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Co-regulation Proteomics Reveals Substrates and Mechanisms of APC/C-dependent Degradation

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Transaction Report:

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

1st Editorial Decision 12 July 2013

Thank you for submitting your manuscript on cell cycle proteomics identification of APC/C substrates for consideration by The EMBO Journal. It has now been seen by three expert referees, whose comments are copied below. As you will see, all referees appreciate the well-desgined and comprehensive mass spectrometry approach and dataset, as well as the follow-up work on phosphorylation-dependent degradation of KIF1C. However, there are also concerns - stated most explicitly by referee 2 - with the overall amount of immediate new biological insights, especially in light of the substantial precedent for both vertebrate and yeast kinesin family members as APC/C substrates, and given the somewhat preliminary follow-up characterization of in vivo APC/C regulation of KIF1C and its cellular consequences.

Given these recommendation and in light of the strong proteomics approach and dataset, we would like to offer publication of a revised manuscript as a 'Resource Article' (note that the 'Resource' labeling is solely intended to point the appropriate target readership to articles that may be of particular methodological or dataset value, and to advertise the journal's interest in receiving and considering such studies). Such a revision should address and incorporate the various specific and minor comments raised by all three referees; but it would not be essential address the major experimental requests raised by referee 2 in full - for detail please see his/her referee report below, where I have added comments on these issues. Additional editorial issues to address will be to include Material & Methods in the main manuscript (as our guidelines do not permit delegating this section entirely into the supplement), and to upload also the Supplementary Table 1 with peptide identifications and quantifications at this stage.

Please note that it is our policy to allow only a single round of major revision, and that it will therefore be important to carefully and comprehensively respond to all points at this stage. We generally allow three months as standard revision time, and it is our policy that competing manuscripts published during this period will have no negative impact on our final assessment of your revised study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension. Also, please do not hesitate to contact me if you should need any other clarifications regarding this decision, the reviews or your revision.

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

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REFEREE REPORTS:

Referee #1:

In this manuscript Singh et al. used a quantitative mass spectrometry-based proteomics approach to obtain cell cycle dependent abundance profiles for >4400 proteins. They monitored the dynamics of known APC substrates, and by using correlation-based protein profiling they identified dozens of novel, putative substrates of APC ubiquitin ligase. Furthermore, the authors used a well-established in-vitro ubiquitylation assay to validate several of the putative substrates identified in their screen. Among novel APC substrates they identify several kinesins. They showed that KIF1C is degraded in an APC-dependent manner and that its degradation regulates the bipolar spindle formation and required for proper cell division. Importantly, the authors identified a D-box degron in KIF1C and identified Ser6 as a CDK1-dependent phosphorylation in this degron.
This is an elegantly performed work, manuscript is well-written, and data are appropriately discussed. The manuscript provides important insights into the dynamics of protein abundance in cell cycle, identifies several novel substrates of APC, and demonstrates the function of phosphorylation-dependent degradation of KIF1C in cell cycle. With a few minor revisions, the manuscript can be recommended accepted for publication.
Comments:
1. The accuracy of TMT-based quantification can be compromised by co-eluting peptides. For quantification of TMT-labeled peptides, what fraction of parent ion intensity was used as cut-off? This is not a major weakness of the manuscript; nevertheless the readers should be made aware of.
2. Figure 1, panels "F" is missing (or it is mislabeled).
3. In figure 2, the panel C is not very clear and not much informative. For example, what it mean by word "like" "highly" "factor." This panel can be removed or presented differently.

Referee #2:

The Anaphase Promoting Complex/Cyclosome (APC/C) is a large E3 ubiquitin ligase that controls mitotic progression by targeting specific proteins for degradation in a temporal manner. Understanding the regulation of the APC/C and knowing its substrates is thus of importance for understanding mitotic regulation. In this study by Singh et al a proteomic approach is used to identify APC/C substrates in an unbiased and global manner. This leads to the identification of the kinesin family of proteins as an abundant class of APC/C substrates. Further characterization of how CDK1 phosphorylation of a D-box in the kinesin KIFC1 controls its degradation is performed.
The understanding of which proteins are substrates of the APC/C is of great importance and the mass spectrometry approach taken by Singh et al is novel and well performed. Given their strong mass spectrometry set-up it would have been informative to combine this with specific APC/C inhibition for instance through depletion of an APC/C subunit or co-activator which in a more global manner could have validated their proposed substrates. Unfortunately the study suffers from novelty as a number of kinesins as well as other microtubule motor proteins have been shown to be APC/C substrates and just expanding the list does not provide much novel insight into APC/C biology. The regulation of KIF1C degradation by CDK1 phosphorylation of its D-box is interesting and further insight into the mechanism would make this a more interesting study in general.

