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Identification of Cdk targets that control cytokinesis

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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 17 June 2014

Thank you for submitting your manuscript on Cdk targets controlling budding yeast cytokinesis for our consideration. It has now been seen by three expert referees, whose comments are attached below for your information. As you will see, all three reviewers consider the study of value and potential interest, however they also raise a number of significant concerns regarding the overall presentation and interpretation, but also regarding the conclusiveness and completeness of some of the data. These issues to be addressed include better explanation of the truly novel aspects of the work on the background of already available knowledge from the literature; inclusion of additional controls, statistical analysis, and more detailed experimental descriptions; some follow-up on the functional/physiological outcome of phospho-site ablations; as well as clarification of several other currently confusing or inconclusive aspects (either through better explanation or additional data). Although I realize that incorporating all these points will likely amount to a considerable amount of revision work, I do feel that addressing these issues would be important to make this study a strong and compelling EMBO Journal paper, not least in light the partial conceptual precedent problems highlighted most clearly by referee 2.

Therefore, should you be able to satisfactorily improve the experimental and presentational issues along the lines suggested by the three reviewers, we shall be happy to consider the manuscript further for publication in The EMBO Journal. Please keep in mind that it is our policy to allow only a single round of major revision, making it important to carefully answer to all points raised 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; should you foresee a problem in meeting this three-month deadline, please let me know in advance and we could discuss the possibility of an extension.

Thank you again for the opportunity to consider this work, and please do not hesitate to contact me

in case you should have any question regarding this decision or the reports. I look forward to your revision!

REFEREE REPORTS:

Referee #1:

Summary. The authors have taken on the challenge of identifying Cdk and Cdc14 substrates that control cytokinesis in budding yeast. This is a timely and important topic in the cell cycle field. There is much debate in higher eukaryotes about the phosphatases that counteract Cdk phosphorylation during mitotic exit. Cdk is known to inhibit cytokinesis but very few of the relevant substrates are known. Likewise, the Cdk counteracting phosphatase Cdc14 is important for cytokinesis yet very few cytokinetic substrates have been identified.
There are essentially 3 independent components to this story, all related to this general theme. First, the authors use a constitutively nuclear Cdc14 construct to assess the importance of cytoplasmic Cdc14 for various aspects of cytokinesis. While a similar approach was published previously (Bembenek et al 2005, Cell Cycle 4:961-71), it is nice to see the basic results corroborated and the authors here go into deeper mechanistic detail. Second, the authors use a phosphoproteomics approach to globally monitor protein dephosphorylation upon ectopic Cdc14 overexpression in mitotically arrested cells in an attempt to identify those proteins whose dephosphorylation correlates with the timing of cytokinesis. Third, the authors use a clever genetic system to identify Cdk targets whose constitutive phosphorylation impedes successful completion of cytokinesis. While the connections between these three sub-stories are not always direct, the authors do a nice job assembling a coherent story. This work emphasizes again the challenge in studying phosphoregulation of cytokinesis, as mutation of phosphorylation sites on individual proteins rarely yields strong phenotypes, demonstrating the required robustness within this biological process. Moreover, conclusive identification of direct kinase and phosphatase targets is challenging. Overall, the experiments are technically sound, the paper is well written, and the results provide useful new insight into the roles of Cdk and Cdc14 in regulating cytokinesis, and the dynamics of protein dephosphorylation during mitotic exit. Given the strong current interest in these topics, I think the paper is well suited for publication in EMBO. There are a substantial number of items of concern noted below though that I recommend the authors consider addressing prior to publication. Only one of them definitely requires additional experimentation though so it should be reasonable to revise the document.

Major Concerns:

While an apparent goal of the authors is to identify Cdc14 substrates whose dephosphorylation is important to activate steps in cytokinesis, the study falls short of this goal. The phosphoproteomics study, while very useful and technically sound, does not distinguish between Cdc14 substrates and substrates of any other phosphatase that might be active in late mitosis as Cdk activity is quenched. The authors provide some analyses of the data with comparisons to what is known about Cdc14 specificity, but this does not provide any solid evidence linking Cdc14 to any given substrate. Since the design of the proteomic experiment causes termination of Cdk activity during the timecourse, it seems very likely that many other phosphatases contribute to the dephosphorylation profiles, as exemplified by the abundance of non-Cdk sites that decrease in abundance. The conclusions of this part of the study should focus primarily on the dynamics of Cdk site dephosphorylation during mitotic exit and cytokinesis and less on the identification of Cdc14 targets (especially in the absence of any validating experiments).

Along those same lines, no evidence is presented that Cdc14 directly dephosphorylates Inn1, Ede1, or Aip1. Any phosphatase active at the time of cytokinesis could in principle be responsible for their dephosphorylation. Additional experiments would need to be conducted to link Cdc14 to these proteins. I don't feel such experiments are necessary for the paper to be published, but they would be necessary to make any conclusions that Cdc14 is involved in their regulation.

