**GigaScience**

**SILAC-based phosphoproteomics reveals new PP2A-Cdc55-regulated processes in budding yeast.**

---Manuscript Draft---

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<td>Funding Information:</td>
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<td>Abstract:</td>
<td>Background: Protein phosphatase 2A (PP2A) is a family of conserved serine/threonine phosphatases involved in several essential aspects of cell growth and proliferation. PP2ACdc55 phosphatase has been extensively related to cell cycle events in budding yeast, however few PP2ACdc55 substrates have been identified. Here, we performed a quantitative mass spectrometry approach to reveal new substrates of PP2ACdc55 phosphatase and new PP2A-related processes in mitotic arrested cells. Results: We identified 626 potential PP2ACdc55 substrates involved in a broad range of mitotic processes. In addition, we validated new PP2ACdc55 substrates such as Slk19 and Lte1, involved in early and late anaphase pathways, and Zeo1, a component of the cell wall integrity pathway. Finally, we constructed docking models of Cdc55 and its substrate Mob1. We found that the predominant interface on Cdc55 is mediated by a protruding loop consisting of residues 84-90, thus highlighting the relevance of these aminoacids for substrate interaction. Conclusions: We used phosphoproteomics of Cdc55 deficient cells to uncover new PP2ACdc55 substrates and functions in mitosis. As expected, several hyperphosphorylated proteins corresponded to Cdk1-dependent substrates, although other kinases' consensus motifs were also enriched in our dataset, suggesting that PP2ACdc55 counteracts and regulates other kinases distinct from Cdk1. Indeed, Pkc1 and Cla4 kinases emerged as novel nodes of PP2ACdc55 regulation, highlighting a major role of PP2ACdc55 in membrane trafficking and cytokinesis, gene ontology terms significantly enriched in the PP2ACdc55-dependent phosphoproteome.</td>
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Reviewer #1: The manuscript by Baro et al, describes the use of 3 different SILAC based proteomic approaches combined with a powerful yeast genetic deletion model. The goal of the project was to identify possible substrates of the PP2A-B55/cdc55 phosphatase. To do this the authors arrested wild-type and Cdc55 deletion mutants in metaphase by also deleting cdc20. The only issue with this approach is that during metaphase PP2A-cdc55 activity is suppressed and the majority of all phosphorylation sites would already be maximally phosphorylated (Olsen et al., 2010). Hence the ability to further enhance phosphorylation during this time is likely to be limited and therefore the increase observed is likely a under representation of the true number of PP2A specific phosphorites.

We have reported previously that budding yeast PP2A-Cdc55 phosphatase activity is high in metaphase and it is inactivated during anaphase (Queralt et al 2006, Queralt and Uhlmann 2008), using the exactly same set-up arresting cells in metaphase by Cdc20 depletion. Under this conditions, we can detect hyperphosphorylation events of PP2A-targets. In fact, this is a well established system in our lab that allowed us to study deeply three candidates substrates Net1 (Queralt et al 2006) and Bfa1 and Mob1 (Baro et al 2013). In the current work we have extended this laboratory system to a global proteomic study.

This likely explains why only 27 sites were specifically increased, while 62 were decreased (Fig 1H).

We are really sorry since we noticed that we have failed to properly explain how we made the phosphopeptide identification. We have amended that in the manuscript and introduce further explanation in Results and Materials sections.

Combining the three methods, we identified 1260 high confidence phosphopeptides with increased phosphorylation (Heavy (wt)/light (cdc55mutant) ratio < 0.75) in the absence of Cdc55 phosphatase; therefore likely to be PP2A-Cdc55 substrates. Those 1260 hyperphosphorylated peptides include 62 (increased) phosphopeptides that were identified in at least 2 (out of 3) enrichment methods (Figure 1H) with a t-test p-value <0.05. Therefore, considering the different nature of the phosphopeptides identified for each method, the 62 phosphopeptide are pretty likely to be PP2A-Cdc55 substrates.

Nevertheless, we cannot consider the remaining 1198 hyperphosphorylated peptides to be all false positives (some phosphopeptides were identified with highly amounts of PSM, and pretty high confidence proteomic statistics). Of course, we also agree that we cannot claim that all are true positives. For this reason we introduced the table of the 62 common hyperphosphorylated peptides and we have soften the conclusions.

Often, proteomics studies are performed using just one of the phosphoenrichment methods using biological duplicates or triplicates. However, it has been reported that
the different phosphopeptide enrichment methods isolated distinct, partially overlapping segments of a phosphoproteome, whereas none of the methods were able to provide a whole phosphoproteome (Zhou et al. 2001, Bodenmiller et al. 2007). Enrichment techniques are complementary, such that a combination of methods greatly enhances the number of phosphopeptides isolated from complex samples (Dunn et al. 2009). So, none of the phosphopeptide enrichment methods provide a whole phosphoproteome. Each method provides varying degrees of selectivity and specificity of phosphopeptide enrichment resulting in the identification of subsets of phosphopeptides of different nature. Therefore, our idea was to use the different phosphopeptide enrichment methods in order to identify the highest spectrum of the PP2A-Cdc55 phosphoproteome. Actually, we really think the strength of our data is indeed based on the 3 different enrichment methods.

To properly assess PP2A-Cdc55 substrates the ideal experiment would be to analyse the kinetics of dephosphorylation for the wt and deleted strains as they exit mitosis, similar to previous studies (Bouchoux and Uhlmann, 2011; Cundell et al., 2016). This may also explain why Mob1, which the authors previously showed to be a PP2A substrate, did not show up as a strong and significant hit in their SILAC approach.

In our experimental conditions, we can detect increase phosphorylation of PP2A-Cdc55 substrates already in metaphase; and in most of the cases since the protein is hyperphosphorylated in absence of Cdc55, its phosphorylation does not increase more during anaphase. Of course, we cannot rule out that it might occurs for some specific substrates, but in general in absence of Cdc55 the substrates are equally hyperphosphorylated in metaphase and in anaphase (see Bfa1 and Mob1 in Baro et al. 2013, Figure 1 and Figure 2).

Mob1 was identified as medium confidence phosphopeptide with increase phosphorylation in absence of Cdc55, already in the first SILAC-Phosphoproteome analysis that we performed (Method 1). In fact, thanks to this identification we proceed to study the physiological relevance of this substrate and we already published it (Baro et al. 2013). At that moment, we looked for protein in “our favourite” candidates list (proteins related to FEAR and MEN pathways), not only in the high confidence peptides but also in the medium confidence ones. However, for a global analysis of the data as we are doing here, we can only include high confidence peptides.

Also without the kinetic data, it is difficult to deconvolve the direct and indirect effects of PP2A deletion. It's possible that many of the increased phosphorylation events could be due to disruption of negative and positive feedback loops that impinge on other phosphatases and kinases (Grallert et al., 2014). It is also unclear exactly how 'increased' phosphopeptides were chose and how these were subsequently analysed and compared especially with regards to the precise number of unique phosphosites that are specifically increased between PP2A wt and deletion strains.

We are really sorry since we noticed that we have failed to properly explain how we made the phosphopeptide identification. We have amended the manuscript in several sections and introduce further explanations in Results and Materials sections. Combining the three methods, we identified 1260 high confidence phosphopeptides with increased phosphorylation (Heavy (wt)/light (cdc55mutant) ratio < 0.75) in the absence of Cdc55 phosphatase; therefore likely to be PP2A-Cdc55 substrates. We agree that we cannot distinguish among direct and indirect effects on PP2A-Cdc55 deletion, and in fact we already discussed that point in the manuscript (Results and discussion). Nevertheless, during anaphase a key mitotic phosphatase in budding yeast, Cdc14 is activated, and in order to exit from mitosis most of the phosphorylation events are erased, mitotic kinases (at least Cdk1 and Polo-kinase) are inactivated, therefore we believe that the feedback loops among phosphatases and kinases will be higher during anaphase. This consideration and the fact that the system of cdc55 deletion in metaphase arrested cells worked pretty well in our laboratory, prompted us to use this set-up.

Despite these concerns, there is still some interesting data which could form the basis
of an interesting publication. The authors should take the above limitations into
account, and alter their conclusions accordingly. They would also benefit to rethink
exactly what the experiment they have performed is actually telling them, and perhaps
consider revising the initial goal to better suit the experimental data.

We thank the reviewer for his/her global evaluation of the manuscript. We think we
have amended most of his/her concerns.

We are really sorry since we noticed that we failed to properly explain how the analysis
was performed. In the new version of the manuscript, we have explained better how the
data was acquired and analyzed (results and methods sections) and now we
provide better statistical parameters details of the phosphopeptides.

Combining the three methods, we identified 1260 high confidence phosphopeptides
with increased phosphorylation (Heavy (wt)/light (cdc55mutant) ratio < 0.75) in the
absence of Cdc55 phosphatase; therefore likely to be PP2A-Cdc55 substrates. Those
1260 hyperphosphorylated peptides include 62 phosphopeptides that were identified in
at least 2 (out of 3) enrichment methods (new Figure 1I) with a t-test p-value <0.05.
Therefore, considering the different nature of the phosphopeptides identified for each
method, the 62 phosphopeptide are pretty likely to be PP2A-Cdc55 substrates.
Nevertheless, we cannot consider the remaining 1198 hyperphosphorylated peptides
to be all false positives (some phosphopeptides were identified with highly amounts of
PSM, and pretty high confidence proteomic statistics). Of course, we also agree that
we cannot claim that all are true positives.

Specific points:
1. The introduction is a bit long and could be shortened, also suggest moving the
discussion about previous mass spec studies to the discussion.

Done. We have shorten the introduction and move previous mass spec studies to the
discussion section.

2. Page 4 Line 87: Perhaps consider citing either the Hunt or Castro labs for the role
   of Greatwall in regulating PP2A. (see 2010 Science papers).

Done

3. Page 5 Lin105-7: “PP2ACdc55 downregulation in anaphase”: I assume the
authors mean increased activity here as PP2A is reactivated during mitotic exit not
repressed.

As we previously published, budding yeast PP2A-Cdc55 is downregulated during
anaphase (Queralt et al 2006). The reactivation of the PP2A-Cdc55 occurs upon exit
from mitosis, during cytokinesis/G1.

4. Figure 1A: It would be nice to have a diagram here where you show the work
   flow. Specifically highlighting the wt and deleted strains being compared, which one
   was grown in which media (Heavy or Light), number of biological replicates etc .as I
   think this may help readers understand what is being compared. You could condense
   and simplify additional figure 1 and insert it here perhaps?

Done

5. Page 8 Line 181: Some greater clarification about the number of
   phosphopeptides that are increased in the deletion mutant are needed. From Figure
   1H it appears that there are only 27 sites. But the text suggest that this number is
   1,260. I assume that 1A is the whole dataset, that is 1260 sites were identified across
   both the wt and del mutant? If so Fig1H is the most important figure and the analysis
   needs to be primarily performed on the 26 sites that are increased compared to the wt.
   Not the complete 1260.

We have explain better this point in Page 8, Results section. We were able to quantify
globally 1491 phosphoproteins, represented by 4467 phosphopeptides. Among them,
we found 1,260 hyperphosphorylated peptides in the cdc55 mutant which show H/L
ratios <0.75 (log2(H/L)<-0.42), corresponding to 628 phosphoproteins. The 1260
phosphopeptides (or 628 phosphoproteins) have increase phosphorylation in absence of Cdc55. Those 1260 hyperphosphorylated peptides include 62 (increased) phosphopeptides that were identified in at least 2 (out of 3) enrichment methods (new Figure 1) with a t-test p-value <0.05. Therefore, considering the different nature of the phosphopeptides identified for each method, the 62 phosphopeptide are considered hyperphosphorylated peptides, which showed similar performance in the different purification protocols used, rather than being more likely to be PP2ACdc55 substrates. Indeed, each approach uncovered a unique subset of hyperphosphorylated peptides useful for downstream analysis (some hyperphosphorylated peptides found in only 1 method were detected with a huge amount of PSM and great scores; therefore, those are also pretty likely to be substrates).

6. Additional File 2: What are the values listed? Are these are log2 H/L ratios as they are all below 1? A full table with the H/L ratios for all identified phosphopeptides along with the p-value would be very helpful. In addition, there should be a comparisons between biological replicates along with p-values to ensure that sites identified are significant. Were label-swaps performed? This data should be presented in a single clearly labelled table. Additional file 3 (old Additional file 2) has been improved following the reviewers considerations.

7. It appears the data has been heavily averaged, and I couldn’t quite follow what was done in order to generate Figure 1A. Why was a H/L ratio of less than 0.75 considered to be increased phosphorylation? Normally a ratio greater than would be the desired cut-off would be used (e.g. >1 for a 2-fold increase). Similarly, a value <1 would be considered significantly decreased with values between -1 and +1 considered unchanged. These need to be clarified.

The first point to clarify is that the phosphopeptides of interest are the ones with H/L ratio < 0.75 or log2(H/L) ratio < -0.42. In the material and methods (section: “Stable Isotope Labelling of Yeast Cells and Preparation of Yeast Protein Extracts for Phosphoproteomic Analysis”) we specify that the wild type (Y858) and the cdc55 (Y859) strains were labeled with the heavy (H) and light (L) aminoacids, respectively (we hope this point will be now much clear with the new Fig. 1A). Therefore, what is expected for the cdc55 substrates is the H/L ratio below 1 (or 0 in log scale). We set our threshold to 0.75 (-0.42 in log scale) as commonly done in the Proteomic laboratories for similar set-ups.