In its present state I do not find it suitable for publication in EMBO J.

Major concerns:

1) At least 2 kinesins have been shown to be APC/C substrates: Kif22 (Feine et al 2007) and Kif18A (Sedgwick et al 2013) and in addition numerous other spindle associated proteins are also reported APC/C substrates (see for example Song & Rape 2010) so their statement that they find kinesins as APC/C substrates surprising and novel is clearly questionable. Furthermore a previous bioinformatic approach looking for D-boxes and KEN boxes suggested that Kif4A, KIFC1, KIF13B and KIF3B are potential APC/C substrates (Liu et al 2012) again questioning the novelty of this statement.
[EDITOR COMMENT: please see also referee 3's point 5 on this, and carefully revise novelty claims regarding kinesin substrates throughout the manuscript. You may also consider a revision of the manuscript title in this light, maybe placing stronger emphasis on the application of protein profile similarity screening]

2) For their validation of a number of kinesins as APC/C substrates they use a HeLa S3 G1 extract were the APC/C-Cdh1 is active and add in vitro translated substrate. It appears to me that there is still on-going translation of the substrates in these assays for instance Figure 4A-B seriously complicating the interpretation. Furthermore the biological relevance of this assay is questionable and instead validation should be performed in an in vivo like assay were co-activators or APC/C activity is inhibited by RNAi and then the temporal degradation pattern of the kinesins explored by western blot in a temporal manner upon mitotic exit. This will also address more precisely which co-activator is responsible for the targeting of the kinesin as for instance Kif18A has been proposed to be an APC/C-Cdc20 substrate and not an APC/C-Cdh1 substrate. Performing these experiments will help strength their arguments.
[EDITOR COMMENT: I feel that this will not be essential for this study, given that the Ayad et al assay used for validation has been successfully applied before, and given that the requested in vivo assays and tests of APC/C activator roles may go beyond the scope of the current work as a primary proteomics study]

3) The experiments in Figure 6A is an end-point assay and should instead be performed as the experiments in Figure 4A-B that is with a time-course and quantification. In its current state one cannot come to any conclusions regarding phosphorylation of Ser6 in degradation by APC/C-Cdh1. Further in vivo validation of the role of Ser6 phosphorylation in regulating KIF1C degradation should be performed for instance by live cell degradation assays using fluorescently labeled proteins to support their conclusions.
[EDITOR COMMENT: while in vivo degradation assays will not be necessary, I think the end-point assays should indeed be complemented by some time course experiments, at least for the key versions WT-DM-S6D-S6A]

4) The fact that according to the authors KIF1C is an APC/C-Cdh1 substrate and their profiling also suggests it being a "late" APC/C substrate conceptually seriously questions the relevance of the experiments performed in Figure 6C-D. How can degradation late in mitosis be relevant for an earlier event? Also previous work using Cdh1 RNAi failed to observe any defects in early spindle formation using live cell assays so this discrepancy needs to be more carefully addressed (see Floyd et al 2008). This is clearly an over-expression assay (as they show in figure S6) that by no means addresses whether the degradation of KIF1C has anything to do with spindle formation. What needs to be done is to look at spindle formation in cells expressing endogenous levels of KIF1C and non-degradable KIF1C and then see if degradation influences spindle formation.
[EDITOR COMMENT: I agree with these concerns, and feel that - unless you are inclined to repeat these experiments in knock-down-rescue conditions without overexpression - the conclusions from these cellular assays should be much more carefully stated and deemphasized. Furthermore, in the absence of such stronger data I feel that the model in Fig 7B is not sufficiently substantiated and should be removed.]