Figure 3A -I can't figure out why the purple dashed line should be different in the experiments on the right and left. If combined Sic1 and Cdc14 induction is sufficient to drive cytokinesis in the

absence of MEN, it shouldn't matter if Cdc15 is inhibited or not, yet on the left the inhibited strain fails to complete cytokinesis while on the right the same strain appears to complete cytokinesis. This is a confusing figure because the justification for the two separate experiments (differing only in whether or not cells are released from metaphase arrest) is not addressed in the text, nor is the reason for using different outputs (FACS vs spheroplasts). Given that the authors are making a very strong conclusion that MEN kinases don't make significant contributions to cytokinesis, despite quite a few publications to the contrary, the reason for this apparent discrepancy needs to be addressed (or the experiment explained more fully - maybe I am just missing something).

Figure 7 - The phenotypic results are fairly clear. However, the authors can't make the conclusion that "Inn1 dephosphorylation promotes actomyosin ring constriction" without an additional control. They would need to show rescue of the phenotype with the Inn1-5A mutant because 1) the entire Inn1-Clb2m fusion cytokinesis defect is not due to Inn1 Cdk phosphorylation and 2) the entire Inn1-Clb2m fusion cytokinesis defect is not due to Cdk activity in general since the Clb2 fusion causes a phenotype as well that is only partially rescued by the 5A mutations (from data in Fig 6A and B). Since this is the only Cdk target that the authors provide mechanistic conclusions for, I think solidifying it with this type of control is important.

Minor Concerns:

The authors should probably acknowledge more that Bembenek and Yu (2005, Cell Cycle) previously provided evidence that cytoplasmic Cdc14 is required for proper completion of cytokinesis. This is not explicitly stated anywhere that I can find.

Early dephosphorylation substrates - The timing for "early", "intermediate" and "late" seems entirely arbitrary. In particular, the lab has previously categorized a handful of Cdk substrates into these groups based on their dephosphorylation in synchronized cultures. It seems like it would be appropriate to base the early, intermediate, and late windows on the dephosphorylation of these "marker" proteins from the prior work. This is important, because I find it very strange that the early dephosphorylated proteins are not enriched for Cdk-type sites. I'd expect this category to be highly enriched in Cdk sites. Maybe 20 minutes is too early for Cdc14 to accumulate (on that note the legend for 3C should define the arrows for Cdc14 bands - I assume the top one is thought to be the induced Cdc14>). Is it possible that the observed dephosphorylation reflects some other physiological change, maybe the response to galactose addition, that is unrelated to Cdc14. Control samples from a strain lacking GAL-CDC14 but treated in the same way would have determined to what extent the observed early profiles were really Cdc14-dependent. This is probably too much to ask to do at this point, but it might be worthwhile revisiting the definition of the time windows and maybe at least looking by western blot under these experimental conditions when, for example, Fin1 gets dephosphorylated to mark the "early" window.

Throughout the entire set of figures I find it hard to distinguish the red and orange colors. I recommend using either yellow in place of orange or selecting shades of red and orange that provide more contrast.

There is a general lack of statistical analyses throughout. This is important in a number of places where phenotypes are weak and apparent differences appear small. Examples are Figures 2E, 6A & B, 7C & D. In addition, the number of cells counted to provide quantitative results should be indicated throughout. It is in some places and is not in others. Error bars should be defined when present.

Please define somewhere that Tub1 is a loading control for all the western blots.

I wouldn't use the word "generic" in the abstract to describe the constitutive phosphorylation strategy. It makes it sound ordinary or standard and undersells the potential power of it. I think the authors mean "widely applicable" or something similar.

Figure E1 - No details are provided on how cytoplasmic versus nuclear localization is quantified. For example, how is nuclear localization distinguished from nucleolar?

Figure 2 - Please provide a color legend for panel D in the figure. The legend refers the reader to

panel C, but it's not clear what this means, since there is white labeling in panel C, but no white bars in D. The black bars in D have no match in C. One can infer the color scheme but a legend would make it easier to interpret.

Figure 2B - authors conclude in text that "no budded +CDC14 spheroplasts persisted" (page 7). This doesn't look technically correct as the values in the graph appear to be above zero.

Figure 4A - there is a technical problem with the graph image - large sections are filled in with black, obscuring many of the data points.

Figure 4F - this panel is confusing. I don't understand what "cumulative fraction of dephosphorylated peptides" means or why these lines should be increasing over time. Why not just provide, for each timepoint, the fraction of dephosphorylated Cdk sites that fall into each category. These should all add up to 1 then. For example, summing the data points for the 120 min timepoints would clearly give a value higher than 1, which doesn't make sense if these are fractional values.

Related to the comments on 4F, for the analysis of Ser vs Thr dephosphorylation preference, have the authors taken into account that phosphoSer Cdk sites appear to be more abundant in general than phosphoThr Cdk sites, by about 3:1 if I remember correctly from the phosphoproteomic study of Cdk phosphorylation sites from the Morgan lab (Holt et al 2009 Science). If so, this should be stated. This would influence the conclusions about whether phosphoSer is really being dephosphorylated preferentially.

Figure 4G - also confusing. What is the basis for the different Spa2 phosphorylation site groupings in the legend? Which sites are Cdk consensus, which are not? One of the black profiles appears to closely match the green ones so it is not clear why it is not part of the same cluster. More detailed info on this panel, in general, is needed for interpretation.