In the figure 1A (new Figure 1B) we show the whole dataset which is the result of combining the three experimental approaches (TiSH, SIMAC and TiO2) and applying the selection criteria described in the last paragraph of the “Data Analysis for Peptide Identification and Quantitation” section in material and methods. This gives us 4,467 phosphosites being 1,260 of them the hyperphosphorylated ones (H/L ratio below 0.75, in linear scale). Moreover, the figure 1H (new Figure 1I), indeed show the statistically significant phosphopeptides which are included in at least 2 out of 3 experimental conditions (TiSH, SIMAC and TiO2). Among them, 62 are the ones that are significantly hyperphosphorylated in absence of cdc55 in more than one approach. We have modified both figures (new Figure 1B and Figure 1I) for clarity.

8. From Figure 1H it appears that only 27 or 62 phosphosites are increased in the deleted vs wt strains. If this is correct, then the analysis should be performed on these 27/62 phosphosites, not the full dataset. This goes for the rest of the analysis, such as the STRING, motif-x and GO-enrichments. This also seems to contradict Figure1A, can the authors explain this better?

The figure 1A (new Figure 1B) was generated averaging all the single phosphopeptides obtained in the three experimental approaches (we considered the three approaches as biological replicates). The total number of phosphosites plotted were 4,467 being 1,260 of them hyperphosphorylated (H/L<0.75) in absence of Cdc55. On the other hand, as we described above, the wild type cells were heavily labeled while the cdc55 KO cells were labeled with light aminoacids. Therefore, in a H/L ratio the phosphopeptides with a ratio below 1 (0 in log scale) are those that are
hyperphosphorylated. We set the significance ratio below 0.75 (-0.42 in log scale) for hyperphosphorylated peptides. We decided to use all the hyperphosphorylated peptides to increase the statistical power of the systems biology analysis (motif-x, GO analysis and STRING). This is an exploratory analysis without the aim to fully characterize the specificity of Cdc55. While we agree that the 1260 hyperphosphorylated peptides might include false positives, the bioinformatics studies allow us to uncover some interesting features:

1. We identified 10 sequences motif susceptible (MotifX) to be recognized by PP2A-Cdc55, while so far only one was properly described (S/TP). This finding is in agreement to previous published PP2A-Cdc55 substrates that do not contain the S/T P site.

2. String analysis identified two nodules, Cdc28 and Cla4, as putative kinases to be counteracted by PP2A-Cdc55. It has been pretty well established in the field that PP2A-Cdc55 is a phosphatase counteracting Cdc28 kinase at the S/T P sites. PP2A-Cdc55 was modestly related to Cla4 in the literature, and we suggest that they might be really functionally linked.

3. The GO suggested some biological processed to be regulated by PP2A-Cdc55. Several published PP2A-Cdc55 functions are included in those GO, confirming previous data. New biological processes like cytokinesis is not surprising due to the cdc55 deletion mutant phenotype and the described localization of PP2A-Cdc55 to the cytokinesis locus. However, its function and targets during cytokinesis in budding yeast have not been examined. We are currently studying the PP2A-Cdc55 function in cytokinesis in our laboratory in detail.

We think that those bioinformatics analysis mostly confirm some published data and we carefully suggested new features of the PP2A-Cdc55 focusing on those with previously reported data.

9. Page 10 Line 233: 562 unique motifs seem very high especially for an input of 721 peptides, it's almost 1:1, and above the number of total kinases in yeast. Also, the authors should clarify exactly which phosphopeptides were used for the analysis. Again if they want to show PP2A specific effects then only the phosphopeptides that are specifically increased in the deletion vs wt should be analysed.

We thank the reviewer to point this out. There is a misunderstanding here and we have amended the text in the Results sections. As shown in figure 2A, there is only 10 unique phosphomotifs (8 for serines and 2 for Threonines). When we mentioned before 562 unique motifs we meant 562 unique aa sequences around the phosphorylated residue. This have been fixed in the text.

10. Page 11: "Novel roles for PP2ACc55 phosphatase in cytokinesis and endocytosis". A 'd' is missing in the title. Also, I think a bit too much is made of the GO-terms. These are most likely reflecting the fact that you have mitotically arrested samples and you cannot conclude that they are PP2A specific, without doing additional assays. It is also not clear what is being compared. The most logical thing here would be to look for differences between the wt and deleted strains, but this doesn't appear to be what was done. Rather, all phosphopeptides identified were analysed. If this is the case, then the analysis is likely only reflecting the mitotic state, and not able to specifically tell you anything about PP2A substrates.

The 1260 hyperphosphorylated peptides are the ones increase in the cdc55 mutant, therefore the analysis is done looking for the differences between the wt and the cdc55 delete strains. We agree that the use of the whole list of hyperphosphorylated proteins (628 proteins from 1,260 phosphopeptides) is a handicap. However, the 62 phosphopetides (55 proteins) identified in more than one approach reflect more the nature of those phosphopeptides to be enrich using different columns, more than to be more likely to be real substrates. Indeed, each approach uncovered a unique subset of hyperphosphorylated peptides useful for downstream analysis (some hyperphosphorylated peptides found in only 1 method were detected with a huge amount of PSM and great scores; therefore, those are also pretty likely to be substrates).
In addition, 55 proteins is a low number in statistical terms, therefore, an analysis only with 62 phosphopeptides will generate more doubts than real questions. Use the whole list, by contrast, is highly confident in terms of statistics and give us a promising starting point in the understanding of the biological meaning of the peptides that appear hyperphosphorylated when cdc55 is knocked-down. In any case, as mentioned above, the systems biology analysis (GO terms, networks and motif-x) is something exploratory focused in opening doors more than answering questions. The GO analysis is a bioinformatic analysis which main goal is to characterize the Gene Ontology terms specifically enriched in a population of genes/proteins when they are compared with the whole genome. Therefore, the biological conclusions must always be taken carefully. In our work we analyzed the Gene Ontology terms enrichment in a set of proteins carrying hyperphosphorylated peptides in a cdc55 knock-down mutant. We agree that our results likely mean that the mitotic state of the yeast is altered. However, it also true that the population of phosphoproteins used in the comparison is deducted from a list of peptides that appeared hyperphosphorylated when cdc55 was knocked-down. Consequently, it seems highly probable that the enrichment of certain functionalities is due to the absence of this protein in the cell. In fact, the GO suggested some biological processed to be regulated by PP2A-Cdc55. Several published PP2A-Cdc55 functions are included in those GO, confirming previous data. New biological processes like cytokinesis is not surprising due to the cdc55 deletion mutant phenotype and the described localization of PP2A-Cdc55 to the cytokinesis locus, but was not described/published for Cdc55 in budding yeast. We are currently studying the PP2A-Cdc55 function in cytokinesis in our laboratory in detail.

11. Figure 4: A non-PP2A substrate should be included to show that any disruption to the timing of mitotic exit potentially caused by PP2A-Cdc55 deletion is not affecting dephosphorylation kinetics. Also timing here would be more appropriate label rather than cell cycle stage, which is subjective. Would also consider aligning all of the blots above and below each other. The phosphatase assay should be labelled as a separate panel (e.g. 4C), and a loading control is needed.

We have included Pgk1 as the loading control. The timing of the experiments is a bit different every day we release the cells from the metaphase arrest. Therefore, we estimate anaphase spindles by immunofluorescence, look for Cib2 protein levels and budding index and identify the cell cycle stages combining all these parameters. We prefer to do not separate the phosphatase assay in another panel since we run all the samples together in the same protein gel. To separate them in two panels we will have to repeat some lanes in two different panels and it will look odd.

A complete demonstration that timing of mitotic exit and that the dephosphorylation kinetics are not disturb in a cdc55 deletion strains was already published from our lab (Baro et al 2013, Figure 3). In this figure we analyzed mitotic progression by Cdh1 dephosphorylation, Cib2 phosphorylation and degradation, Cdc5 degradation and accumulation of Sic1. As already demonstrated in the paper, the timing and general features of mitotic exit are similar in the wt and cdc55 deleted strain.

References:
Reviewer #2: The authors report on the identification of new substrates for PP2A Cdc55 phosphatase during mitosis using mass spectrometry by comparing the phosphoproteome of a wild type yeast strain to that for a cdc55 lacking mutant strain. The manuscript is well-written and appears comprehensive in reporting how the current literature relates to their results on PP2A Cdc55 phosphatase substrates.

Since I only have a layman's knowledge of proteomics and cell cycle signalling pathways, the main aim of this review is to check the consistency of the data supporting the results reported in the manuscript by the authors. In general, I feel Baro et al have been comprehensive in disseminating the data associated with their manuscript and I have only minor suggestions for improvements.

The manuscript requires an, "Availability of supporting data and materials" section. In this section, information about the raw data stored in PRIDE should be provided.

Done

With regards to the data itself, the dataset contains *.raw files which have been generated by the Orbitrap mass spectrometer. The dataset also contains *.msf files - what are these?

The msf file is the report generated by the Proteome Discoverer Software and include a compilation of the different technical replicates in one unique report. It includes the search in databases (filtered by Percolator algorithm using a FDR < 1%), the normalized quantification values of the features (peptides) as well as the list of the proteins. This file can be opened with the Proteome Discoverer software (and other proteomic software) so that the user can easily visualize the data and use different settings for the filters.

I think it would be helpful if the dataset provides information on the files it contains such as what they are and which experiments they correspond to. For example, are the files from the large-scale identification of PP2A Cdc55-dependent phosphoproteome work, TAP- or HA-tagging experiments? Is it possible to add this information into their PRIDE archive dataset entry webpage or in a README text file that is added to their dataset?

Done

Large-scale identification of PP2A Cdc55-dependent phosphoproteome in metaphase-arrested cells

Can the authors check that Fig. 1C is referred to in the manuscript text?

We thank the reviewer for this point. We missed to referred to Fig.1C in the previous version of the manuscript and it has been fixed now (new Figure 1D).

The list of peptides listed in PDF files in Additional files 3 and 4 could be provided in an Excel file or text format. This will aid the re-use of the data instead of users having to copy and paste or even type out them manually if the copying function does not work properly in their PDF document applications.

Done. We extended the reviewers comments to all figures, and all the Additional file containing list of elements presented before as pdf have been changed to xlsx.

Novel roles for PP2A Cdc55 phosphatase in cytokinesis and endocytosis

Are the protein interactions of the network shown in Additional file 7 available as a text file as well as a screenshot? This would be more useful if people wanted to re-use it.

We have added a complete list of the protein interactions nodes in the new Additional file 10.

Validation of novel PP2A cdc55 substrates in mitosis

Page 15 first paragraph: Is the reference to Additional file 1 correct on line 337? Maybe Additional file 3 is the correct file to be referred to? If so, where is Kin4 in this file?
Again we thank the reviewer to point this mistake. It should be new Additional file 3.

Data Analysis for peptide identification and quantification

This Section mentions the use of R with the rvest, vennerable, venneuler packages - page 27 line 640. The R scripts should be provided along with documentation on how to use it. Since R scripts are source code then the manuscript will need an, "Availability of source code and requirements" section as detailed in the authors instructions (https://academic.oup.com/gigascience//pages/research).

Done. Included in Additional file 15.

Structure prediction of Cdc55

I do not have experience of the tools used in this section. Are HHpred and MODELLER command line tools? If so, then any scripts involving their use should be make available.

HHpred, MODELLER and HADDOCK are software or/and online tools available (websites) to perform this kind of analysis.

Sampling the binding interface of the CDC55/Mob1 complex

If HADDOCK is a command line tool then any script involving its use should be made available.

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Peter Li, GigaScience

Reviewer #3: Baro et al conducted an analysis of PP2A-Cdc55 substrates and interacting proteins under mitotic arrest conditions in budding yeast by proteomics and quantitative phosphoproteomics. They validated differences in phosphorylation for proteins involved in the MEN and FEAR pathway as well as a different PP2A regulatory subunit, and generated a docking model for Cdc55 and Mob1.

PP2A is a phosphoprotein phosphatase consisting of catalytic, scaffolding, and regulatory subunit. The regulatory subunit confers substrate specificity. The PP2A regulatory subunit Cdc55 is the yeast homolog of B55 in humans and thought to be responsible for counteracting Cdk1 phosphorylation during mitotic exit. In yeast, PP2A-Cdc55 acts in concert with the Cdc14 phosphatase during mitotic exit.

The authors indicate that while their study was under preparation, another study was published that conducted PP2A-Cdc55 substrate analyses in G1, S, and G2. A comparison of the datasets should be included.

We have compared of our dataset with Godfrey et al. We have found 69% (128 common/186 proteins in Godfrey et al) of the proteins identified in Godfrey et al indicating a high degree of overlapping in both studies. We have discussed it in the discussion section and included the common proteins in Additional file 13.

Baro et al uses a Cdc55-delete strain to identify potential substrates of PP2A-Cdc55 in mitotic arrest which is induced by Cdc20 depletion. The authors state that 95% of cells were arrested in metaphase but do not show data to support this. This should be included.

The metaphase arrest in a cdc55 deletion strains is a well-established system in our laboratory leading to a 95-100% arrest of the cells in metaphase. The characterization of the mutant and mitotic exit kinetics were carefully studied in our laboratory and published previously (Baro et al 2013).

To detect differences in phosphorylation between wild-type and Cdc55-delete strains,
the authors use a SLIAC strategy. It would be helpful if the authors could include a scheme of the experimental design and clearly indicate which strain was labeled heavy and which strain was labeled light.

Done. Included in new Figure 1A.

Interestingly, the authors used three different strategies for phosphopeptide enrichment but do not provide an explanation for this unusual combination. Please explain.