Minor points:
1) In Abstract they use APC/C but in the manuscript APC. This should be consistent throughout.
2) In the abstract the line "KIF1C degradation is dependent on a stabilizing CDK1 phosphorylation" should be rephrased.
3) Page 4: In the statement concerning the temporal degradation of SECURIN and CYCLIN the

original papers from the Pines lab should be cited as they were the ones determining the exact timing.
4) Page 5: "function of kinesins in mitosis made these proteins as..." should be rephrased.
5) Page 6: The claim that following a release from a double thymidine release can generate defined cell populations reflecting metaphase to anaphase etc should be removed and rephrased as there is no way this is the case. [EDITOR COMMENT: please rephrase with the appropriate cautionary notes]
6) Page 9: Kif18A has also been described as an APC/C substrate so first line is incorrect.
7) Page 11: Holt et al and Littlepage and Ruderman references are at the moment separate.
8) In their discussion on D-box and KEN box recognition the latest work from the Barford lab (He et al 2013) should be cited.
9) Page 19: Last line. What is supporting this "growing notion"?
10) Page 20: Arg6 should be Arg5.

Referee #3:
In this manuscript, Singh et al. do a comprehensive mass spec analysis of synchronized cells progressing through the cell cycle. They then identify proteins with profiles matching that of APC substrates. Analysis of this set of substrates identifies a large set of kinesins. They go on to do a very thorough analysis of these potential substrates and show that several are APC substrates in vivo and in vitro. This is an extremely well done study. The experiments are largely beautifully performed, and they have gone pretty deep in their analysis. In fact, while one can always come up with more to do, in my opinion, the MS is suitable for publication without any experiments. Arguably, a reviewer could ask them to show that the S6A mutant is prematurely degraded, but I don't think this is necessary given all the work already included. I do however, have a few small comments:

1. Figure 1F seems to be missing (and isn't really referred to). I assume this is just a "lettering" error.

2. While I understand the basic idea to figure 1G-J, it seems like I must be missing something for the following reason. My understanding is that each of the gray lines is one peptide. However, even if they observed 10 unique peptides, and they graphed the three runs of the experiment separately, this would be only 30 gray lines. 1I and 1J look like hundreds. This is only an issue in that it makes me doubt that i understand what was done.

3. In figure 2C, the word "protein" was scored twice, with different occurrences. It isn't clear how this could be the case. Also, this is a rather unorthodox way to search. It seems better to search for functional domain (kinase, g protein, metabolism, etc)

4. In the legend for figure 3A it says that "various" kinesins were ranked. They should be more specific. How were these chosen. Is this all the kinesins for which peptides were detected?

5. The authors might want to note that the kinesins Cin8 and Kip1 and the kinesin-associated proteins Cik1 have been shown to be APC substrates in yeast, as have the spindle proteins Ase1 and Fin1.

1st Revision - authors' response 11 October 2013

Detailed point by point response to the reviewers' comments:

Referee #1:
1. The accuracy of TMT-based quantification can be compromised by co-eluting peptides. For quantification of TMT-labeled peptides, what fraction of parent ion intensity was used as cut-off? This is not a major weakness of the manuscript; nevertheless the readers should be made aware of.
Response to 1.1: We did not apply any cut-offs in the MS1 or MS2 domain, which we now clarify in the Materials and Methods section. Although the co-elution can have an effect on the accuracy of TMT, we are less concerned with the absolute accuracy as we are clustering based on normalized, relative abundance profiles in order to identify APC substrate candidate. We did not derive accurate

quantitative information (e.g. the abundance of protein X was decreased by 70% from phase 1 to phase 2), which would have required accurate quantification.

2. Figure 1, panels "F" is missing (or it is mislabeled).
Response to 1.2: We apologize for this oversight. Figure 1 was mislabeled resulting in the apparent 'loss' of panel F. We completely revised Figure 1 in order to streamline the manuscript and in the process corrected the mislabeling.

3. In figure 2, the panel C is not very clear and not much informative. For example, what it mean by word "like" "highly" "factor." This panel can be removed or presented differently.
Response to 1.3: Following the suggestion from Reviewer 1, we moved this panel to the supplementary material and instead clarify the result of our text analysis in the main text ("This analysis resulted in 'kinase' (25 occurrences, rank 6) and 'kinesin' (22 occurrences, rank 7) as the two most frequent protein family names following generic terms including 'protein', 'isoform', 'similar', 'flj', 'cdna', 'like', and 'highly' co-occupying ranks 1 to 6.")