Figure 5C - the authors state that "...which was not observed after fusion to Clb2m Cdk" (page 13). This is too strong a statement. For Inn, Chs2, and Srv2 there is clearly an enhanced DNA content greater than 2C compared to controls.

Figure 5E - the GFP images don't appear consistent with the graph data. I clearly see open bud necks in the m Cdk image, but not the Clb2m image. Are they reversed and mislabeled?

Figure 6 - Complementation of the phenotypes of Ede1 and Aip1 is very weak. In fact, one could make perhaps a stronger argument from this data that the Cdk sites in Ede1 and Aip1 do not contribute significantly to the Clb2 fusion-induced phenotype. At a minimum, some statistics should be included to convince readers that these differences are meaningful.

Figure 7 - Statistics needed in panels C and D to support conclusions.

Figure 7B - What is the nature of the microscopic imaging? Indirect immunofluorescence? This is never stated anywhere.

Mass spec dataset - It would useful to the readers to include the common names for the proteins in the dataset, and also to provide tables listing all the proteins that fall into each of the categories from Figure 4B.

Referee #2:

The central theme of this paper is that dephosphorylation of Cdk substrates is a key mechanism driving the events of cytokinesis in budding yeast. Much of the emphasis is on the phosphatase, Cdc14, which is known to dephosphorylate numerous Cdk substrates when Cdks are inactivated in late mitosis. The first part of the paper (Figures 1-2) demonstrates that Cdc14 activation in the cytoplasm, where it is exposed to the proteins of the bud neck, is required for multiple steps in cytokinesis; this is followed by evidence in Figure 3 that Cdk inactivation synergizes with Cdc14 activation to promote cytokinesis. Next, the authors search for phosphoregulated cytokinesis proteins by using mass spectrometry to identify proteins that are dephosphorylated in late mitosis.

This leads to a method to identify the critical phosphoproteins by fusing candidate proteins to a cyclin, thereby promoting constitutive phosphorylation - leading to evidence that dephosphorylation of the cytokinesis protein Inn1 is required for normal cytokinesis.

The paper includes some innovative technical approaches. The quantitative mass spectrometry method produced a list of potential Cdc14 substrates that will be a useful resource to complement previous phosphoproteomic studies in budding yeast. Many readers will also be interested by the cyclin fusion method to screen for phenotypes of phosphorylated proteins.

Specific points:

1. Throughout the paper, the text implies that we know little about the mechanisms coupling the cell cycle to cytokinesis, and that the role of Cdc14 in this process is not clear. On the contrary, there is considerable previous evidence that dephosphorylation of Cdk substrates, resulting from a combination of Cdk inactivation and Cdc14 activation, is important for yeast cytokinesis, and there are numerous previous publications supporting this case for specific Cdk/Cdc14 targets, including the protein Inn1, which the authors focus on here. Many of these previous publications are either not cited or cited but not discussed in any detail (see specific comments below). The authors should adjust the text to provide a more realistic and comprehensive overview of current published knowledge. Specific examples of this issue include:

a. Figures 1-3 show that Cdc14 acts together with Cdk inactivation to promote cytokinesis. This section ends with the following sentences: "These observations suggest that Cdk downregulation and Cdc14 phosphatase activation, and therefore most likely the dephosphorylation of Cdk target proteins, drive cytokinesis. We therefore hypothesize that the main contribution of the MEN to cytokinesis is that of promoting Cdk downregulation and Cdc14 activation." This conclusion has been made in previous papers and is already widely believed. Here, for example, is the final sentence of the abstract from Sanchez-Diaz et al, published in EMBO J in 2012: "The dephosphorylation of CDK targets is therefore central to the mechanism by which the MEN and Cdc14 initiate cytokinesis and block polarised growth during late mitosis." Unfortunately, the Sanchez-Diaz paper is cited only in passing in the introduction.

b. Figure 3A is cited to show that Sic1 and Cdc14 synergize to drive cytokinesis, but the figure shows primarily that induction of these proteins drives rebudding, which is not cytokinesis. Is the y-axis labeled correctly on the right-hand plot? The photos at right do not provide a quantitative view of the effects on cytokinesis. In any case, these data fit with previous studies showing that premature cytokinetic events are promoted by Cdk inhibition in a Cdc14-dependent manner in budding yeast (Sanchez-Diaz et al 2012), and that Cdk inhibition has similar effects in fission yeast (Dischinger et al., 2008) and mammalian cells (Niiya et al., 2005). The authors should also cite and comment on the recent phosphoproteomic analysis of Cdc14 targets in yeast (L Kao et al., Mol Cell Proteomics 2014).