We have included the explanation in the manuscript in Results section. Often, proteomics studies are performed using just one of the phosphoenrichment methods using biological duplicates or triplicates. However, it has been reported that the different phosphopeptide enrichment methods isolated distinct, partially overlapping segments of a phosphoproteome, whereas none of the methods were able to provide a whole phosphoproteome (Zhou et al 2001, Bodenmiller et al 2007). Enrichment techniques are complementary, such that a combination of methods greatly enhances the number of phosphopeptides isolated from complex samples (Dunn et al 2009).

In addition, the TiSH method (a combination of enrichment and fractionation methods abbreviated TiSH for TiO2-SIMAC-HILIC) has some others peculiarities: extensive HILIC fractionation of the monophosphorylated peptides increases the phosphopeptide coverage (Engholm-Keller et al 2012). Moreover, the ratio of monophosphorylated and multiphosphorylated peptides detected for each method also varies considerably:

- Mono-phosphorylated/multiphorshorylated (in our dataset)
  - 42%/58% TiO2
  - 83%/17% SIMAC
  - 90%/10% TiSH

So, none of the phosphopeptide enrichment methods provide a whole phosphoproteome. Therefore, our idea was to use the different phosphopeptide enrichment methods in order to identify the biggest PP2A-Cdc55 phosphoproteome. The set-up of our experiment was to use the three different phosphoenrichment methods available at the moment using SILAC. We performed a biological replicate for each method with technical duplicates or triplicates (triplicates for SIMAC and duplicates for TiO2 and TiSH). Due to the intrinsic and distinct nature of the phosphoenrichment methods, we do not expect to increase the reproducibility of the three different phosphopeptide enrichment methods and the overlapping will remain low. To perform the sample data analysis we used the raw files from the technical replicas of each experimental method (described above). A unique peptide and protein list was obtained from the compilation of all the replicates’ raw files for each phosphoenrichment method used. We have explained those points better in the manuscript.

A table of all identified peptides or at least phosphopeptides is not included, please do so.

The list of hyperphosphorylated peptides is included in new Additional file 3. The whole set of peptides identified in the wt and cdc55 strains are deposited in PRIDE as msf.files. A summary can be found in new Additional file 2 containing 10069 peptides.

In their analyses, they identify 1,260 phosphorylation sites that increase in phosphorylation upon Cdc55 deletion. While a table of hyperphosphorylated sites is included, the description of this table (additional file 2) needs to be improved to be understandable for the reader. Are the values in the TiSh, SIMAC, TiO2 column ratios? If so of what? The statistical analysis is missing. It would also be helpful if either the site of phosphorylation would be indicated in the peptide sequence in column c or if the peptide sequence would be represent as centered around the phosphorylated residue +/- 6 amino acid for instance. P-values for the quantification of each phosphopeptides should be included.

Done. New Additional file 3 (old Additional file 2) has been improved following the
Only the hyperphosphorylated peptides are included in additional file 2, however, the volcano plot in Figure 1H shows all data. This data needs to be added.

The volcano plot includes the 1260 hyperphosphorylated peptides in absence of Cdc55 and not all the peptides identified. We have modified the volcano plot for clarity (new Figure 1I).

To exclude that differences detected in phosphorylation are due to do difference in protein abundance, the authors conduct an analysis of protein abundance differences in both strains. However, the authors do not conduct a subtractive analysis to correct phosphopeptide ratios by protein abundance differences, just state that most proteins don't change. Such an analysis should be included.

As the reviewer suggested, correct phosphopeptide ratios by protein abundance differences will be the perfect escenario. For that you need to be able to detect “ALL” the protein and normalize by the their abundance. But the reality in proteomics is that only a fraction of the protein identified in our enrichment fractions is also identified in the whole extract (without enrichment). We have separate the whole cell extract in 8-10 fractions in order to increase the chances to identify more proteins, but still not all the proteins are detected. Therefore, the analysis we show is the best we can do at this point with the limitations of the proteomics. For instance, we were able to detect 45 proteins out of the 55 (corresponding to 62 phosphopeptides) hyperphosphorylated proteins (new figure 1J); and as included in the manuscript most of them do not change their protein abundance.

In addition, after several years of working with cdc55 mutant, we did not observe global changes in protein abundance in the cdc55 mutant. Moreover, all the proteins we have deeply studied previously do not change protein levels especifically in a cdc55 mutant (Queralt et al 2006, Queralt and Uhlmann 2008, Baro et al 2013).

As the reviewer stated, the protein analysis results are not included. Only a list of protein names is provided in additional file 3. The complete list of all quantified protein ratios and their identification metrics needs to be added.

Done. Included as new Additional file 4.

Only 145 sites are identified in 2 out of 3 replicates and only 30 in all three replicates. The low reproducibility of identifications and quantifications is potentially due to the use of three different enrichment methods, each of which likely selects for a different subset of phosphopeptides. Because the overlap of the three or even two out of three datasets is very low, subsequent analyses based on phosphopeptides that are identified as statistically significantly increased are limited. To improve the dataset, the authors need to improve the overlap of the phosphoproteomic analyses for instance by using the same phosphopeptide enrichment strategy in replicate to ensure greater reproducibility in phosphopeptide identification and quantification.

As the reviewer noted, none of the phosphopeptide enrichment methods provide a whole phosphoproteome and they purified peptides of different nature. Each method provides varying degrees of selectivity and specificity of phosphopeptide enrichment. Therefore our idea was to use the different phosphopeptide enrichment methods in order to identify the biggest PP2A-Cdc55 phosphoproteome. We actually think, that the strength of our data is indeed based on the 3 different enrichment methods. Due to the intrinsic and distinct nature of the phosphoenrichment methods, we do not expect to increase the overlapping of the 3 different enrichment methods increasing the amount of biological replicates. To perform the sample data analysis we used the raw files from the technical replicas of each experimental method (described above). A unique peptide and protein list was obtained from the compilation of all the technical replicates' raw files for each phosphoenrichment method used. We have now clarified all these points in the manuscript.

We are unable to provide biological triplicates of each phosphoenrichment method.
Currently, the three proteomics units are using different and new mass-spectrometers; therefore, it is technically impossible to perform biological triplicates in the same conditions as we did several years ago.

Instead of concentrating on the 62 phosphopeptides with statistically significant increases in phosphorylation, the authors conduct their subsequent analyses on all increased phosphorylation sites. Because the abundance change of these phosphopeptides is not supported by statistical analysis, these analyses are not informative. For instance, the authors state that they find a preference of PP2A-Cdc55 for the dephosphorylation of phosphothreonine over phosphoserines as reported by others. However, the enrichment in threonine-directed phosphorylation sites is not observed in just the 62 statistically significant increased phosphorylation sites. The same strategy of using all increased sites is applied for all subsequent analysis, including assigning counteracting kinases, GO and STRING network analysis, and needs to be revised using a dataset with a higher degree of overlap in phosphopeptide identifications and quantifications.

Just note that the 62 phosphopeptides of the volcano plot are not the most statistically significant ones. They reflect the phosphopeptides being enriched in more than one approach due to their intrinsic nature. Considering the different nature of the phosphopeptides identified for each method, the 62 phosphopeptide are pretty likely to be PP2A-Cdc55 substrates. Nevertheless, we cannot consider the remaining 1198 hyperphosphorylated peptides to be all false positives (some phosphopeptides were identified with highly amounts of PSM, and pretty high confidence proteomic statistics). Of course, we agree that we cannot claim that all are true positives.

We agree that the use of the whole list of hyperphosphorylated proteins (628 proteins from 1,260 phosphopeptides) instead of the repeated ones is a handicap. However, this number is low in statistical terms, therefore, an analysis only with 55 proteins will generate more doubts than real questions. Use the whole list, by contrast, is highly confident in terms of statistics and give us a promising starting point in the understanding of the biological meaning of the peptides that appear hyperphosphorylated when cdc55 is knocked-down. In any case, as we mentioned above, the systems biology analysis (GO terms, networks and motif-x) is something exploratory focused in opening doors more than answering questions. The GO analysis is a bioinformatic analysis which main goal is to characterize the Gene Ontology terms specifically enriched in a population of genes/proteins when they are compared with the whole genome. Therefore, the biological conclusions must always be taken carefully. In our work we analyze the Gene Ontology terms enrichment in a set of proteins carrying hyperphosphorylated peptides in a cdc55 knock-down system. We agree that our results likely mean that the mitotic state of the yeast is altered. However, it also true that the population of phosphoproteins used in the comparison is deducted from a list of peptides that appeared hyperphosphorylated when cdc55 was knocked-down. Consequently, it seems highly probable that the enrichment of certain functionalities is due to the absence of this protein in the cell. In fact, the GO suggested some biological processed to be regulated by PP2A-Cdc55. Several published PP2A-Cdc55 functions are included in those GO, confirming previous data. New biological processes like cytokinesis is not surprising due to the cdc55 deletion mutant phenotype and the described localization of PP2A-Cdc55 to the cytokinesis locus, but was not described/published for Cdc55. We are currently studying the PP2A-Cdc55 function in cytokinesis in our laboratory in detail.

The authors also perform Cdc55 interactome studies. Again only protein names are provided in additional file 8, no information on peptide counts versus controls or parameters/metrics of identification are provided. Surprisingly, for the analysis of HA-tagged Cdc55 this information is available in additional file 9. Here the authors perform two replicates, however, it is not determined if the identifications were reproducible. There is no explanation why the authors performed TAP as well as HA purifications. The authors concluded that the interactomes analysis in total are not informative because only a few proteins identified in the phosphoproteomics analysis are captured here.
The purification of Cdc55 was an alternative strategy that we use in our laboratory in order to identify processes related to PP2A-Cdc55. We tried two purification strategies (HA and TAP) that were optimized in our laboratory for other purposes. We agree that some of the raw data is pretty poor (HA purification), but sadly is what we get at that moment from the Proteomic services performing the analysis. For the TAP strategy, the proteomic service provided us with the raw data (Excel file) and it is included in the PRIDE repository.

Finally, the authors perform an in silico analysis of Cdc55 and Mob1 to identify interaction surfaces which recapitulates a previously identified interface on Cdc55 but does not identify a corresponding interface on Mob1. In general the identification of PP2A holoenzyme specific substrates is of high interest and could be a great resource for the community. However, the analyses conducted here lack rigor and need to be improved upon to be valuable.

We thank the reviewer for his/her insights and truly believe that we have addressed most of his/her concerns in the new version of the manuscript.

We are really sorry since we noticed that we failed to properly explain why we used the three different phospho-enrichment methods. In the new version of the manuscript, we have explained better why we used the three phospho-enrichment methods and we provided better statistical parameters details of the phosphopeptides.

Combining the three methods, we identified 1260 high confidence phosphopeptides with increased phosphorylation (Heavy (wt)/light (cdc55 mutant) ratio < 0.75) in the absence of Cdc55 phosphatase; therefore likely to be PP2A-Cdc55 substrates. Those 1260 hyperphosphorylated peptides include 62 phosphopeptides that were identified in at least 2 (out of 3) enrichment methods (Figure 1H) with a t-test p-value < 0.05. Therefore, considering the different nature of the phosphopeptides identified for each method, the 62 phosphopeptide are pretty likely to be PP2A-Cdc55 substrates. Nevertheless, we cannot consider the remaining 1198 hyperphosphorylated peptides to be all false positives (some phosphopeptides were identified with highly amounts of PSM, and pretty high confidence proteomic statistics). Of course, we also agree that we cannot claim that all are true positives.

Often, proteomics studies are performed using just one of the phospho-enrichment methods using biological duplicates or triplicates. However, it has been reported that the different phospho-enrichment enrichment methods isolated distinct, partially overlapping segments of a phosphoproteome, whereas none of the methods were able to provide a whole phosphoproteome (Zhou et al. 2001, Bodenmiller et al. 2007). Enrichment techniques are complementary, such that a combination of methods greatly enhances the number of phosphopeptides isolated from complex samples (Dunn et al. 2009). In addition, the TiSH method (a combination of enrichment and fractionation methods abbreviated TiSH for TiO2-SIMAC-HILIC) has some other peculiarities: extensive HILIC fractionation of the monophosphorylated peptides increases the phosphopeptide coverage (Engholm-Keller et al. 2012). Moreover, the ratio of monophosphorylated and multiphosphorylated peptides detected for each method also varies considerably:

Mono-phosphorylated/multiphosphorylated (in our dataset)
- 42%/58% TiO2
- 83%/17% SIMAC
- 90%/10% TiSH

So, none of the phosphopeptide enrichment methods provide a whole phosphoproteome. Each method provides varying degrees of selectivity and specificity of phosphopeptide enrichment resulting in the identification of subsets of phosphopeptides of different nature. Therefore our idea was to use the different phosphopeptide enrichment methods in order to identify the highest spectrum of the PP2A-Cdc55 phosphoproteome. Preliminary results with one biological replicate in one of the approaches did not increase the reproducibility of the three different phosphopeptide enrichment methods. The intrinsic reproducibility of a given phospho-enrichment methods is high, but do not increase the matching phosphopeptides in more than one approach. Due to the intrinsic and distinct nature of the phospho-enrichment methods, we do not expect to increase the reproducibility of
the three different phosphopeptide enrichment methods and the overlapping will remain low. Actually, we really think the strength of our data is indeed based on the 3 different enrichment methods.

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Running Head: Targets of PP2A\textsuperscript{Cdc55} phosphatase

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Text word count: 13283
Key words: mitosis, PP2A<sup>Cdc55</sup> phosphatase, Pkc1, Cla4, mitotic exit network (MEN), Mob1, phosphoproteomics, SILAC.