Referee #2:
1) At least 2 kinesins have been shown to be APC/C substrates: Kif22 (Feine et al 2007) and Kif18A (Sedgwick et al 2013) and in addition numerous other spindle associated proteins are also reported APC/C substrates (see for example Song & Rape 2010) so their statement that they find kinesins as APC/C substrates surprising and novel is clearly questionable. Furthermore a previous bioinformatic approach looking for D-boxes and KEN boxes suggested that Kif4A, KIFC1, KIF13B and KIF3B are potential APC/C substrates (Liu et al 2012) again questioning the novelty of this statement.
[EDITOR COMMENT: please see also referee 3's point 5 on this, and carefully revise novelty claims regarding kinesin substrates throughout the manuscript. You may also consider a revision of the manuscript title in this light, maybe placing stronger emphasis on the application of protein profile similarity screening]
Response to 2.1: The manuscript was revised as requested to reference the kinesin paper(-s) mentioned. Furthermore, we deemphasized the aspect of kinesins as a family of APC/C substrates. This is also reflected by a new title. Changes to this effect will be found throughout the revised manuscript.
With respect to the Liu et al. paper, we would like to highlight the fact that their tool is not barely screening tool to identify novel APC substrates, but instead is an excellent bioinformatic tool to identify D- and KEN-boxes post hoc in identified substrates. This notion is underscored by the fact that GPS-ARM identified >3,800 proteins with relevant APC substrates and >1400 proteins with relevant KEN boxes. It remains to be seen whether indeed >5000 proteins are indeed APC substrates as predicted by GPS-ARM.

2) For their validation of a number of kinesins as APC/C substrates they use a HeLa S3 G1 extract were the APC/C-Cdh1 is active and add in vitro translated substrate. It appears to me that there is still on-going translation of the substrates in these assays for instance Figure 4A-B seriously complicating the interpretation. Furthermore the biological relevance of this assay is questionable and instead validation should be performed in an in vivo like assay were co-activators or APC/C activity is inhibited by RNAi and then the temporal degradation pattern of the kinesins explored by western blot in a temporal manner upon mitotic exit. This will also address more precisely which co-activator is responsible for the targeting of the kinesin as for instance Kif18A has been proposed to be an APC/C-Cdc20 substrate and not an APC/C-Cdh1 substrate. Performing these experiments will help strength their arguments.
[EDITOR COMMENT: I feel that this will not be essential for this study, given that the Ayad et al assay used for validation has been successfully applied before, and given that the requested in vivo assays and tests of APC/C activator roles may go beyond the scope of the current work as a primary proteomics study]
Response to 2.2: Following the suggestion from the Editor, we consider this request as beyond the scope of this manuscript.

3) The experiments in Figure 6A is an end-point assay and should instead be performed as the experiments in Figure 4A-B that is with a time-course and quantification. In its current state one cannot come to any conclusions regarding phosphorylation of Ser6 in degradation by APC/C-Cdh1. Further in vivo validation of the role of Ser6 phosphorylation in regulating KIF1C degradation

should be performed for instance by live cell degradation assays using fluorescently labeled proteins to support their conclusions.
[EDITOR COMMENT: while in vivo degradation assays will not be necessary, I think the end-point assays should indeed be complemented by some time course experiments, at least for the key versions WT-DM-S6D-S6A]
Response to 2.3: We thank the reviewer for this suggestion. We carried out degradation experiments with time course sampling for the different phosphorylation mutants instead of end point assays. We updated Figure 6 to incorporate the new data. The results of two additional biological repeats of the time course degradation assay are shown in Figure S5A.

4) The fact that according to the authors KIF1C is an APC/C-Cdh1 substrate and their profiling also suggests it being a "late" APC/C substrate conceptually seriously questions the relevance of the experiments performed in Figure 6C-D. How can degradation late in mitosis be relevant for an earlier event? Also previous work using Cdh1 RNAi failed to observe any defects in early spindle formation using live cell assays so this discrepancy needs to be more carefully addressed (see Floyd et al 2008). This is clearly an over-expression assay (as they show in figure S6) that by no means addresses whether the degradation of KIF1C has anything to do with spindle formation. What needs to be done is to look at spindle formation in cells expressing endogenous levels of KIF1C and non-degradable KIF1C and then see if degradation influences spindle formation.
[EDITOR COMMENT: I agree with these concerns, and feel that - unless you are inclined to repeat these experiments in knock-down-rescue conditions without overexpression - the conclusions from these cellular assays should be much more carefully stated and deemphasized. Furthermore, in the absence of such stronger data I feel that the model in Fig 7B is not sufficiently substantiated and should be removed.]
Response to 2.4: Following the reviewer's advice we removed Figure 7B and revised the text accordingly to include the requested changes and references. The revised paragraph reads now: "Overexpression of wildtype KIFC1 in cells disturbs the stoichiometric balance between KIFC1 and its counterforce generating kinesin EG5/KIF11. This imbalance may be responsible for the increased number of monopolar spindles observed. However, this phenotype was even more profound in cells overexpressing non-degradable D-box or S6D of KIFC1 (Figure 6C), which was not observed with other phosphorylation site mutants. We consider these results as consistent with the notion that the down-regulation of the KIFC1 antagonist EG5/KIF11 induces a monopolar spindle phenotype and that the decrease of KIFC1 by RNA interference can induce multipolar spindles (Zhu et al, 2005). However, it remains an unanswered question as to how degradation late in mitosis will show an effect early in the following mitosis as CDH1 knockdown experiments did not show any defects in early spindle formation (Floyd et al, 2008). More detailed experiments are required to answer this question."