2. The label-free quantitative mass spectrometry method provides nice evidence for ordered dephosphorylation of likely Cdc14 targets in late mitosis (Figure 4A). However, many of the analyses in Figure 4 and in the supplementary dataset are poorly explained, making it difficult to understand exactly what was done and how to interpret the results. Specifically:

a. The supplemental Excel spreadsheet is not formatted in a useful way: systematic gene names are given but not the more useful gene names, and there should be columns or groupings indicating which of the various categories that peptide has been classified as (early, intermediate, late, rest; Cdk vs non-Cdk). As it is, the reader is required to figure out if the peptide levels decline or are constant. The peptides are arranged in alphabetical order by amino acid sequence, not by the name of the protein they are found in. A key function of this database is for the reader to get a quick view of the specific proteins in each of the major classes, and it is difficult to use the current dataset for that purpose. I also can't find a legend or other information describing this dataset and stating precisely what is found in each column. For example, what does 'nan' signify in many of the rows?

b. As the authors point out, reduced levels of a phosphopeptide in these studies can result from a decrease in the amount of the protein, not dephosphorylation. The authors carry out a control experiment (referred to as 'shotgun analysis' here) in which they measure the total levels of large

numbers of proteins during the same time course used in the phosphopeptide analysis. The description of these experiments is very brief and unclear, but the authors seem to be stating that they obtained stability data for only 49 of the proteins containing a disappearing phosphopeptide. We are not told which 49 proteins, or which category they are from (early vs late; Cdk vs non-Cdk). The authors then make an argument that most of these 49 proteins are stable, and therefore that most of the proteins containing a disappearing phosphopeptide are stable. This is not a particularly satisfying argument because the numbers are so small: at a minimum, the reader should be told the names of the 49 proteins and which ones are stable or not (perhaps by adding a column to the spreadsheet?). Ideally, the shotgun analysis should include far more proteins.

c. Figures 4D and E are poorly explained, and a general reader might struggle to understand what these plots signify.

d. Figure 4G is provided as evidence that multiple phosphopeptides from a single protein tend to have similar dephosphorylation timing. However, it doesn't look that way at all: there is clearly a wide variation in the different phosphopeptides from Spa2. Here again, the description is lacking: What does the numbering of the Spa2 peptides mean? In the excel dataset, there are 12 peptides listed from Spa2, with 9 phosphorylation sites: how do these relate to the 11 sites in Figure 4G?

3. The experiments in Figures 5-7 use the cyclin fusion approach to identify proteins whose constitutive phosphorylation causes defects in cytokinesis. This is a potentially exciting method and the authors find several proteins that seem to have an impact when fused to cyclin. As a negative control, the authors also test the effects of a cyclin fusion carrying mutations that reduce its binding to Cdk1, and in several experiments this mutation decreases the impact of the fusion. Better still, mutation of specific Cdk1 phosphorylation sites reduces the impact of the cyclin fusion in at least one case (Inn1), and so the paper ends (in Figure 7) with studies of the effect of the Inn1-cyclin fusion protein on various aspects of cytokinesis. Inn1 has already been demonstrated to be a target of Cdk1 and Cdc14, and so the studies here tend to provide a relatively small advance over previous studies (Palani et al 2012). The most novel result here is that the Inn1-cyclin fusion protein causes cytokinesis defects that were not seen when Palani et al made a phosphomimetic mutant. The cyclin fusion approach therefore seems to be a more effective way to generate a true 'phosphomimetic'.

a. Figure 7A shows that the Inn1-cyclin fusion does not seem to affect Cyk3 localization, in contrast to minor effects seen by Palani et al. However, Figure 7A does not include the wild-type (unrecombined) control. Given the data in Figure 7B, where the unrecombined control is clearly a better control than the 'delta' mutant, it would seem important to include that control in Figure 7A.

c. Figure 7B is not well explained: is this at a specific time point after release from G1 arrest? Is it possible to show a time course of Inn1 intensity and spindle assembly rather than just a single time point? In this experiment, the delta mutant seems to have little effect on the cyclin fusion phenotype.

d. Figures 7C and D: Are these the means of multiple cells (how many?), and what do the error bars represent? Are these differences statistically significant? How is the time from anaphase onset to Inn1 appearance quantified, when Inn1 intensity at the bud neck (like that of many cytokinesis proteins) rises gradually?

e. The images in Figure 7E are too faint. Can this experiment be more rigorously quantitated?

Referee #3:

In this manuscript entitled "Identification of Cdk targets that control cytokinesis" Kuilman et al. describe series of experiments demonstrating that Cdk activity represses cytokinesis by directly phosphorylating at least four independent proteins involved in cytokinesis. First, the authors show that preventing the phosphatase Cdc14 to spread in the cytoplasm upon mitotic exit has no impact on mitotic exit but leads to a penetrant inhibition of cytokinesis. Hence, substrates of these phosphatase must be dephosphorylated in order for cytokinesis to take place. Cdc14 preferentially targets the same consensus sites as Cdk1. Thus, the role of Cdc14 in cytokinesis suggests that Cdk1 has a specific function in inhibiting cytokinesis. Accordingly, repressing Cdk1 activity enhances the ability of Cdc14 to promote cytokinesis upon over-expression in metaphase cells. Second, a

phospho-proteomic search for protein phospho-sites that are dephosphorylated upon Cdc14 overexpression identifies a large number of sites that share the Cdk consensus site and many of which are known Cdk targets involved in cell cycle progression. This study also identifies late targets of Cdc14. These targets are likely to be involved in late stages of cell division, such as mitotic exit and cytokinesis. Accordingly, the GO terms cell cytokinesis is enriched in these hits. Third, the authors developed a very innovative strategy to induce constitutive phosphorylation of some proteins identified as being dephosphorylated by Cdc14 and being possibly involved in cytokinesis, and test whether maintaining such constitutive phosphorylation causes cytokinesis defects. Constitutive phosphorylation is achieved by constitutive targeting of Cdk1 to the target proteins through fusion of a cyclin moiety to these proteins. This approaches identifies Inn1, Chs2, Ede1, and Aip1 as proteins that need to be dephosphorylated in order for cytokinesis to progress properly. Further analysis of the effect of Inn1 phosphorylation indicates that it delays the recruitment of the protein to the plan of cleavage and the onset of actomyosin contraction. However, unlike previously suggested, it has no effect on the localization of the cytokinetic proteins Cyk3.