Abstract

Background: Protein phosphatase 2A (PP2A) is a family of conserved serine/threonine phosphatases involved in several essential aspects of cell growth and proliferation. PP2A<sup>Cdc55</sup> phosphatase has been extensively related to cell cycle events in budding yeast, however few PP2A<sup>Cdc55</sup> substrates have been identified. Here, we performed a quantitative mass spectrometry approach to reveal new substrates of PP2A<sup>Cdc55</sup> phosphatase and new PP2A-related processes in mitotic arrested cells. Results: We identified 626 potential PP2A<sup>Cdc55</sup> substrates involved in a broad range of mitotic processes. In addition, we validated new PP2A<sup>Cdc55</sup> substrates such as Slk19 and Lte1, involved in early and late anaphase pathways, and Zeo1, a component of the cell wall integrity pathway. Finally, we constructed docking models of Cdc55 and its substrate Mob1. We found that the predominant interface on Cdc55 is mediated by a protruding loop consisting of residues 84-90, thus highlighting the relevance of these aminoacids for substrate interaction.

Conclusions: We used phosphoproteomics of Cdc55 deficient cells to uncover new PP2A<sup>Cdc55</sup> substrates and functions in mitosis. As expected, several hyperphosphorylated proteins corresponded to Cdk1-dependent substrates, although other kinases’ consensus motifs were also enriched in our dataset, suggesting that PP2A<sup>Cdc55</sup> counteracts and regulates other kinases distinct from Cdk1. Indeed, Pkc1 and Cla4 kinases emerged as novel nodes of PP2A<sup>Cdc55</sup> regulation, highlighting a major role of PP2A<sup>Cdc55</sup> in membrane trafficking and cytokinesis, gene ontology terms significantly enriched in the PP2A<sup>Cdc55</sup>-dependent phosphoproteome.
Background

Protein phosphorylation is a key regulatory mechanism of protein function that governs cell cycle progression (reviewed in (1)). The highly conserved and specific family of cyclin-dependent serine/threonine kinases, the Cdks, were considered the main component of the cell cycle control system once they were discovered. Nowadays, it has become clear that the opposing phosphatases also play a key role in setting the net phosphorylation state of each substrate, thereby being the other side of the coin controlling phosphorylation waves during cell cycle progression. Cdk1-cyclin activity progressively increases as the cell cycle progresses, reaching its maximum in metaphase. At the end of mitosis, high Cdk1 activity needs to return to lower levels in order to enter into a new G1 phase, and activation of Cdk1-counteracting phosphatases is required for this transition.

Type 2A phosphatases (PP2A) is a family of conserved protein serine/threonine phosphatases involved in several essential aspects of cell growth and proliferation. PP2A is a major Cdk1-counteracting phosphatase during cell cycle progression, which works solely as a multimeric enzyme (2). The PP2A core enzyme consists of a scaffold subunit and a catalytic subunit. The heterodimeric complex interacts with a variable regulatory subunit (B subunit) to assemble into a holoenzyme. Although highly conserved within the same family, these regulatory subunits share little sequence similarity across families, and their expression levels vary greatly in different cell types and tissues (3). Several studies have shown that PP2A regulatory subunits confer exquisite substrate specificity to PP2A holoenzymes in vivo (4–12).
PP2A is highly conserved from yeast to humans. Knockdown of either the catalytic or a subset of regulatory subunit genes of PP2A holoenzymes results in unviable cells (13–17). In *S. cerevisiae*, the PP2A scaffold subunit is known as Tpd3. The catalytic subunit of the core enzyme is either Pph21 or Pph22, two highly homologous proteins sharing 95% sequence identity (18, 19). Mutation of both *PPH21* and *PPH22* eliminates the majority of PP2A activity in the cell and drastically reduces growth. Strains lacking *PPH21*, *PPH22*, and a third related gene, *PPH3*, are completely unviable (19). The regulatory subunits comprise Cdc55 (B-type in vertebrates), Rts1 (B’-type in vertebrates) and the predicted B-subunit Rts3. In this work, we refer to Tpd3, Pph21 or Pph22, and Cdc55 holoenzyme as PP2A<sup>Cdc55</sup>.

PP2A<sup>Cdc55</sup> and its mammalian homolog, PP2A<sup>B55</sup>, have been extensively studied for their role in mitotic entry regulation (reviewed in (20)). The regulatory axis of Greatwall and PP2A inhibitors, endosulfins (Igo1/2 in budding yeast), govern mitotic entry in both yeast and in higher eukaryotes (21–24), illustrating the strong conservation of PP2A regulatory mechanisms across eukaryotes. One of the first known functions of PP2A<sup>Cdc55</sup> in cell-cycle regulation was its key role affecting Swe1 and Mih1 activity at the G2/M transition (25–30) (Wee1 and Cdc25 in vertebrates). More recently, signals regarding the status of membrane traffic have been shown to be integrated into mitosis progression through PP2A<sup>Cdc55</sup> via a signaling cascade that includes Rho1, Pkc1 and Zds1/2. Pkc1 binds to PP2A<sup>Cdc55</sup>-Zds1/2, which directly controls the phosphorylation states of Mih1 and Swe1 (31–36).

However, PP2A<sup>Cdc55</sup> substrates and functions during mitotic exit are less understood, since another phosphatase, Cdc14, which is essential and specifically activated at anaphase-onset, has been considered the principal Cdk1-counteracting phosphatase during mitotic exit in
budding yeast. In contrast, in vertebrates, although *CDC14* homologues are present (37), their functions seem less conserved (38), and PP2A-B55 and PP1 phosphatases are considered the major Cdk1-counteracting phosphatases during mitotic exit (39, 40).

Indeed, yeast PP2A<sub>Cdc55</sub> has also been shown to play a major role during mitotic exit. PP2A<sub>Cdc55</sub> counteracts Cdk1-dependent phosphorylation of Net1, which is crucial for Net1-Cdc4 dissociation (41). Zds1/2 proteins cooperate with separase to downregulate PP2A<sub>Cdc55</sub> at anaphase-onset (42, 43) which leads to Cdc4 activation and release. Thus, Zds1/2 are common PP2A<sub>Cdc55</sub> modulators, participating in both entry and exit from mitosis. It has recently been described that PP2A<sub>Cdc55</sub> downregulation in anaphase also initiates the Mitotic Exit Network (MEN) by dephosphorylating the MEN components Bfa1 and Mob1 (44). In addition, PP2A<sub>Cdc55</sub> downregulation at anaphase-onset facilitates separase proteolytic activity towards Scc1, which triggers sister-chromatid segregation (45). Finally, PP2A<sub>Cdc55</sub> as well as its homologue, PP2A-B55, has been shown to counteract Cdk1-dependent phosphorylation of APC/C during mitosis (46–49). In conclusion, PP2A<sub>Cdc55</sub> is also a major Cdk1-counteracting phosphatase during mitotic exit in budding yeast.

Quantitative mass spectrometry analysis has been used to identify Cdk-dependent phosphorylation sites in a large number of substrates *in vivo*, by comparing the phosphoproteome of wild-type cells and Cdk1 defective cells (50, 51). More recently, a global analysis of Cdc14 dephosphorylation sites was performed using a similar approach (52, 53). In this study, we performed a systematic quantitative phosphoproteomic analysis of PP2A<sub>Cdc55</sub> deficient cells to identify novel PP2A<sub>Cdc55</sub> substrates and regulated processes. Since drug inhibition by Okadaic acid in budding yeast only works at high concentration,
which also inhibits other Ser/Thr phosphatases, and due to the specificity that the regulatory
subunits confer to PP2A (54), in our approach we used a cdc55 deletion mutant to explore
the PP2A<sub>Cdc55</sub>-dependent phosphoproteome. Hence, cdc55 deficient cells lack PP2A<sub>Cdc55</sub>
activity but not the other PP2A complexes, PP2A<sub>Rts1</sub> or PP2A<sub>Rts3</sub>. We identified both
known and potentially new substrates for PP2A<sub>Cdc55</sub> as well as their phosphorylation sites.
Our dataset is consistent with PP2A<sub>Cdc55</sub> being a serine/threonine phosphatase and having a
major role in counteracting Cdk1 activity, since S/T-P sites were the most abundant motif
enriched in the absence of Cdc55. But, interestingly, we also identified other kinase
consensus sequences corresponding to ERK/MAPK kinases, Cdc5 Polo kinase and AGC
kinases; suggesting that PP2A<sub>Cdc55</sub> counteracts other kinases apart from Cdk1, and/or
regulates their activities. Finally, we were able validate up to 9 targets by protein-protein
interactions and/or by western blot, which strongly support the validity of our study. We
assume that the substrates of the PP2A<sub>Cdc55</sub> phosphatase identified might not be all direct
targets; however, as well as this, our work also uncovered valuable new PP2A-related
processes.

Data description
To screen for potential new substrates of the PP2A<sub>Cdc55</sub> phosphatase during mitosis, we
performed a quantitative phosphoproteomic analysis based on the Stable Isotope Labelling
by Amino Acids in Cell Culture (SILAC) technique. To study the PP2A<sub>Cdc55</sub>-dependent
phosphoproteome, we compared the phosphoproteome of a wild-type strain and a cdc55Δ
mutant strain, which lacks the activity of PP2A<sub>Cdc55</sub> but not other PP2A complexes. The
PP2A regulatory subunits confer substrate specificity to PP2A. Therefore, in our approach
we specifically studied the PP2A$^{\text{Cdc55}}$ and no other PP2A complexes (with Rts1 or Rts3).

To minimize compensatory mutations that might accumulate over time in the gene deletion strain, we freshly prepared the $cdc55\Delta$ mutant. Wild-type and $cdc55\Delta$ cells were grown in methionine-free minimum media containing $^{13}\text{C}_6$-lysine and -arginine (heavy) or unmodified arginine and lysine (light), respectively. Both strains expressed $CDC20$ under the control of the repressible MET3 promoter and were synchronized at the metaphase-to-anaphase transition by adding methionine to the media, which causes Cdc20 depletion. At the time of harvesting, more than 95% of cells in each culture were arrested in metaphase.

Protein extracts were prepared as described in methods.

We used three different strategies to enrich for phosphopeptides: SIMAC, TiO$_2$ and TiSH-based (TiO$_2$-SIMAC-HILIC). A schematic representation of the different strategies used is shown in Fig. 1A (more details in Additional file 1 and Methods). Different phosphopeptide enrichment methods isolated distinct, partially overlapping segments of a phosphoproteome, whereas none of the methods were able to provide a whole phosphoproteome (55, 56). Phosphopeptide enrichment strategies are complementary, such that a combination of methods greatly enhances the number of phosphopeptides isolated from complex samples (57). Analysis of the heavy/light labelled phosphopeptides was performed by LC-MS/MS (see methods for more details). Global analysis of the data led to the identification of 10,069 peptides (Additional file 2), including 4,467 phosphopeptides. Only peptides identified with high confidence (< 1% FDR) were used for further analysis. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium with the dataset identifier PXD007613.
Analyses

Large-scale identification of PP2A<sup>Cdc55</sup>-dependent phosphoproteome in metaphase-arrested cells

To study the PP2A<sup>Cdc55</sup>-dependent phosphoproteome, we selected the hyperphosphorylated peptides averaging all the single phosphopeptides obtained in the three experimental approaches (SIMAC, TiO2 and TiSH), according to the filtering parameters described in methods (Fig. 1B; H/L ratio <0.75 or log2(H/L) ratio <-0.42). Analysis of this subset of data led to the quantification of 1491 phosphoproteins, represented by 4467 phosphopeptides. Among them, we found 1,260 hyperphosphorylated peptides which show H/L ratios <0.75 (log2(H/L)<-0.42), corresponding to 628 phosphoproteins. The hyperphosphorylated peptides selected and statistical parameters used are shown in Additional file 3. In addition, already known PP2A<sup>Cdc55</sup> substrates such as Net1, Mob1, Gis1 and Whi5 were identified as being hyperphosphorylated in the cdc55∆ mutant, which strongly supports the validity of our approach (41, 44, 58, 59).

Since phosphorylation changes measured by the heavy/light ratio could be affected by changes in protein abundance due to absence of Cdc55, we analyzed one aliquot of the protein extract without phosphopeptide enrichment (see methods) and determined the heavy/light ratio to account for protein abundance. A full list of the peptides and proteins identified are summarized in Additional file 4. We could quantify a total of 18,592 peptides, of which 15,640 peptides contained a heavy/light ratio >0.8 and 2,952 peptides which had a heavy/light ratio <0.8. Therefore, only 15.8% of the peptides had reduced protein abundance due to the absence of Cdc55 (Fig. 1C). In fact, we identified 286 matching proteins to the selected hyperphosphorylated dataset (see Additional file 5), and most of them had similar protein abundance between the wild type and the cdc55∆ mutant.
(heavy/light ratio >0.8 in non-enriched analysis). Therefore, we conclude that most of the hyperphosphorylated proteins selected with a heavy/light ratio <0.75 (log2(H/L) ratio <-0.42) correspond to phosphorylation changes and not to protein abundance changes. Nevertheless, we cannot rule out that, for some proteins, changes in protein abundance might affect the heavy/light ratio, since we could not identify all the hyperphosphorylated peptides in the non-enriched fraction.