Minor points:
1) In Abstract they use APC/C but in the manuscript APC. This should be consistent throughout.
Response to 2.5.1: We apologize for this inconsistency. We changed all occurrences of APC to APC/C.

2) In the abstract the line "KIF1C degradation is dependent on a stabilizing CDK1 phosphorylation" should be rephrased.
Response to 2.5.2: We rephrased this sentence to "Additional experiments showed that the KIFC1 degradation is modulated by a stabilizing CDK1-dependent phosphorylation site within the degradation motif of KIFC1."

3) Page 4: In the statement concerning the temporal degradation of SECURIN and CYCLIN the original papers from the Pines lab should be cited as they were the ones determining the exact timing.
Response to 2.5.3: We added the two requested papers from the Pines lab.

4) Page 5: "function of kinesins in mitosis made these proteins as..." should be rephrased.
Response to 2.5.4: The sentence was rephrased: "Kinesins are microtubule-associated motor proteins, some of which are known to regulate microtubule dynamics during cell division. As such, some kinesins have been described as potential targets for cancer drug development."

5) Page 6: The claim that following a release from a double thymidine release can generate defined

cell populations reflecting metaphase to anaphase etc should be removed and rephrased as there is no way this is the case. [EDITOR COMMENT: please rephrase with the appropriate cautionary notes]
Response to 2.5.5: Following the advice from the reviewer, we decided to remove the detailed description of these time points from the main text. In addition, Figure 1A was changed featuring now the label "tentative cell cycle stage".

6) Page 9: Kif18A has also been described as an APC/C substrate so first line is incorrect.
Response to 2.5.6: Kif18A has indeed been described as APC substrate during the preparation of this manuscript. The manuscript has been changed to incorporate the results of this publication.

7) Page 11: Holt et al and Littlepage and Ruderman references are at the moment separate.
Response to 2.5.7: This formatting issue has been corrected.

8) In their discussion on D-box and KEN box recognition the latest work from the Barford lab (He et al 2013) should be cited.
Response to 2.5.8: The suggested reference was integrated into the discussion on D-box and KEN box recognition: "Recent structural data of the C-terminal WD40 domain of S. cerevisiae CDH1 in complex with the APC/C inhibitor, Amd1, reveal indeed hydrogen bond interactions between the D-box arginine of Amd1, and two acidic residues (glutamine and aspartate) from CDH1 (He et al, 2013), which is consistent with the model of phosphorylation inhibition proposed above. While this structural study provides some insight into the structural underpinnings of the observed phenotypes of our KIFC1 mutants, only more in-depth structure-guided mutational analysis can test the precise mechanism behind the KIFC1 D-box phosphorylation and binding to the APC/C."

9) Page 19: Last line. What is supporting this "growing notion"?
Response to 2.5.9: The published work by Littlepage and Ruderman as well as Holt et al. support this growing notion. For clarification, we add these two references after this statement.

10) Page 20: Arg6 should be Arg5.
Response to 2.5.10: We apologize for this mistake and corrected it in the main text.

Referee #3:
1. Figure 1F seems to be missing (and isn't really referred to). I assume this is just a "lettering" error.
Response to 3.1 (see also Response to 1.2): We apologize for this oversight. Figure 1 was mislabeled resulting in the apparent 'loss' of panel F. We completely revised Figure 1 in order to streamline the manuscript and in this process corrected the mislabeling.