Together, the data presented are very convincing and the approach used are well controlled and their validity is carefully established. These studies clearly establish that one of the functions of Cdc14 in late mitosis is to de-repress the cytokinetic machinery, which is kept in check by Cdk1-dependent phosphorylation earlier in mitosis. Furthermore, the data presented convincingly show that phosphorylation of Inn1 and Chs2 represses cell division. Finally, the -Clb2m fusion method introduced here is going to be very helpful for many experimentalists in other studies. However, addressing some of the points below (listed with decreasing importance) would still greatly strengthen the conclusions of the manuscript.

1- The physiological importance of the findings described is not particularly well addressed in the manuscript. For example, it is unclear whether expression of the phospho-ablating alleles individually or in combination has any impact on cellular physiology and cell division? Do any of these combinations help restore some aspects of cytokinesis in the context of the CDC14-NLS strain? Do they cause premature cytokinesis and possibly damage of the segregating chromosomes or the mitotic spindle? It would be expected that in such assays the phospho-ablating mutants act in a dominant manner.

2- Expression of Cdc14-NLS is synthetic lethal with the hof1 but not with the cyk3 deletion. This suggests that Cdc14 is particularly required for the activation of the Cyk3 pathway. The authors should discuss this observation in regard to the genes that they identify as being target of Cdk1-dependent inhibition. Related to that, the CDC14-NLS seems to have a penetrant phenotype, yet all substrates identified are not essential. Are the deletions of these genes showing synthetic lethal interactions?

3- In the Figure 5E the authors score the ability of the constitutively phosphorylated alleles to cause the formation of cell chains. In the text, it is indicated that this figure reports on the number of cells that have a continuous cytoplasm. However, the details provided do not allow the reader to judge at which steps these cells have aborted cytokinesis and whether the cytoplasms are indeed continuous. A more specific analysis of this question scoring the open, constricted, or resolved status of the membrane (an assay originally introduced in Norden et al., 2006 and used earlier in the present manuscript) would help understand more precisely the step affected by the indicated mutations.

Minor point:
page 9 the authors tell us that expression of Cdc14 and Sic1 induces cytokinesis in mitotic cells. From the figure, we understand that the cells where more precisely arrested in metaphase using the microtubule-destabilizing drug Nocodazole. To simplify the reading, this precision should be introduced in the text as well.

1st Revision - authors' response 12 September 2014

We thank the three reviewers for their constructive criticism of our manuscript and also for acknowledging the potential impact of our study.

Reviewer #1:
The reviewer finds that our contribution is "a timely and important topic in the cell cycle field" and thinks "the paper is well suited for publication in EMBO". However, the reviewer raises concerns and suggests additional experiments. We have performed those, as detailed in our point-by-point response to the reviewer's comments.

Major Concerns (numbered by paragraph)
1./2. We agree with the referee that we have not provided formal proof that Cdc14 is the phosphatase that dephosphorylates Inn1, Ede1 and Aip1 during mitotic exit *in vivo*. Although a number of observations support dephosphorylation of the identified phosphoproteins by Cdc14, it is far from trivial to obtain formal evidence for this. Therefore, we have changed the manuscript at several places to make this clear. E.g. see our paragraph on page 11 "Protein dephosphorylation in response to Cdc14 expression could be due to direct dephosphorylation by Cdc14, or due to dephosphorylation by other phosphatases…"

3. We apologize for an error in the labeling of Figure 3A, which led to a misunderstanding. The cells in the left half of the figure were released from metaphase by Met3-Cdc20 re-induction, with or without 1NM-PP1 treatment to inhibit *cdc15-as1*. This shows that the *cdc15-as1* allele can be efficiently inhibited to block mitotic exit under our conditions. In contrast to what we indicated, there was no galactose induction of Sic1 or Cdc14 in this part of the experiment. Rather, the right half of the experiment shows the response of the cells to Sic1 and/or Cdc14 induction, while Cdc20 remains repressed.

In addition to correcting this mistake, and as suggested by the reviewer, we have repeated this experiment and used the same readout for cytokinesis in both parts of this comparison, namely the loss of budded cells after spheroplastation. This comparison confirms more clearly that Sic1 and Cdc14 together efficiently drive cytokinesis independently of the mitotic exit network.

4. The reviewer is right that a control experiment using Inn1-5A fused to Clb2m would strengthen our conclusion that Inn1 dephosphorylation promotes actomyosin ring constriction. We have now performed and included this control experiment. As expected, it shows that most of the difference between Inn1-Clb2m and Inn1-Clb2mΔCdk cells is due to phosphorylation on the 5 phosphorylation sites mutated in the Inn1-5A-mutant. These results are now included in Figure 7D.