The overlap of hyperphosphorylated peptides (n=1260) and their corresponding phosphoproteins found in the three different approaches (SIMAC, TiO2 and TiSH) are shown by Venn diagrams (Fig. 1F-G). Common proteins found in the three experiments are summarized in Fig. 1H and the common peptides are shown in Additional file 6. The volcano plot of the common phosphopeptides (at least in two of the three approaches) showed a higher amount of hyperphosphorylated peptides (n=62) compared to the hypophosphorylated ones (n=27) (Fig. 1I), in accordance with enrichment in PP2A$^{Cdc55}$ potential substrates. We managed to identify and quantify the amount of protein of 55 proteins (out of 62) in the whole cell extract (Fig. 1J). In 76% (42/55) of the cases the amount of protein did not change significantly and, therefore, we can be certain that the H/L ratio is due to hyperphosphorylation of the peptides rather than a change in protein abundance. However, since SIMAC, TiO2 and TiSH-based enrichments have different capacity and specificity, common hits are considered hyperphosphorylated peptides which showed similar performance in the different purification protocols used, rather than being more likely to be PP2A$^{Cdc55}$ substrates. Indeed, each approach uncovered a unique subset of hyperphosphorylated peptides useful for downstream analysis.
We next analyzed the phosphorylated residues found in the hyperphosphorylated peptides (n=1260) dataset using the non-enriched sample as background. From 1,375 unique phosphosites identified, 78.25% corresponded to phosphoserine, 20.65% to phosphothreonine and 1.09% to phosphotyrosine (Fig. 1D), which is consistent with PP2A<sup>Cdc55</sup> being a Ser/Thr phosphatase. This phosphosite distribution is also consistent with the recently reported PP2A<sup>Cdc55</sup> preference for threonine residues in mitotic substrates (60, 61), since the global <i>S. cerevisiae</i> phosphoproteome consists of only 13-15% phosphothreonine. Our next analysis regarding phosphomotifs enriched in the cdc55Δ mutant also highlighted PP2A<sup>Cdc55</sup> preference for phosphothreonines (see below).

**PP2A<sup>Cdc55</sup> dependent phosphorylation sites of known kinases.**

We were interested in studying the kinases counteracted by PP2A<sup>Cdc55</sup>. It has been shown that PP2A<sup>Cdc55</sup> phosphatase can counteract Cdk1 phosphorylation (41) and Cdc5 phosphorylation (44, 45). We found that 32.95% of the phosphosites correspond to SP/TP (minimum Cdk1 consensus sequence), consistent with PP2A<sup>Cdc55</sup> mainly counteracting Cdk1 phosphorylation (Fig. 1E).

In order to identify consensus phosphorylation sites of other known protein kinases, enriched sequence motifs surrounding the phosphosites in the hyperphosphorylated dataset were analyzed via Motif-X (62). For this analysis, only phosphorylated residues identified with high confidence were considered (peptides with a pRS probability > 95%; see Additional file 7). The 721 unique hyperphosphorylated peptides contained 562 unique aminoacids sequence containing the phosphorylated residue. We obtained 10 representative unique phosphomotifs (8 for serines and 2 for threonines). As expected, the most represented motif found was S-P (Fig. 2A), present in 23.95% of the dataset, which
corresponds to the minimum consensus site of Pro-directed kinases, such as ERK1, p38MAPKs, Cdk1, Cdk2, Cdk4 and Cdk5 (63). Interestingly, the second phosphorylation consensus sequence found was R-x-x-S of AGC kinases, which include the PKC, PKA, Sch9, Ypk1 and Ypk2 kinases. This motif was present in 9.58% of the dataset. With a similar abundance, we found S-x-x-S and T-P motifs (9.29% and 8.99%, respectively). Finally, we found the motif S-x-x-E, one of the consensus sites described for polo kinase-dependent phosphorylation, present in 5.16% of the dataset. Within this consensus site we can infer the D/E/N-x-S motif described for the budding yeast polo-like kinase Cdc5 (64).

Motif sequences, their scores and fold increase are shown in Fig 2B. Interestingly, T-P motif presented the highest fold-increase, followed by R-x-x-S-x-x-S and S-P motifs. Our results suggest a greater regulation of T-P sites over S-P sites by PP2ACdc55 in mitotic cells, as recently reported (60, 61). The motifs uncovered also suggest PP2ACdc55 could counteract other kinases apart from Cdk1 and Cdc5 Polo-like kinase. Several of these kinases are also found hyperphosphorylated in our study, as well as some substrates of these newly identified PP2ACdc55-counteracted kinases, suggesting that PP2ACdc55 could directly regulate their kinase activity (Table 1).

On the other hand, Cdk1-dependent phosphoproteome was uncovered in a similar study, where approximately 314 proteins containing Cdk1 consensus sites were identified as likely Cdk1 targets in budding yeast (50). Since Cdk1 is the main kinase counteracted by PP2ACdc55 phosphatase, we compared our list of potential PP2ACdc55 substrates containing the S/T-P motif to the Cdk1-dependent data set and we found 74 proteins that were common in both datasets (Fig. 2C). These common proteins corresponded to GO processes such as cell cycle and mitotic cell cycle (Fig. 2C right panel) as expected, and they are more likely to be regulated by both Cdk1 and PP2ACdc55.
Novel roles for PP2A$^{Cdc55}$ phosphatase in cytokinesis and endocytosis

Functional clustering of proteins that displayed enhanced phosphorylation in our dataset is presented in Additional file 8 and summarized in Table 2. We found a strong enrichment for cell cycle related functional categories such as cell cycle, mitotic cell cycle, cell growth, budding, cell polarity, actin cytoskeleton, cytokinesis and endocytosis. Most of these processes are related to mitosis events, consistent with a PP2A$^{Cdc55}$ role in mitosis and our analysis of mitotic arrested cells.

PP2A$^{Cdc55}$ has been recently shown to monitor membrane trafficking and bud growth, integrating several cues to the mitotic entry regulators Swe1 and Mih1 (34) (Wee1 and Cdc25 in mammals). Budding impinges a dramatic re-arrangement of the cell wall and cell morphogenesis, GO categories that were found in our study. Interestingly, we found components of the cell wall integrity pathway, the Pkc1, Bck1, Ypk1/2 and Pkh1 kinases, and Zeo1. Indeed, Pkc1 consensus motif was enriched in our PP2A$^{Cdc55}$-dependent phosphosites. Moreover, we have been able to identify a physical interaction between Zeo1 and Cdc55 (see below), suggesting that Zeo1 is likely to be a PP2A$^{Cdc55}$ substrate. We also identified other proteins related to budding such as Bud3, Bud6, Gin4 and Nap1.

Interestingly, many proteins required for cytokinesis like Inn1, Boi1, Shs1, Bni4, Cdc11, Cdc12, Cdc3 and Iqg1 were also found among the PP2A$^{Cdc55}$-dependent phosphoproteome, as well as proteins involved in the general organization of the actin cytoskeleton like Sla1, Bud6, Bni1 and Spa2. On the other hand, we also found proteins related to vesicle-mediated transport and endocytosis. Control of membrane structures, cell membrane trafficking and endocytosis have recently been linked to cytokinesis processes (65) and mammalian homolog, PP2A-B55, has been related to the reformation of the nuclear
envelope and the Golgi apparatus during telophase (40). Finally, we also identified proteins related to osmotic stress and nutrient response. Thus, PP2A\textsuperscript{Cdc55} phosphatase seems to play a key role sensing several cues of the environmental conditions, cell growth and cell structure, and integrating them into cell cycle regulation. In our screen, we also found proteins related to signal transduction, transcription, chromatin organization and organelle organization, all processes that are monitored and/or coordinated within the cell cycle (see Additional file 8).

A String Network Analysis of our hyperphosphorylated proteins is showed in Additional file 9 and the list of interactions in Additional file 10. We plotted the number of interactions found for each protein, and we identified 6 proteins with more than 15 interactions: Cdc28, Cla4, Pkc1, Snf1, Stb1 and Swi4 (Fig. 3). Cdc28 and Pkc1 (34, 35) had been previously linked with PP2A\textsuperscript{Cdc55}. Pkc1 is a serine/threonine kinase involved in cell wall organization that has recently been related to PP2A\textsuperscript{Cdc55}, as it controls the binding of Igo1/2 proteins to PP2A (36). As we just mentioned, we uncovered several proteins from the cell wall organization pathway, and the Pkc1 kinase consensus site was found enriched in our PP2A\textsuperscript{Cdc55} dependent phosphoproteome.

On the other hand, Snf1 is an AMP-activated serine/threonine kinase involved in the regulation of transcription of glucose-repressed genes. It regulates filamentous growth in response to starvation. Stb1 regulates the MBF-G1/S specific transcription factor; while Swi4 together with Swi6 forms the second G1/S specific transcription factor, SBF.

Therefore, Snf1, Stb1 and Swi4 have essential roles in G1, and are potential regulation nodes of PP2A\textsuperscript{Cdc55}, highlighting the importance of this phosphatase in G1 regulation, in agreement with recent studies uncovering G1-related functions (58, 59, 66, 67).
Finally, Cla4 also emerged as a potential node of PP2ACdc55 regulation in our study.

Strikingly, Cla4 is a PAK kinase that regulates septin ring assembly during cytokinesis and vacuole inheritance. Cla4 and the related Ste20 kinase consensus motif were found enriched among our PP2ACdc55 dependent phosphoproteome and many proteins related to cytokinesis were identified. Altogether, our results suggest that Cla4 and PP2ACdc55 might have more related functions in cytokinesis than previously anticipated.

Validation of novel PP2ACdc55 substrates in mitosis

cdc55Δ cells exhibit elevated tyrosine 19 phosphorylation on Cdk1 due to dysregulation of Swe1 and/or Mih1 (27, 29, 31, 68). We first confirmed that we could detect this hyperphosphorylation of Cdk1-Y19 in cdc55Δ cells in our phosphoproteome screen (VGEGETGVVYK, Y6 phosphoRS site probability > 89%).

We next searched for already known PP2ACdc55 substrates (Fig. 4A), as we previously published an extended study about Net1 being a PP2ACdc55 substrate and its functional relevance for mitotic exit regulation (41). Net1 was identified as being hyperphosphorylated in the cdc55Δ mutant, suggesting our approach to broadly identify substrates worked. In addition, Mob1 protein was identified in this phosphoproteomic study as a low confidence phosphopeptide, which we recently validated as a new PP2ACdc55 substrate and demonstrated functional relevance for MEN activation (44). Based on that result, we looked for other MEN components in our PP2ACdc55-dependent phosphoproteome, and we found Lte1 hyperphosphorylated in the cdc55Δ mutant. We further explored Lte1 phosphorylation at the metaphase to anaphase transition (Fig. 4B). Wild-type and cdc55Δ cells were arrested in metaphase by Cdc20 depletion and released into synchronous anaphase by Cdc20 re-introduction. In wild-type cells, Lte1 was
dephosphorylated in anaphase and transition to G1 (M/G1). In contrast, Lte1 was hyperphosphorylated in cdc55Δ cells at the indicated times, suggesting is likely to be a PP2A<sup>Cdc55</sup> substrate. Native protein extracts from metaphase samples were treated with alkaline phosphatase as a control of phosphorylation. Additional MEN components, Cdc14 and Kin4, were also identified in our phosphoproteome analyses as putative new substrates of PP2A<sup>Cdc55</sup> (Additional file 3), suggesting a closer regulation of the whole MEN pathway by PP2A<sup>Cdc55</sup> phosphatase.

On the other hand, one component of the FEAR pathway was also identified in our PP2A<sup>Cdc55</sup>-dependent phosphoproteome, Slk19, which is a kinetochore-associated protein involved in chromosome segregation and Cdc14 release. We explored Slk19 protein modifications in the metaphase to anaphase transition as we had done for Lte1. In wild type cells, Slk19 is phosphorylated in metaphase and, upon anaphase entry, undergoes cleavage. In contrast, Slk19 was hyperphosphorylated in cdc55Δ cells throughout anaphase, and although it underwent cleavage, Slk19 showed an altered migration pattern of the cleaved form. This result suggests that PP2A<sup>Cdc55</sup> is required to dephosphorylate Slk19.

In addition, Rts1 the second regulatory subunit of PP2A<sup>Cdc55</sup> was also identified in our phosphoproteome analysis. PP2A<sup>Rts1</sup> is located at the centromeres during mitosis and prevents cohesin cleavage by separase (69), and it is also required for cell size control (70). Rts1 was dephosphorylated in M/G1 in wild-type cells (Fig. 4B). In contrast, Rts1 was hyperphosphorylated in cdc55Δ cells at the indicated times. Native protein extracts from metaphase samples were treated with alkaline phosphatase as control. These results indicate that Rts1 is hyperphosphorylated in the absence of PP2A<sup>Cdc55</sup>, suggesting that PP2A<sup>Cdc55</sup> is required to dephosphorylate Rts1.
Zeo1 and other potential PP2A<sup>Cdc55</sup> substrates interact with the PP2A<sup>Cdc55</sup> phosphatase in vivo

Finally, we used Cdc55 pull-down strategies to further validate new potential substrates of PP2A<sup>Cdc55</sup> and further explore specific binding partners of this phosphatase. We first used tandem affinity purification (TAP) to find new Cdc55 interactors, using a strain expressing a TAP-epitope tagged Cdc55 (TAP-Cdc55). TAP involves fusion of the TAP epitope (protein A from Staphylococcus aureus and the calmodulin binding peptide [CBP] arranged in tandem and separated by a TEV protease cleavage site) to the target protein of interest. The fusion protein and their associated components were then recovered by two rounds of affinity purifications. Eluted fractions were then directly processed by high sensitive LC-MS/MS methods. A strain without the TAP epitope was used as control. The peptides identified in the TAP-Cdc55 pull-down that are not found in the negative control purification are considered novel Cdc55 associated proteins (Additional file 11). Among them, 4 proteins Zeo1, Apa1, Dnm1 and Set1 were also found hyperphosphorylated in our PP2A<sup>Cdc55</sup>-dependent phosphoproteome (Fig. 4C), suggesting they are likely to be PP2A<sup>Cdc55</sup> substrates.