2. While I understand the basic idea to figure 1G-J, it seems like I must be missing something for the following reason. My understanding is that each of the gray lines is one peptide. However, even if they observed 10 unique peptides, and they graphed the three runs of the experiment separately, this would be only 30 gray lines. 1I and 1J look like hundreds. This is only an issue in that it makes me doubt that i understand what was done.
Response to 3.2: We thank the reviewer for spotting this error and apologize for this oversight. We incorrectly state in the figure legends that the 'sum normalized reporter ion traces from the peptides from CDC20…' are shown. In fact, we show the sum normalized reporter ion traces from the MS2-spectra (peptide-spectrum matches). We corrected the figure legend accordingly which reads now as follows: "(E to G) All complete sum normalized reporter ion traces from the MS2 spectra (peptide-spectrum matches) associated with NUSAP1 (E), TPX2 (F) and GAPDH (G) are shown in various shades of grey."

3. In figure 2C, the word "protein" was scored twice, with different occurrences. It isn't clear how this could be the case. Also, this is a rather unorthodox way to search. It seems better to search for functional domain (kinase, g protein, metabolism, etc)
Response to 3.3 (see also Response to 1.3): We removed this panel and added a clarification in the main text. Though 'unorthodox', we felt that this simple word counting approach provided the desired completely unbiased analysis of the protein names.

4. In the legend for figure 3A it says that "various" kinesins were ranked. They should be more specific. How were these chosen. Is this all the kinesins for which peptides were detected?
Response to 3.4: We thank the reviewer for spotting this unclear formulation. The reviewer is correct in that we analyzed the identified kinesins. This fact is now clarified in the revised figure legend: "(A) Heat map reflecting the percentile (%) in which the kinesins identified in our analysis are ranked in the six reference clusters (C1 to C6)."

5. The authors might want to note that the kinesins Cin8 and Kip1 and the kinesin-associated proteins Cik1 have been shown to be APC substrates in yeast, as have the spindle proteins Ase1 and Fin1.
Response to 3.5:We added the requested references for the yeast kinesins known to be degraded by the APC ("Only a single kinesin family member in higher eukaryotes, KIF22, had been described as an APC/C substrate previous to this study (Feine et al, 2007); in addition, two kinesins (KIP1 and CIN8) had already been described as substrates in yeast (Gordon & Roof, 2001; Hildebrandt & Hoyt, 2001).").

2nd Editorial Decision 06 November 2013

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by two of the original reviewers, and I am pleased to inform you that both of them are largely satisfied with the revisions made. However, there are still various editorial/presentational issues that will need to be addressed before the study would be ready for ultimate acceptance and publication.

- Referee 3 asks for some specific corrections, including a clarification and better explanation of the presentation in Fig1E/F/G that would be accessible also to general readers, and that will justify the connecting lines that have been drawn there.

- Supplementary Table S1, despite being referred to in the text, is still missing and needs to be uploaded.

- Part of the Material and Methods section (last paragraph on p.21, first paragraph on p.22) reads like a near-verbatim copy from another recent publication of yours. Please slightly reword this to avoid potential problems such as self-plagiarism accusations at a later stage.

- please provide source image data for all gels, blots and autoradiographs, in order to make the primary data more accessible and to allow readers to judge the full gels from which the cropped, sometimes rather pixelated and possibly also digitally assembled panels were derived. We would ask for a single PDF/JPG/GIF file per figure comprising the original, uncropped and unprocessed scans of all gel/blot panels used in the respective figures. These should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. A ZIP archive containing these individual files can be uploaded upon resubmission (selecting "Figure Source Data" as object type) and would be published online with the article as a supplementary "Source Data" file.

- gel/blot/autorad panels that may be composites of various assembled fragments/lanes (e.g. Fig 4A?) will need justification of any such digital assembly procedures based on the source data, as well as re-drawing to clearly indicate any separation lines. Please also re-make panels such as those in Fig 4B, Fig 6A or Fig S3, which currently suffer from low resolution and other apparent digital assembly artifacts (e.g. horizontal lines in as in the middle row of Fig 4B). When remaking Fig 6, please also note that it currently contains a second page with an apparent duplication of the panels used in Fig 6A.