Minor Concerns:
1. We have initially performed experiments using the NES mutant created by Bembenek 2005. However, in line with what has been observed and reported from Fred Cross's lab (Bloom et al. 2011), this *cdc14-nes* mutant does not support efficient Clb2 degradation. It thus appears that, in deviation from the conclusions in the Bembenek 2005 study, the *cdc14-nes* allele is a general hypomorphic loss of function allele. We therefore had to look for a new method to separate the mitotic exit and cytokinesis functions of Cdc14, leading us to create the *CDC14-NLS* allele. Nevertheless, we do acknowledge other findings from the Bembenek 2005 paper e.g. relating to Cdc14 localization to the site of cytokinesis (bottom of page 4).

2. We agree that better benchmarking of our dephosphorylation time windows would be appropriate and we have done this in our revised manuscript. We show in Figure E4 the 'early' disappearance of Fin1 phosphopeptides, in line with the 'early' classification of Fin1 by Bouchoux and Uhlmann 2011. In addition, Figure 3C exemplifies the dephosphorylation timing of Orc6 in our timecourse, which was used to define the 'late' category in line with Bouchoux and Uhlmann 2011.

We share the reviewer's surprise that Cdk sites are not more highly enriched in the early category. Because the focus of our current study was on late dephosphorylated substrates, we have to leave an in depth analysis of the early category substrates, which will no doubt be interesting, to a future study.

Following the reviewer's suggestion the bands corresponding to the endogenous and exogenous forms of Cdc14 are now indicated in Figure 3C.

3. The contrast between the orange and red colors might have been insufficient, and this has been improved in the figures accordingly.

4. Statistical analyses have now been included throughout the manuscript, which includes all of the figures mentioned by the reviewer, Figures 2E, 6A & B, 7C & D, as well as Figures 5C and 7G. Details of the statistical analyses are found, as appropriate, either in the Figure legends or the Materials and methods.

In addition, we now provide details of the number of cells counted to provide quantitative results, as appropriate in the figures or their legends. Error bars are now also defined in relevant figure legend, where they usually represent standard deviations.

5. A complete description of the antibodies used for Western blotting, including those used for the loading controls, is now included.

6. In line with the reviewer's good suggestion, we replaced 'generic' in the abstract with 'new and widely applicable' to describe our constitutive phosphorylation strategy.

7. When the nucleolar release assays were first used, a counterstain with a nucleolar marker like Nop1 or Net1 was included. Meanwhile, this has become a workhorse assay in the budding yeast mitotic exit field. Nucleolar localization can be reliably distinguished as a DAPI-weak, crescent-shaped segment of the nucleus, as compared to the DAPI strong and round remainder of the nucleus.

8. A color legend is now included to make Figure 2 easier to interpret.

9. The reviewer is correct that the fraction of +CDC14 spheroplasts that persist is greater than zero. We therefore changed our statement to say that 'no budded spheroplasts persisted above background levels'.

10. We thank the reviewer for alerting us to what must have been a PDF conversion problem after uploading our original PDF files for review. The revised Figure 4 will by uploaded as an EPS file and we will take extra care to ensure that this can be successfully and faithfully converted.

11. The 'cumulative fractions' shown in Figure 4F indeed corresponds to the fraction of dephosphorylated Cdk sites that fall into each category. Because the categories are not mutually exclusive, the total of the fractions adds up to more than 1. This is better explained in the revised figure legend.

12. The reviewer points to an ambiguity in our description of the results in Figure 4F. Our analysis indeed detected more phosphoSer compared to phosphoThr sites. Plotted is the fraction of those sites that are dephosphorylated, thus representing a difference in the dephosphorylation likelihood of the respective phosphopeptides. This has been clarified in the text.

13. All of the Spa2 phosphorylation sites analyzed in Figure 4G are Cdk consensus sites. It is correct that one of the profiles shown in black (stable) appears to closely match one of the profiles in green (late dephosphorylated). The basis for the groupings is the same as in Figure 4A and 4B, which is now made clear in the revised figure legend. The filters for these groupings are explained in the Materials and methods and have been consistently applied throughout the manuscript. On visual inspection, the black Spa2 peptide in question could be considered a late dephosphorylated peptide, however it narrowly missed some of the 'fold change' criteria required for 'late' annotation. It might be considered a false negative annotation, cases of which are unavoidable when implementing automated annotation of large datasets.

14. The statement regarding Figure 5C, that a greater than 2C DNA content 'was not observed after fusion to Clb2mΔCdk' was toned down to 'was not as pronounced or even absent after fusion to Clb2mΔCdk '

15. We thank the reviewer for pointing out that we had indeed erroneously swapped the two photographs in Figure 5E, this has been corrected.

16. The reviewer considers the complementation of Ede1 and Aip1 Clb2m fusion by phosphosite mutations weak, and asks that statistics be included in Figure 6. This has been done, as suggested. We also now represent the individual values of two biological replicates to show that the reproducibility of our FACS-based cytokinesis assays is very high. Thus, although some of the

differences in cytokinesis defects between wild-type and non-phosphorylatable mutants are small, they are reproducible and significant.