We next performed a second Cdc55 purification using HA-Cdc55 tagged strain and HA-affinity columns. The eluted fractions were subjected to TiO<sub>2</sub> enrichment to search for proteins that are undergoing phosphorylation modifications among the newly identified Cdc55 associated proteins. The enriched peptides were subjected to LC-MS/MS. Peptides identified are shown in Additional file 12. Among them, Psh1, Tgl1, Hos3 and Sro9 were identified as Cdc55-interacting proteins. Peptide and protein modifications were obtained using the Mascot search engine. Interestingly, Tgl1 and Psh1 were also found in our quantitative phosphoproteomic study of potential PP2A<sup>Cdc55</sup> substrates (Fig. 4D).
Considering that those proteins interact physically with \(\text{PP2A}^{\text{Cdc55}}\) and are found hyperphosphorylated in \(\text{cdc55}^{\Delta}\) cells, they are likely new \(\text{PP2A}^{\text{Cdc55}}\) substrates.

We observed little overlap between our SILAC study with the pull-down experiments. This is consistent with the long-held notion that kinase-substrate interactions are commonly weak and transient, thus difficult to detect by purification-based protein interaction screens.

**Docking models of \(\text{PP2A}^{\text{Cdc55}}\) and Mob1 highlight potential binding interfaces for Cdc55 and Mob1**

To explore the interaction surface of Cdc55 and its Cdk1-dependent substrates, we performed rigid-body computational docking using HADDOCK (71) (version 2.2). Except for the previously validated substrate Mob1 (44), none of the other substrates have structural data for regions with Cdc55-dependent phosphosites. As such, we built a homology model of Cdc55 based on the crystal structure of the mammalian homologue B55 and used the published crystal structure of Mob1 to build 100,000 models of the Cdc55/Mob1 complex, using knowledge of a Tau binding region on B55 to restrict the search space of the docking calculations on the Cdc55 surface.

The best 10,000 models, ranked by intermolecular energy, cluster into 437 representative binding poses that show a smooth distribution of Mob1 across the surface of the \(\beta\)-propeller of \(\text{PP2A}\) (Figure 5A). Filtering these models for those where Mob1 adopts a binding pose compatible with dephosphorylation by the catalytic subunit of \(\text{PP2A}\), measured by the distance between a known phosphosite (S80) and the proton donor on \(\text{PP2A}\) (H118), narrows down the possible interaction nodes to 294 models (12 clusters) with a very similar interaction surface (Figure 5B). In these models, the predominant interface on Cdc55 is
mediated by a protruding loop consisting of residues 84-90, which were shown to be critical for Tau binding and more recently to the binding of mitotic substrate PRC1; therefore, the Cdc55 residues interacting with its substrates seem to be conserved. This is shown more clearly by a statistical analysis of per-residue interface propensities where the residues 84-90 (marked in red) appeared concentrated in the more frequent interfaces (Figure 5C). On Mob1, there is no such conserved narrow interface (represented as red residues broadly spread throughout the interphases), even among the binding poses consistent with the dephosphorylation function, although one face of the protein seems to be more favorable for interaction (Figure 5D). Interestingly, most of these models are located in between the regulatory B55 subunit and the catalytic subunit of PP2A, which would be compatible with an open-close conformational change of the scaffold subunit. Indeed, a substantial degree of flexibility of the scaffold subunit has been observed upon formation of the core enzyme alone (72).

Discussion

Mitotic exit depends on phosphatase activation in all organisms studied so far. PP2A\textsuperscript{Cdc55} is a major Cdk1-counteracting phosphatase during cell cycle progression and a principal mitotic regulator. To uncover new PP2A\textsuperscript{Cdc55} targets and functions during mitosis, we depleted \textit{CDC55} in budding yeast and screened for hyperphosphorylated peptides enriched in metaphase-arrested cells in a quantitative SILAC-based approach. Non phospho-enriched control samples indicated that most of the phosphorylation changes found can be attributed to PP2A\textsuperscript{Cdc55} inactivation and not to changes in protein abundance in the \textit{cdc55\Delta} mutant. None of the different phosphopeptide enrichment methods available provide a whole
phosphoproteome. Each method provides varying degrees of selectivity and specificity of phosphopeptide enrichment resulting in the identification of phosphopeptides of different nature. In fact, the ratio of monophosphorylated and multiphosphorylated peptides detected in each method varies considerably (42% and 58% in TiO2; 83% and 17% in SIMAC and 90% and 10% in TiSH, respectively). Due to the intrinsic and distinct nature of the phosphoenrichment methods the overlapping among the three strategies is modest (Fig. 1); but the combination of the methods greatly enhances the number of phosphopeptides isolated of different nature.

While preparing this manuscript, two SILAC-based studies targeting PP2A$^{Cdc55}$ (61) and the mammalian, PP2A-B55 (60), were published. By comparing the phosphorylation status of Cdk1 substrates in the absence of PP2A$^{Cdc55}$ at different cell cycle phases (G1, S and G2/M) (61), they deciphered how PP2A$^{Cdc55}$ contributes to determining the progressive phosphorylation of Cdk1 substrates. In contrast, our study focused on metaphase-arrested cells (when the PP2A$^{Cdc55}$ activity is higher during mitosis), and we considered not only the Cdk1-counteracted substrates but all Cdc55-dependent phosphorylation sites for downstream analysis. Nevertheless, when we compare both dataset, we found 69% of the proteins identified in (61) in our data (128 common/186 proteins in (61), Additional file 13), indicating a high degree of overlapping in both studies. Interestingly, although phosphorylated serines were more abundant, threonines showed the most dramatic fold-increase in our X-motif analysis, in agreement with the two published studies showing that this phosphatase has a threonine preference (60, 61).
Although the increased phosphorylation of the proteins identified in the cdc55Δ mutant is either a direct or indirect effect of PP2A<sup>Cdc55</sup> inactivation, new regulated PP2A<sup>Cdc55</sup> processes can be discovered. In fact, gene ontology analysis of our phosphoproteome study identified several processes related to mitosis, actin cytoskeleton organization, budding and cytokinesis. Budding impinges a dramatic re-arrangement of the cell structure, and morphogenesis changes, GO categories that were also found in our study. In addition, we identified proteins related to osmotic stress and nutrient response. Thus, PP2A<sup>Cdc55</sup> phosphatase seems to play a key role in sensing several cues of the environmental conditions, cell growth, cell polarity and cell structure, and integrating them to regulate the cell cycle.

We identified several kinases hyperphosphorylated in the absence of PP2A<sup>Cdc55</sup>, as well as some of their substrates, suggesting that processes regulated by these kinases are potentially regulated by PP2A<sup>Cdc55</sup> phosphatase as well. Indeed, we found a major set of peptides containing Cdk1 consensus sites. In addition to Cdk1 consensus sites, we found other kinase consensus motifs enriched in the Cdc55-dependent phosphoproteome. It has been shown that Cdc5 kinase phosphorylation of Scc1 (45) and Bfa1 is counteracted by PP2A<sup>Cdc55</sup> phosphatase (44). In accordance, we identified a group of proteins containing the proposed Cdc5 polo-like kinase consensus sites (D/E/N-x-S/T) (Additional file 14). Strikingly, we found the R-X-X-S phosphorylation motif to be enriched in the absence of Cdc55. This motif corresponds to the consensus phosphorylation motif of Pkc1, which also emerged as a node of interactions in the PP2A<sup>Cdc55</sup>-dependent phosphoproteome.
PP2A<sup>Cdc55</sup> has been shown to integrate membrane growth into mitosis regulation via Rho1 and Pkc1 (34–36), regulators of the cell wall integrity pathway. Indeed, kinases of this pathway like Pkc1, Bck1, and the closely related Pkh1, Ypk1 and Ypk2, were found hyperphosphorylated in the absence of Cdc55. Regulation of Cdc55 activity by Pkc1 phosphorylation in the context of blocking membrane trafficking has also been uncovered (35). Thus, mutual regulation of Pkc1 and Cdc55 seems to occur and they might share several substrates. Interestingly, we found Zeo1, an upstream negative regulator of the cell integrity pathway, to be hyperphosphorylated in the absence of Cdc55 phosphatase. In addition, we showed that Cdc55 and Zeo1 potentially interact through co-purification assays, thus, we conclude Zeo1 is likely a new PP2A<sup>Cdc55</sup> substrate.

On the other hand, the yeast casein kinase Yck2 was identified in our phosphoproteome screening and has been described to present a genetic interaction with Cdc55 (73). Yck2 shows cell cycle-specific localization to sites of polarized growth and it is required for proper septin organization and cytokinesis, functional groups identified in our GO analysis (74).

Moreover, the PAK kinase phosphorylation motif R-R-x-S (a subset of R-x-x-S) was also enriched in the absence of phosphatase PP2A<sup>Cdc55</sup> and two Pak-like kinases, Ste20 and Cla4, were found hyperphosphorylated (as well as their identified substrates) in the PP2A<sup>Cdc55</sup>-dependent phosphoproteome. Ste20 and Cla4 have been linked to cytokinesis (65, 75–79) and we identified Cla4 as a node of interactions in the PP2A<sup>Cdc55</sup>-dependent phosphoproteome, suggesting a functional link between Cla4 and PP2A<sup>Cdc55</sup>. Indeed, Nap1, a septin regulator in fission yeast is phosphorylated by Cla4 and dephosphorylated by PP2A (80) indicating that PP2A counteracts Cla4 phosphorylations. Altogether, we conclude that
PP2A<sup>Cdc55</sup> could counteract other kinases separate from Cdk1 and Cdc5, like Pkc1 and Cla4, as well as regulate their activity.

In previous studies, we identified a dual regulation of the Mitotic Exit Network (MEN) by PP2A<sup>Cdc55</sup> phosphatase, which dephosphorylates Bfa1 and Mob1. Here, we found that other MEN components were hyperphosphorylated in the Cdc55-dependent phosphoproteome, and we validated Lte1 as a likely substrate of PP2A<sup>Cdc55</sup> phosphatase. Thus, PP2A<sup>Cdc55</sup> seems to closely regulate the MEN pathway, by dephosphorylating other elements apart of Bfa1 and Mob1. In addition, we validated Slk19, a component of the Cdc14 early anaphase release (FEAR) pathway, as likely PP2A<sup>Cdc55</sup> substrates during mitotic exit, as well as other potential substrates Apa1, Dnm1, Set1, Psh1 and Tgl1 by co-purification with Cdc55.

To better understand how PP2A<sup>Cdc55</sup> interacts with its substrates, we built computational docking models of Cdc55 with its recently described Cdk1-dependent substrate, Mob1. Interestingly, residues 84-90, located at the Cdc55 groove structure, were predicted to interact with Mob1. This same interface has been shown to be critical for Tau and PRC1 binding to mammalian B55 in vivo. Further studies, including substrates regulated by other PP2A<sup>Cdc55</sup>-counteracted kinases, could help elucidate how this specific phosphatase recognizes and interacts with its substrates.

This work attempts to bring new insight into the mitotic exit regulation picture, with a special focus on PP2A<sup>Cdc55</sup> functions in this critical phase of cell division. A profound understanding of mitotic exit regulation could set the stage for new therapeutic strategies, since failure to progress normally through mitotic exit can induce cell death and could be exploited to kill hyper-proliferating cancer cells. The study of phosphatase holoenzymes,
and especially, the regulatory phosphatase subunits such as Cdc55, provides valuable information for the development of new pharmacological inhibitors or modulators that selectively target specific phosphatase complexes.

Potential implications

Dysregulation of PP2A phosphatases have been found in many solid cancers and leukemias. PP2A-B55, and its highly-conserved homolog in budding yeast, PP2A<sub>Cdc55</sub>, regulate the cell cycle and are required for efficient mitotic exit. Budding yeast is thus a powerful model to gain insight into mitotic exit regulation, specifically, to the activities of PP2A phosphatase holoenzymes, which could promote the design of new therapeutic strategies, since failure to progress normally through mitotic exit may be exploited to kill hyper-proliferating cancer cells. Here, we used phosphoproteomics of Cdc55 deficient cells to uncover new PP2A<sub>Cdc55</sub> substrates and functions in mitosis. We also reveal new kinases potentially counteracted and regulated by PP2A<sub>Cdc55</sub> phosphatase. In particular, Pkc1 and Cla4 kinases were discovered as significant PP2A<sub>Cdc55</sub> regulation nodes. Finally, we attempted to gain insight into Cdc55-substrate interaction using docking models of Cdc55 and Mob1 substrate, which suggest a specific interface for substrate interaction.

Methods

Yeast strains, plasmids and cell cycle synchronization procedures

All yeast strains used in this study were derivatives of W303. Epitope tagging of endogenous genes was performed by gene targeting using polymerase chain reaction (PCR)
products. Endogenous CDC55 was N-terminal-tagged as previously described (41).

Metaphase arrest by Cdc20 depletion was also performed as previously described (81).