- finally, I am concerned by the fact that several responses made in your point-by-point response letter do not appear to be reflected in the final manuscript. For example, your response to Ref 2 Point 5.3 states "We added the two requested papers..." but this is not the case, as this respective introductory section appears to have been removed completely. I find it difficult to understand why the (originally quite informative) introduction has been so drastically shortened, given that we have no strict character limit for EMBO Journal articles and given that only the penultimate introduction

paragraph on kinesins would have needed to be moved in relation to the shifted emphasis - you may want to consider at least partially reverting this change. Similarly, I don't find the response to referee 2 point 5.6 'Kif18 has indeed been described as APC substrate during preparation of this manuscript' very appropriate, given that the Sedgwick et al paper was published a full five months before submission of your study to our journal - I would strongly suggest to alter the statement on the bottom of p.7 "During the preparation of this manuscript..." into something like "During the course of this work..." to be more accurate and scholarly.

At this stage, I am therefore returning the manuscript to you for a final round of revision, hoping that you will be able to address/clarify all these remaining issues. When resubmitting, please therefore make sure to directly respond to all the points I made above.

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REFEREE REPORTS:

Referee #1 (Report):

In the revised manuscript the authors have adequately addressed all the concerns raised. As far as this reviewer is concerned, the manuscript can now be recommended for publication.

Referee #3 (Report):

Overall, I think the new version of the manuscript merits publication. Most of my original critiques were reasonably modest and most of these issues have been corrected. There are still quite a few small items in the text that need correcting as mentioned below.
The only significant item is that I still do not entirely understand what each of the grey/blue lines are in figures 1E-1G.
I can understand how the authors could plot points at these time points, but I do not understand how they are able to connect individual points. I have the feeling that this is in no way critical, but I think this should be clearer.

Small corrections:
1. On the bottom of page 5, the authors note that "spectra derived from three known APC/C substrates, NUSAP1 and TPX2.." Should this be "two" substrates??
2. On the top of 9, the authors appear to use "e.g." in the text in a nonstandard way. This should be corrected.

2nd Revision - authors' response 05 December 2013

Please find attached the revised version of our manuscript entitled "*Co-regulation Proteomics Reveals Substrates and Mechanisms of APC/C-dependent degradation*". We apologize for our oversights when preparing the previous revised version and hope that we manage to adequately address all remaining issues.

We would also like to thank you for your guidance and advice while preparing this revised version of the manuscript, which is now hopefully acceptable for publication in *The EMBO Journal*.

Please see below for a detailed point by point response to the reviewers' comments.

We look forward to hearing back from you.

Detailed point by point response to the reviewers' comments:

- Referee 3 asks for some specific corrections, including a clarification and better explanation of the presentation in Fig1E/F/G that would be accessible also to general readers, and that will justify the connecting lines that have been drawn there.

In order to clarify Figures 1E to G and to justify the connecting lines, we revised the main text, the figure legend and the corresponding Materials and Methods section. These revised section read as follows:

Main text: The very good reproducibility was confirmed by extracting all complete TMT-based peptide abundance profiles from the MS/MS spectra associated with NUSAP1 (24 complete peptide abundance profiles, Figure 1E), TPX2 (55 complete peptide abundance profiles, Figure 1F), and the "house-keeping" protein GADPH (371 complete peptide abundance profiles, Figure 1G), with the red trace corresponding to the mean profile, i.e. the respective protein abundance profile. The NUSAP1 and TPX2 profiles clearly represent the canonical abundance profile for APC/C substrates in contrast to GAPDH serving as negative control.

Figure legend: (E to G) The complete TMT-based peptide abundance profiles (after sum normalization) from all MS/MS spectra (peptide-spectrum matches) associated with NUSAP1 (E), TPX2 (F) and GAPDH (G), respectively, are shown in various shades of grey. The red traces indicate the mean.

Materials and Methods: Peptide-spectrum matched TMT ion intensities were extracted from the fragment ion spectra without applying MS- or MS/MS-based cut-offs. Prior to further data analysis we corrected for loading variation. Next, each TMT ion channel intensity was divided by the sum of all sister channel intensities (sum normalization), which resulted in the peptide abundance profiles. As it has been recently shown that the Model-Based clustering based on finite mixture models (Banfield & Raftery, 1993; Fraley & Raftery, 2002) can be successfully applied to time series data analysis (Fröhwirth-Schnatter & Kaufmann, 2008) such that each time series yi, i = 1,…,N, in a panel of N time points is considered to be a single entity connected by a line (Figures 1E to 1G). The protein abundance profile was calculated based on the average TMT-based peptide abundance profiles from all associated peptide-spectrum matches.