17. As suggested, statistical analysis is now also included in Figure 7C and D, confirming that the differences in time from anaphase onset to Inn1 recruitment or start of Myo1 ring contraction are indeed significant.

18. Details that the images in Figure 7B have been acquired using indirect immunofluorescence are now included in the figure legend.

19. Inclusion of the common names for each protein, as well as the dephosphorylation categories, will indeed make the mass spectrometry dataset more useful. This has now been included.

Reviewer 2
The reviewer finds that the paper 'includes some innovative technical approaches' and that many readers 'will also be interested by the cyclin fusion method'. However the reviewer raises a number of concerns.

1. The reviewer cautions that we have not adequately acknowledged previous work in the field, and gives specific examples. We concede that we should have better placed our work into the context of previous studies and we have rectified this in our revision.

After having done this, we nevertheless would like to lay out the advance that our study brings to the field. While others have of course implicated Cdc14 in cytokinesis, it is really our *CDC14-NLS* allele, that for the first time fully uncouples mitotic progression from cytokinesis. All previous studies have not been able to exclude the possibility that effects of Cdc14 on cytokinesis could have been indirect through affecting other aspects of mitotic exit.

Similarly, the reviewer is right that a handful of cytokinesis proteins are known Cdk targets, and that some of these have been shown to be dephosphorylated by Cdc14. In some cases, dephosphorylation has been correlated with localization changes of other cytokinetic factors. An actual functional impact on cytokinesis has to our knowledge been previously shown only in the case of Chs2. Taken together, we think that our study offers both technical and conceptual advances over what was previously known.

a. We agree with the reviewer and apologize for the unintended omission of a reference to the Sanchez-Diaz 2012 paper on page 9, when we discuss the consequences of simultaneously expressing Sic1 and Cdc14. We have added an explicit mention of this important study.

b. Shown on the graphs in Figure 3A is the percentage of remaining large-budded cells, the disappearance of which equates to completion of cytokinesis. We did not observe rebudding under the conditions of our experiment. References to all the publications mentioned by the reviewer have now been included.

2a. We have reformatted the supplemental Excel spreadsheet, that reports the results of the phospho-proteome analysis, to make it easier to use. Following the reviewer's suggestion, standard yeast names have been included, as well as the timing categories of peptide disappearance. In addition, the data have been sorted based on the standard yeast name in all sheets. Finally, we have included a legend to indicate the meanings of the columns and the values in the fields.

b. We have now also included the protein stability score for every phosphopeptide (if available) in the spreadsheet. The protein stability analysis was performed to estimate the contribution of protein degradation to phosphopeptide disappearance. Our shotgun approach covered approximately 1/3 of all budding yeast proteins. It would go beyond the scope of this manuscript to aim for providing an even more comprehensive protein stability dataset. We have changed the text to better explain how the analysis has been performed.

c. The legends and explanations for the data shown in Figures 4D and E have been improved in the revised version of the manuscript.

d. The reviewer is right that Figure 4G shows that not all phosphopeptides from the same protein are dephosphorylated with the same kinetics, however it exemplifies that a significant majority do. The numbering of the 11 unique peptides of Spa2 in this analysis simply follows their dephosphorylation timing. Two of the 12 peptides in the dataset differ only in the charge of the peptide, while in this figure we report on the 11 phospho-peptides with distinct sequences. Looking at all proteins for which we detected more than one phospho-peptide, we find that there is statistically highly significant, but of course not absolute, clustering of dephosphorylation timings. This is now more clearly explained.

3. a. As requested by the reviewer, an unrecombined control is now included in the quantification of Cyk3 accumulation at the bud neck, shown in Figure 7A, This control further confirms that the level of Cyk3 accumulation is unaltered by the Inn1 phosphorylation status.

c. Figure 7B is now better explained. The Inn1 bud-neck staining as a function of spindle morphogenesis was assessed in synchronized cells at two time points while cells progressed through mitosis. We also followed the reviewer's suggestion to observe Inn1 accumulation in live cells. This data is contained in Figure 7C, confirming delayed recruitment to the bud neck of persistently phosphorylated Inn1.

d. Figures 7C and D and their description have now been improved. The number of cells that these measurements are based on is indicated (between approximately 20 and 50 in each case). Shown are the means and standard deviations. The onset of anaphase is the last time frame before the two spindle pole bodies start poleward movement. The time to the first detectable Inn1 accumulation at the bud neck (Figure 7C) or the first detectable change in the size of the actomyosin ring (Figure 7D) have been scored. Although the Inn1-signal increases gradually, this happens within a short time-span (Palani et al. 2012), shorter than the difference in the recruitment timings between Inn1-Clb2m and Inn1-Clb2mΔCdk. Thus, the gradual increase in Inn1-signal at the bud neck does not constitute a limitation to the accuracy with which we measure Inn1-recruitment.

e. We have improved the quality of the images shown in Figure 7E. In addition, we had mistakenly stated that Figure 7F is a quantification of Figure 7C, while it is of course the quantification of Figure 7E. The actomyosin phenotypes were blind scored in at least 20 cells for each genotype to ensure an unbiased result. This exemplifies the clearly distinct behavior of the actomyosin ring in Inn1-Clb2m cells.