**Stable Isotope Labelling of Yeast Cells and Preparation of Yeast Protein Extracts for Phosphoproteomic Analysis**

For each biological replicate, yeast cells were labelled with stable isotopes and protein extracts prepared as previously described (82). In brief, cells were grown in minimum media containing either 100 mg/L arginine and 100 mg/L $^{13}$C$_6$-arginine and 100 mg/L $^{13}$C$_6$-lysine (Cambridge Isotope Laboratories Inc.). Y859 ($^\text{MAT} a^{\text{lys2}::\text{TRP1}, \arg^4\Delta::\text{HIS3 MET-Cdc20::LEU2}}$) and Y858 (as Y859 but $^{\text{cdc55}\Delta}$) cells were grown in free-methionine minimum media containing $^{13}$C$_6$-lysine and -arginine (heavy) or unmodified arginine and lysine (light), respectively. Both strains were synchronized at the metaphase-to-anaphase transition by adding methionine to the media.

Protein extracts were prepared by mechanical lysis using glass beads in presence of protein inhibitors (Complete EDTA-free, Roche) and 2X phosphatase inhibitors PhosStop (Roche). Cell lysates were mixed 1:1 and digestion with trypsin was performed. Approximately 400 μg of the mixed heavy/light protein sample were processed for in-solution digestion as previously described (83). Proteins were reduced with 5 mM DTT for 30 min at 37°C and alkylated with 10 mM iodoacetamide for 30 min at 30°C. Samples were diluted five times with 25 mM ammonium bicarbonate, trypsin (Promega, ratio enzyme:protein 1:10) was added and incubated overnight at 37°C. Digestion was stopped by addition of formic acid.

**Phosphopeptide enrichment**
Three strategies were used for phosphopeptide enrichment. In the first approach, phosphopeptide enrichment by sequential elution from IMAC (SIMAC) was done as previously described (82). Peptides samples were added to an immobilized metal affinity chromatography suspension (Phos-Select, Sigma) and were incubated for 1h at room temperature. The flow-through was collected, and the immobilized metal affinity chromatography resin was washed once with 50 μl 50% ACN and 0.1% TFA. The wash fraction was pooled with the flow-through. Acid elution was then carried out by adding 50 μl 30% ACN and 1% TFA and incubating for 5 min at room temperature. After this step, alkaline elution was done with 50 μl 0.5% NH₄OH pH 10.5, followed by 30 min incubation at room temperature. For further enrichment of phosphopeptides, the flow-through fraction and the acid eluate were incubated with TiO₂ beads (GL Sciences, Tokyo, Japan) and incubated with shaking for 1 h at 30 °C. The TiO₂ beads were washed twice with 80% ACN and 1% TFA and once with water. Bound peptides were eluted from the beads with 0.5% NH₄OH pH 10.5 for 30 min at 30 °C. Eluted peptides were dried via centrifugal evaporation, resuspended with 1 μl formic acid and 15 μl water and analyzed using nano-LC-MS/MS on an LTQ-Orbitrap (Themo Scientific) mass spectrometer.

In the second strategy, phosphopeptide enrichment was done using TiO₂ chromatography following the product specifications (TiO₂ Mag Sepharose, GE Healthcare). An aliquot of 100 μg was separated to be further processed and analyzed without phosphopeptide enrichment. All samples (enriched and non-enriched for phosphopeptide) were dried via centrifugal evaporation and subjected to fractionation with a high pH reversed phase peptide fractionation kit (Pierce). The peptides were eluted in 9 fractions of increasing acetonitrile (ACN) concentration of 5% ACN to 75% ACN. The 9 eluted fractions were
dried via centrifugal evaporation, resuspended in 1% FA and analyzed in a nanoAcquity liquid chromatographer (Waters) coupled to an LTQ-Orbitrap Velos (Thermo Scientific) mass spectrometer.

In the third approach, a combination of enrichment and fractionation methods was used (The “TiSH” method: TiO$_2$-SIMAC-HILIC) as previously described(84). Briefly, peptide digest was first pre-enriched in phosphopeptides using TiO$_2$ chromatography (85) (5 μm, GL Sciences Inc, Japan) followed by SIMAC purification (86). The mono-phosphorylated peptide fraction from the SIMAC enrichment was further subjected to a second TiO$_2$ purification. The mono-phosphorylated fraction was then pre-fractionated by HILIC chromatography (Hydrophilic Interaction Liquid Chromatography, Column TSK Gel Amide 80 15 cm 0.3mm ID) using a 40 min gradient from 90% B buffer (95% acetonitrile, 0.1% TFA) to 60 % B buffer. Twenty-five fractions were collected, which were pooled into a final five fractions that were then analyzed by reverse phase LC-MS/MS. The multi-phosphorylated fraction from SIMAC was directly analyzed by LC-MS/MS after desalting and concentration using a Poros Oligo R3 (ABSciex) Reversed phase (RP) micro-column.

**LC-MS/MS Analysis**

For the first approach, the peptides were analyzed using nano-LC-Ms/MS on an LTQ-Orbitrap Velos (Thermo Scientific) mass spectrometer. Peptides were separated on a BioBasic C-18 PicoFrit column (75 μm Øi, 10 cm, New Objective, Woburn, MA) at a flow rate of 200 nL/min. Water and ACN, both containing 0.1% formic acid, were used as solvents A and B, respectively. Peptides were trapped and desalted in the trap column for 5 min. The gradient was started and kept at 10% B for 5 min, ramped to 60% B over 60 min or 120 min, depending on the sample complexity, and kept at 90% B for another 5 min.
Peptides (m/z 400-1400) were analyzed on the LTQ-Orbitrap velos in full Scan MS mode with a resolution of 60,000 FWHM at 400m/z; up to the 7 most abundant peptides were selected from each MS scan and then fragmented using collision induced dissociation in a linear ion trap using helium as collision gas at 7500 FWHM and 30 sec exclusion time. Generated .raw data files were collected with Thermo Xcalibur v.2.2.

For the second approach, the peptides (enriched and non-enriched) were resuspended in 1% FA and were injected for chromatographic separation. Peptides were trapped on a Symmetry C18™ trap column (Waters), and were separated using a C18 reverse phase capillary column (75 µm Øi, 25 cm, nano Acquity, 1.7µm BEH column; Waters). The gradient used for the elution of the peptides was 1 to 35 % B in 90 min, followed by a gradient from 35% to 85% in 10 min (A: 0.1% FA; B: 100% ACN, 0.1%FA), with a 250 nL/min flow rate. Eluted peptides were subjected to electrospray ionization in an emitter needle (PicoTip™, New Objective) with an applied voltage of 2000V. Peptide masses (m/z 300-1700) were analyzed in data dependent mode where a full Scan MS was acquired in the Orbitrap with a resolution of 60,000 FWHM at 400m/z. Up to the 10 most abundant peptides (minimum intensity of 500 counts) were selected from each MS scan and then fragmented using CID (Collision induced Dissociation) in the linear ion trap using helium as collision gas. Multistage activation was enabled to favor the detection of phosphopeptides. The scan time settings were: Full MS: 250 ms and MSn: 120 ms. Generated .raw data files were collected with Thermo Xcalibur v.2.2.

For the third strategy, the peptides were resuspended in 0.1 % TFA and analyzed using an Easy-nanoLC (Thermo Fisher Scientific, Proxeon, Denmark) coupled to an LTQ-Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific). Peptides were loaded onto a pre-column of 2cm Reprosil –Pur C18 AQ 5 µm RP material (Dr. Maisch, Ammerbuch-
Entrigen, Germany) using the EASY-LC system and eluted directly onto a 20 cm long fused silica capillary column (75 µm ID) packed with Reprosil- Pur C18 AQ 3 µm RP material. The peptides were separated using a gradient from 0-34% B (A buffer: 0.1 % formic acid (FA); B buffer: 90% ACN/0.1% FA) at a flow rate of 250 nL/min over 30-60 min depending on the UV trace of the HILIC fractions. The peptides (m/z 400-1400) were analyzed in full MS mode using a resolution of 120.000 FWHM at 200 m/z and the peptides were selected and fragmented using helium as collision gas and the fragment ions were recorded in the LTQ with low resolution (rapid scan rate). A maximum of 3 sec were allowed between each MS and for MSMS the ion filling time was set to 40 ms and an AGC target value of 2E4 ions. Raw data was viewed in Xcalibur v2.0.7.

**Data Analysis for Peptide Identification and Quantification**

To perform the sample data analysis we compile the raw files from the technical replicates of each phosphoenrichment method obtaining a unique list of peptides and proteins for each method. Peptide identification was performed using Proteome Discoverer v1.4.1.14 (Thermo Scientific) and search against Swiss Prot /Uniprot *Saccharomyces cerevisiae* database (v. January 2016) with SequestHT search engine. Both a target and a decoy database were searched to obtain a false discovery rate (FDR). To improve the sensitivity of the database search, Percolator (semi-supervised learning machine) was used to discriminate correct from incorrect peptide spectrum matches. The PhosphoRS node was used to provide a confidence measure for the localization of phosphorylation in the peptide sequences identified with this modification.

Database search were performed with the following parameters: precursor mass tolerance 10 ppm, fragment mass tolerance 0.6 Da, cysteine carbamidomethylation as fixed
modification and 2 missed cleavage for trypsin. Variable modifications considered were phosphorylation on S/T/Y and K/R label,$^{13}\text{C}_6$ and oxidation (M).

Only peptides with high confidence Percolator q of 0.01 (FDR<1%) were considered for further analyses.

Peptide quantification from SILAC labels was performed with Proteome Discoverer v1.4. The log2-ratio value associated with each peptide was calculated as a weighted average of the scans used to quantify the peptide, as described elsewhere (58, 59) and the data were normalized based on the median. Only quantified peptides detected as statistically significant (high confidence FDR< 0.01) were selected. The processing of the data was performed in R (v.3.3.1) with the help of the ‘rvest’, ‘Vennerable’ and ‘Venneuler’ packages. Briefly, the H/L ratios from the samples (TiSH, SIMAC and TiO$_2$) were averaged for every phosphopeptide. The resulting list was filtered to keep only the phosphopeptides of interest. That is, peptides with a coefficient of variation (CV) between samples below 40%, peptides without CV (peptides only appearing in one sample) and peptides with a CV above 40% which show a H/L ratio in all the samples below 0.75. Statistical significance was assessed at 5% (two-tailed Student’s t-test; p<0.05). The R Script used for the analysis can be found in Additional file 15. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (87) via the PRIDE (88) partner repository with the dataset identifier PXD007613 (Username: reviewer50711@ebi.ac.uk, Password: Tv9GFPI2).

**Phosphorylation motif analysis**
The Phosphorylation Motifs Enrichment Analysis (PMEA) was performed with the motif-X web tool ([http://motif-x.med.harvard.edu/](http://motif-x.med.harvard.edu/)) (89). Before the analysis, the phosphosites were aligned so that the phosphosite is centered. All the peptides identified in our 3 SILAC approaches were used to search for enriched motifs against the SGD yeast proteome database as a background.

**TAP purification**

Protein extracts were prepared by mechanical lysis using glass beads in presence of protein inhibitors (Complete EDTA-free, Roche) and 2X phosphatase inhibitors PhosStop (Roche). TAP (Tandem Affinity Purification of Protein A and CBP (calmodulin binding protein) epitopes), fusion proteins and associated proteins were recovered from cell extracts by affinity chromatography using an IgG-sepharose matrix. After washing, the Tobacco Etch Virus (AcTEV, Life technologies) protease was added to release the bound material. The eluate was incubated with calmodulin-coated beads in the presence of calcium. This second affinity step was required to remove not only the AcTEV protease but also traces of contaminants remaining after first affinity purification. After washing, the bound material was released with ethylene glycol tetra acetic acid (EGTA). The calmodulin eluates from the TAP-purified complexes were precipitated with trichloroacetic acid (TCA) and directly subjected to LC-MS/MS. Pellets were dissolved with 20 μL of 50 mM ammonium bicarbonate (ABC). Cysteine residues were reduced by 2 mM DTT (DL-Dithiothreitol) in 50 mM ABC at 60º for 20 min. Sulphydryl groups were alkylated with 5 mM iodoacetamide (IAM) in 50 mM ABC in the dark at RT for 30 min. IAM excess was neutralized with 10 mM DTT in 50 mM ABC 30 min at RT. 5 μL of each sample were
loaded onto a trap column (nanoLC column, 3 μ C18-CL, 75 μmx15cm; Eksigen) and desalted with 0.1% TFA at 2 μL/min during 10 min. The peptides were then loaded onto an analytical column (LC Column, 3 μ C18-CL, 75 μmx15cm; Eksigen) equilibrated in 5% acetonitrile 0.1% FA (formic acid). Elution was carried out with a linear gradient of 5-35% B in A for 120 min (A: 0.1% FA; B: AN 0.1% FA) at a flow rate of 300 nL/min. Peptides were analyzed in a mass spectrometer nanoESI qQTOF (5600 TripleTOF, ABSCIEX). The tripleTOF was operated in information-dependent acquisition mode, in which a 0.25-s TOF MS scan 350-1250 m/Z, was performed, followed by 0.05-s product ion scans from 100-1500 m/z on the 50 most intense 2-5 charged ions. Protein identification was performed using ProteinPilot v4.0.8085 (ABSciex) or Mascot v2.3 (Matrix Science) search engines. Protein Pilot default parameters were used to generate peak list directly from 5600 TripleTOF wiff files. The Paragon algorithm of ProteinPilot was used to search Expasy protein database (1072964 sequences). The proteomic analysis was carried out in the SCSIE_university of Valencia Proteomics Unit, a member of ISCIII ProteoRed Proteomics Platform. Peptides identified in two TAP-Cdc55 biological replicates and the untagged control had been deposited to the ProteomeXchange Consortium with the dataset identifier PXD007613.

