. First, we used knockout rather than knockdown of *CAND2* because, as previously reported (PMID: 29499133), even low levels of CAND can be sufficient to support SCF-mediated protein degradation. Given the time and effort required for CRISPR-based knockouts (e.g., single colony isolation, mutant identification and confirmation), it was more practical to focus on a smaller number of cell lines. In addition, cell lines with long doubling time were not chosen for this work. Second, following the reviewer's previous suggestion, we sought a cell line whose relative levels of CAND2 vs. CAND1 notably differ from that in HEK293. As detailed in the manuscript (starting at Line 147), we began by comparing CAND2/CAND1 transcript ratios across various cell lines using data from the Human Protein Atlas. This ratio is 0.28 in HEK293 (defined as 1x), and is 1.2x in TOV-112D, 2.1x in JMSU-1, and 2.7x in JHOS-4. We then examined the protein ratios in these cell lines and found that TOV-112D and JHOS-4 had modestly elevated CAND2/CAND1 protein ratios (1.8x and 1.7x of HEK293, respectively), while JMSU-1 showed 4.3x of HEK293 (Fig. S2H-I). Based on these findings, we selected JMSU-1 cells and generated *CAND2*-KO lines, which subsequently revealed altered protein degradation rates as described in the manuscript.

We do not plan to repeat the IRP2 degradation experiment in TOV-112D or JOHS-4 cells, because these additional results would likely either echo those in HEK293/JMSU-1 or indicate cell-type-specific effects of CAND2 on protein degradation in cancer cell lines, neither of which is expected to provide significant new insights beyond our current findings.

2) In Figure 2, using the new cell line JMSU-1, to identify additional substrates whose turnover rates are regulated by CAND2, the authors performed a dynamic SILAC proteome analysis. This is also a great addition but I also have a couple of comments regarding this experiment: A) The number of replicates, at least I have not seen it, seems to be missing and should be included. B) It would be interesting to know if the proteins whose halflives are affected by CAND2 are involved in certain biological/cellular processes. There are several bioinformatic tools to perform such types of analysis such as string.db, Panther GO, and others.

Response: We appreciate the reviewer's helpful comments and suggested bioinformatic tools. A) In addition to the description in the Method section, we have now added in the figure legend for Fig. 2D that "cell samples were prepared in duplicate". B) We analyzed the proteins with altered $t_{1/2}$ in *CAND2*-KO cells using STRING. The results are presented in Fig. 2F and Table S3, and the relevant text is updated (starting at Line 160). In summary, "these proteins were categorized into a broad spectrum of biological pathways based on GO terms", which aligns with the understanding that CAND2 regulates various CRLs and influences a wide range of biological processes.

I believe that addressing these points would provide a more comprehensive context for the study's findings and enhance its impact. Thank you again for your efforts in revising this manuscript.

Thank you for your constructive feedback and for helping us improve our study and manuscript!

Reviewer #2 (Remarks to the Author):

The authors did a good job addressing my concerns with additional experiments. I support publication of the study but suggest that for figures 1E, 2C, and 2G some statistical evaluation is included, such as providing standard deviations for the calculated half-life and p-values when T1/2s are compared.

Response: Thanks for the support and the helpful suggestion. We have now added the statistical analyses as new figures (Fig. S2G, S2J, and S2M). In line with the reviewer's earlier suggestion, we graphed the kinetics of protein degradation in Fig. 1E, 2C and 2H (previously 2G). These graphs show average protein levels at different time points, fit to a single-exponential decay model, yielding one $t_{1/2}$ per experimental group. As such, these $t_{1/2}$ values cannot be used for statistical analyses. To address this limitation, we re-analyzed the datasets by fitting the data individually for each replicate to estimate $t_{1/2}$ values for each experimental group. This approach allowed us to calculate standard deviations and perform statistical analyses to obtain p-values. Source data for these new figures are also included.

Reviewer #3 (Remarks to the Author):

All my comments have been addressed sufficiently well and the manuscript is of high enough quality and sufficiently broad interest to the ubiquitin and TPD fields to merit publication.

Thank you very much!