Reviewer 3

The reviewer finds our data presented 'very convincing and the approach used […] well controlled'. However, the reviewer adds suggestions to improve the manuscript.

1. The reviewer makes the important prediction that phospho-ablating mutants of cytokinesis regulators should have a dominant effect on advancing cytokinesis. In principle this is of course correct and we have been thinking of ways to make conditional phospho-ablating alleles of these proteins. We have analyzed individual non-phosphorylatable variants of Inn1, Ede1 or Aip1, but those do not produce an obvious cytokinetic phenotype. A likely reason for this is that cytokinesis is a concerted event with several contributing reactions. Disrupting any of them by the use of our constitutive phosphorylated alleles, will readily disrupt cytokinesis. On the other hand, reconstituting ectopic cytokinesis using phospho-ablating proteins might require further knowledge of most, if not all, regulated pathways. To explore this further, starting to combine our phospho-ablating mutants will be an interesting avenue to follow.

2. We have followed the reviewer's very good suggestion and analyzed synthetic genetic interactions between our newly identified and previously known cytokinesis regulators. Cells lacking Ede1 display a weak tendency to form cell chains, consistent with a role of Ede1 in cytokinesis. Ede1 deletion in strains either depleted of Inn1 or lacking Hof1 caused a significant synthetic growth defect and increased cytokinesis failure. This confirms a role for Ede1 in cytokinesis and suggests that it might act in a pathway parallel to Cyk3 and Hof1, possibly in concert with Myo1 or in an additional pathway.

Cells lacking Aip1 did not show a detectable cytokinesis defect. However, *aip1* deletion substantially rescued the cytokinesis defects observed in the absence of Inn1 or Hof1. This suggests that Aip1, probably in its phosphorylated form, acts as a negative regulator of cytokinesis. As an actin regulator, its placement in the Myo1 pathway in parallel to Cyk3 and Hof1, is conceivable. The new data is contained in Figures 6C-E.

3. According to the reviewer's suggestion, we have used the Norden et al.-assay to characterize the cytokinesis defect following Inn1-, Chs2-, Aip1-, Ede1 or Srv2-Clb2m fusion. The data is presented in Figure E4 and shows a marked defect in membrane constriction in all cases. We also note the development of a 'widened' bud neck in many cases, the cause of which is currently unclear.

Minor point:
We apologize for the lack of detail in our description of the mitotic arrest used in Figure 3. The mitotic arrest was achieved by depletion of the APC coactivator Cdc20 under control of the repressible *MET3* promoter. This is explained in the revised manuscript.

2nd Editorial Decision 07 October 2014

Thank you for submitting your revised manuscript on cytokinesis Cdk targets for our consideration. Referees 1 and 2 have now looked at it once more, and I am happy to inform you that they consider their original concerns satisfactorily addressed and have no more objections towards publication in The EMBO Journal. They mention a few necessary remaining modifications, which I would kindly ask you to incorporate in a final round of minor revision. After that, we should be able to proceed swiftly with formal acceptance and publication of the paper!

I look forward to receiving your final version.

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REFEREE REPORTS:

Referee #1:

The authors have thoroughly addressed my concerns and suggestions from the initial submission. I recommend publication. I have just a few minor comments on the revised manuscript.

1. "Spheroplasts" is mis-spelled in the y-axis legends in Figure 3A
2. The abbreviation "ns" is not defined in Figure 7D. I assume it means not significant but this is different than the symbols used in the other figures to indicate lack of statistical significance.
3. Although the authors claim they added to the legend of Fig. 7B a statement that indirect immunofluorescence was used to acquire images, I dot not see this addition.
4. I'm still not clear on the main point of the collection of Spa2 phosphopeptide profiles shown in Figure 4G. While this figure does support that clusters of co-regulated sites exist, it also quite clearly indicates that within a single protein there can be sites that are regulated differently. In my opinion the real power of proteomic approaches like this is to reveal differences in the regulation of distinct sites or subsets of sites that are undetectable using more common methods that only report on the overall phosphorylation state. The authors could get more mileage out of this panel by making this point as well, which I did not notice mentioned elsewhere.
Overall, nice work on a challenging topic and a commendable job on the revisions.

Referee #2:

My previous concerns have been thoroughly addressed by a wide range of improvements to the text and experimental results. In my opinion, the paper is now ready for publication.

One very minor point: 'spheroplasts' is misspelled in Figure 3A. Is the top blue line supposed to be a dashed line?

2nd Revision - authors' response 08 October 2014

Thank you for your and the reviewers' additional comments on our manuscript, Kuilman et al. 'Identification of Cdk targets that control cytokinesis', and for your decision to proceed to a final round of further minor revisions.

We thank the reviewers for their attention to detail and have in the included files addressed your and the referees' points.

We have corrected the textual issues raised by reviewer 1 in his/her points 1-3. We also have taken onboard the reviewer's point 4 and have added a sentence on page 12 of the manuscript, to say that exceptions to the observed tendency of coordinated dephosphorylation seen in Figure 4G could point to distinct modes of regulation affecting individual phosphorylation sites. The two mistakes spotted by reviewer 2 have also been corrected.

We hope that with these final changes you now find that our manuscript is acceptable for publication