- Supplementary Table S1, despite being referred to in the text, is still missing and needs to be uploaded.

This supplementary table will be uploaded with this resubmission of the manuscript.

- Part of the Material and Methods section (last paragraph on p.21, first paragraph on p.22) reads like a near-verbatim copy from another recent publication of yours. Please slightly reword this to avoid potential problems such as self-plagiarism accusations at a later stage.

This particular 'Material and Methods' section was reworded to avoid potential issues of self-plagiarism.

- please provide source image data for all gels, blots and autoradiographs, in order to make the primary data more accessible and to allow readers to judge the full gels from which the cropped, sometimes rather pixelated and possibly also digitally assembled panels were derived. We would ask for a single PDF/JPG/GIF file per figure comprising the original, uncropped and unprocessed scans of all gel/blot panels used in the respective figures. These should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. A ZIP archive containing these individual files can be uploaded upon resubmission (selecting "Figure Source Data" as object type) and would be published online with the article as a supplementary "Source Data" file.

- gel/blot/autorad panels that may be composites of various assembled fragments/lanes (e.g. Fig 4A?) will need justification of any such digital assembly procedures based on the source data, as well as re-drawing to clearly indicate any separation lines. Please also re-make panels such as those in Fig 4B, Fig 6A or Fig S3, which currently suffer from low resolution and other apparent digital assembly artifacts (e.g. horizontal lines in as in the middle row of Fig 4B). When remaking Fig 6, please also note that it currently contains a second page with an apparent duplication of the panels used in Fig 6A.

The indicated figures were revised to ensure consistent resolution throughout the manuscript. Furthermore, composite images were clearly labeled and annotated source files are provided for all figures containing gel/blot/autoradiography images.

- finally, I am concerned by the fact that several responses made in your point-by-point response letter do not appear to be reflected in the final manuscript. For example, your response to Ref 2 Point 5.3 states "We added the two requested papers..." but this is not the case, as this respective introductory section appears to have been removed completely. I find it difficult to understand why the (originally quite informative) introduction has been so drastically shortened, given that we have no strict character limit for EMBO Journal articles and given that only the penultimate introduction paragraph on kinesins would have needed to be moved in relation to the shifted emphasis - you may want to consider at least partially reverting this change.

We profusely apologize for this confusion on our side. It seems that we took the 55,000 character limit mentioned in the "Guide for Authors" too seriously and thus we decided at a fairly late stage to remove this particular section from the introduction – unfortunately, I did miss updating this particular response to reflect these last minute changes. Following your advice, we reversed most of the changes/deletions, which also ensured that the two references from the Pines lab are back in the manuscript (see Clute & Pines, 1999 and Hagting et al, 2002 on page 5). In order to reflect the shifted emphasis away from kinesins, we deleted the introductory paragraph about kinesins.

Similarly, I don't find the response to referee 2 point 5.6 'Kif18 has indeed been described as APC substrate during preparation of this manuscript' very appropriate, given that the Sedgwick et al paper was published a full five months before submission of your study to our journal - I would strongly suggest to alter the statement on the bottom of p.7 "During the preparation of this manuscript..." into something like "During the course of this work..." to be more accurate and scholarly.

We did not mean to be disrespectful or unscholarly. We do apologize if our wording came across as such. Following your advice, we changed the wording such that the sentence now reads: "*During the course of this work, KIF18A was described as an APC/C substrate by Sedgwick et al., independently confirming our co-regulation proteomics-based strategy and its results (Sedgwick et al, 2013).*"
In addition, we used a similar formulation when discussing the Liu et al. paper on page 18.

Reviewer 3:
The only significant item is that I still do not entirely understand what each of the grey/blue lines are in figures 1E-1G.
I can understand how the authors could plot points at these time points, but I do not understand how they are able to connect individual points. I have the feeling that this is in no way critical, but I think this should be clearer.

See response to point 1.

Small corrections:
1. On the bottom of page 5, the authors note that "spectra derived from three known APC/C substrates, NUSAP1 and TPX2.." Should this be "two" substrates??
2. On the top of 9, the authors appear to use "e.g." in the text in a nonstandard way. This should be corrected.

We thank the reviewer for very thoroughly reading the manuscript. These two issues are indeed typos and have been corrected in the revised version of the manuscript.