HA Purification

Protein extracts were prepared by mechanical lysis using glass beads in presence of protein inhibitors (Complete EDTA-free, Roche) and 2X phosphatase inhibitors PhosStop (Roche). HA-Cdc55 fusion proteins and associated proteins were recovered from cell extracts by HA-agarose beads (Sigma). The eluates were precipitated with trichloroacetic acid (TCA)
and proteins were separated in a protein gel. After trypsin digestion, peptide was desalted by Strata X C18 column (Phenomenex) and vacuum-dried. A total of 1µg dried peptide was reconstituted in a solution containing 65% ACN, 2% TFA and was saturated with glutamic acid (20 mg/ml, pH 2.0-2.5). Then the peptide solution was added to TiO$_2$ (GL Science, Saitama) and was incubated for 20 min. The peptides were eluted once with 1.1% NH$_4$OH solution in 50% ACN and once with 3% NH$_4$OH solution in 50% ACN (diluted from 25% NH$_4$OH solution). Two elute fractions were combined and vacuum-dried. Then, phosphopeptides were subjected to nanoelectrospray ionization followed by tandem mass spectrometry (MS/MS) on a Q-Exactive mass spectrometer (ThermoFisher Scientific). Peptide and protein modification were obtained using Mascot software. HA purifications experiments were performed using BGI proteomic services and BGI bioinformatics department.

Western Blot validation of cell cycle-dependent phosphorylated substrates

Cell synchronization by Cdc20 depletion and entry into synchronous anaphase by Cdc20 re-introduction were also performed as previously described (44). Protein extracts for western blots were obtained by TCA protein extraction. Gels of 8-10% were used for electrophoresis. Antibodies used for protein staining were α-HA clone 12CA5 (Roche) and α-Pk clone SV5-Pk1 (Serotec).

Availability of supporting data and materials

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (87) via the PRIDE (88) partner repository with the dataset identifier
PXD007613 (Username: reviewer50711@ebi.ac.uk, Password: Tv9GFPI2). The files uploaded correspond to: (1) Madrid Phospho Analysis 2016.msf. This file contains the proteins and peptides detected in the SIMAC-based enrichment assay (Method 1). It is generated (and can be open) by the "Proteome Discoverer" software with the following raw data: Elu1-12_75.raw, Elu1-12_75_bis.raw, Elu2-12_75.raw, FT-12_75.raw, FT-12_75_bis.raw. (2) reg1418_TiO2_13raw.msf. This file contains the proteins and peptides detected in the TiO2-based enrichment assay (Method 2). It is generated (and can be open) by the "Proteome Discoverer" software with the following raw data:

- reg1418_TiO2_FTwash.raw, reg1418_TiO2_f1f8_160426190328.raw,
- reg1418_TiO2_f1f8_160503121600.raw, reg1418_TiO2_f2f9.raw,
- reg1418_TiO2_f2f9_160503145752.raw, reg1418_TiO2_f3.raw,
- reg1418_TiO2_f3f7FTwash.raw, reg1418_TiO2_f4.raw, reg1418_TiO2_f4f6.raw,
- reg1418_TiO2_f5.raw, reg1418_TiO2_f5_160503221654.raw, reg1418_TiO2_f6.raw,

**Interaction maps and Gene ontology**
The networks were created with the STRING database (http://string-db.org/) by using the proteins obtained from the 1260 hyperphosphorylated peptides (628 phosphoproteins) (90). Only high-confidence interactions from experiments or databases were extracted and binary interactions were also discarded. Classification into functional clusters and gene ontology was performed with the DAVID bioinformatics tools using the 628 hyperphosphorylated proteins (https://david.ncifcrf.gov/) (91). Only clusters with an Enrichment Score higher than 1.5 and GO terms with a p<0.001 were considered.

**Structure Prediction of Cdc55**

A structural model of full-length yeast Cdc55 (Uniprot AC: 2ABA_YEAST) was built by homology modeling. HHpred (92) identified the regulatory B55 subunit of the heterotrimeric human protein phosphatase PP2A (Uniprot AC: 2ABA_HUMAN; PDB: 3dw8_B) as a suitable template and provided a pairwise alignment. We then used the loop model protocol implemented in MODELLER 9v18 (93) to build 50 models of CDC55, which were assessed and ranked with the DOPE statistical potential (94).

**Sampling the binding interface of the CDC55/Mob1 complex.**

Models of the interaction between CDC55 and Mob1 were calculated using the data-driven docking software HADDOCK (version 2.2) (71). As initial structures, we used the Cdc55 homology model with the lowest (best) DOPE score and the available crystal structure of Mob1 (PDB: 2HJN_A). We restricted the search on Cdc55 to solvent accessible residues within a 10 Å radius of the Tau binding region identified by NMR and mutagenesis.
experiments on the homologous B55 (3). All residues are strictly conserved between the
two proteins: E24, K45, F75, D76, Y77, L78, K79, S80, L81, E84, E85, K86, Y185, H186
and D204. For Mob1, we defined the entire surface of the protein as a possible interaction
site. A residue was defined as solvent accessible if its main-chain or side-chain atoms had
a relative solvent accessibility equal to or greater than 15% as calculated by FREESASA
(95) and the NACCESS scale.
We calculated 100.000 models using the data-driven rigid-body docking protocol in
HADDOCK and kept the best 10.000 (top 10%) ranked by HADDOCK score for further
analysis. We then superimposed these models on the heterotrimeric PP2A structure and
calculated the distance between residues P81 in Mob1 (proxy for the phosphosite S80, not
resolved in the crystal) and H118 (proton donor) in the catalytic subunit of PP2A. Using a
threshold of 10 Å as filtered, we obtained a list of 294 models, which we then grouped in
12 representative clusters using a fast contact-based interface similarity algorithm (96). We
also used these 294 models to calculate propensities for each individual residue to be part
of the Cdc55/Mob1 interface. A residue was defined as part of the interface if any of its
atoms was within 5 Å of any atom of the partner protein.

Acknowledgements
We wish to thank Brendan Kelly, Priscilla Aquino, and all the members of our laboratory
for discussion and their critical reading of the manuscript.

Funding information:
Work in our laboratory is supported by the Spanish Ministry of Science and Innovation (BFU2011-27568), Spanish Ministry of Economy and Competitively (BFU2013-43132-P and BFU2016-77975-R AEI/FEDER, UE cofounded by FEDER funds/European Regional Development Fund- a way to build Europe). MRL was supported by the Lundbeck foundation (Junior Group Leader Fellowship). This work was supported by a generous grant from the VILLUM Foundation to the VILLUM Centre for Bioanalytical Sciences at the University of Southern Denmark. SBB is a recipient of ISCIII grant 13FIS037. IDIBELL Proteomics Unit belongs to ProteoRed, PRB2-ISCIII, and is supported by grant PT13/0001/0033.

Author contribution: BB, SJ, JV, IC and EQ performed the experiments. CG, MLH, SBB, CDLT and MRL performed the SILAC experiments. JJBS, BB and EQ performed and discussed the bioinformatics analysis. BB and JR did the in silico docking experiments. BB and EQ design the experiments, interpreted the data and wrote the manuscript. All authors read and discussed the manuscript.

Conflict of interest

The authors declare that they have no conflicts of interest.

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1127 Figure legends

1128 **Figure 1. Potential substrates of PP2A<sup>Ge<sup>55</sup></sup> phosphatase.** (A) Scheme of the three
phosphoenrichment approaches performed in our phosphoproteome study. (B) The
normalized heavy/light (H/L) ratio of all phosphopeptides. The number of phosphopeptides
(n=1260) with H/L ratios <0.75 (corresponding to the hyperphosphorylated peptides) is
shown. (C) Frequency distribution of the H/L ratios from an aliquot of the whole protein
extracts before phosphopeptide enrichment. The protein abundance is unchangeable for
most of the peptides. Red lines mark the lower and upper limits, which are set to 0.75
(log2=-0.42) and 1.3 (log2=0.42), respectively. (D) Distribution of the Ser, Thr and Tyr residues among the hyperphosphorylated peptides in the cdc55Δ mutant. All the peptides (10,069) identified in our 3 SILAC approaches were used as background. (E) Distribution of the S/TP sites within the hyperphosphorylated peptides. (F-G) Venn diagrams representing overlapping hits from the three approaches, for both hyperphosphorylated peptides and proteins. (H) A list of the overlapping proteins identified in more than one approach. (I) Volcano plot representing the common phosphopeptides (present in at least 2 out of 3 approaches) generated from two-tailed Student’s t-test (p<0.05). Green dots represent the significant proteins. (J) Analysis of the protein abundance of the 62 common proteins from I.

**Figure 2. Consensus phosphorylation sites found hyperphosphorylated in absence of PP2A<sup>Cdc55</sup>.** (A) Motifs logo found using Motif-X, for either central residue phospho-Serine or phospho-Threonine. (B) Phosphomotif consensus sequence, motif score and fold increase for each consensus motif. (C) Common elements between Cdk1 and PP2A<sup>Cdc55</sup> targets. Venn diagrams, GO processes from the common Cdk1-PP2A<sup>Cdc55</sup> targets and common protein targets are shown. The 155 proteins containing S/P consensus site in our phosphoproteomic data are used.

**Figure 3. The Interaction Network analysis identified 6 protein nodes related to PP2A-Cdc55.** Distribution of the number of interactions identified 6 protein nodes with more than 16 interactions. The proteins present in these 6 protein nodes with 16 or more interactions are shown.
Figure 4. In vivo validation of PP2A<sup>Cdc55</sup> novel substrates. (A) Summary of already known PP2A<sup>Cdc55</sup> substrates identified in our SILAC experiments. (B) Validation of PP2A<sup>Cdc55</sup> substrates. Strains Y1223 (MAT a LTE1-3PK::LEU2 MET-CDC20::LEU2), Y1224 (as Y1223, but <em>cdc55</em>Δ), Y1240 (MAT a RTS1-6PK::TRP1 MET-CDC20::LEU2), Y1241 (as Y1240, but <em>cdc55</em>Δ), Y1277 (MAT a SLK19-HA6::HIS3 MET-CDC20::LEU2) and Y1278 (as Y1277, but <em>cdc55</em>Δ) were arrested in metaphase by Cdc20 depletion and synchronously release in anaphase by Cdc20 re-introduction. Lte1, Rts1 and Slk19 phosphorylation status were identified by western blot. Native protein extracts from metaphase samples were treated with alkaline phosphastase (CIP lane) as dephosphorylation controls. (C) Proteins identified as PP2A<sup>Cdc55</sup> physical-interactors proteins after TAP purification experiments. Protein extract from Y614 strain containing a TAP-Cdc55 (MAT a, <em>CDC14-HA6::HIS3 TAP::CDC55 GAL1-CDC20::URA3</em>) was prepared and TAP purification assay was performed as described in methods. (D) Proteins identified phosphorylated and co-eluted with HA-Cdc55. Protein extract from Y2541 strain containing an HA-Cdc55 (MAT a HA::CDC55 GAL1-CDC20::LEU2) was prepared, HA-Cdc55 was purified and phosphopeptide enrichment was performed as described in methods.

Figure 5. Docking models of PP2A<sup>Cdc55</sup> and Mob1 highlight potential binding interfaces for Cdc55 and Mob1

(A) Representatives of the best 10,000 models of the CDC55/Mob1 complexes superimposed on the human heterotrimeric PP2A structure (PDB 3dw8). Red spheres
represent the centers of mass of representative models. The regulatory B55 subunit, homologous to Cdc55, is shown in green, while the catalytic subunit is shown in blue. Residues previously identified as interacting with Tau are represented as green spheres. (B)

Representatives of the filtered subset of 294 models of Cdc55/Mob1, after filtering for catalytic subunit distance. (C) and (D) Per-residue interface propensities (log2 scaled, red showing higher values) calculated on 294 filtered models of Cdc55/Mob1, respectively.

List of Additional files

Additional file 1.pdf
Workflow for SILAC analysis of PP2A-Cdc55 dependent phosphoproteome. Three different methods were used for phosphopeptide enrichment: SIMAC, TiO2 and TiSH-based approach. A detailed scheme of each methodology is presented. LC-MS/MS analysis of the eluted fractions was performed in order to identify and quantify the heavy/light labelled peptides. Identification and quantification was analysed using Proteome Discoverer.

Additional file 2.xlsx
Summary of all the peptides identify in the three approaches. 10,069 peptides were identified: 2,696 peptides in Method 1, 2,662 peptides in Method 2 and 4,711 in Method 3.

Additional file 3.xlsx
Hyperphosphorylated peptides corresponding to putative PP2A-Cdc55 regulated proteins. List of the 1,260 quantified hyperphosphorylated peptides identified in our three
SILAC experiments.

Additional file 4.xlsx

Complete list of proteins and peptides identified in the whole cell extract. List of 2,674 proteins and 27,957 peptides identified in the whole cell extract.

Additional file 5.xlsx

Common peptides and proteins quantified in the whole cell extract and in the hyperphosphorylated list. List of the 286 matching proteins identified in the whole cell extract (non-enrich analysis) and in our hyperphosphorylated dataset. All the matching proteins had similar protein abundance between the wild type and the cdc55Δ mutant (heavy/light ratio >0.8 in the non-enriched analysis).

Additional file 6.xlsx

Common peptides found in the phosphoproteomic study. List of the hyperphosphorylated peptides found in the three different phospho-enrichment approaches.

Additional file 7.xlsx

Hyperphosphorylated peptides with a pRS probability >95%. List of hyperphosphorylated peptides containing residues identified with very high confidence (peptides with a pRS probability > 95%). We identified 721 unique hyperphosphorylated peptides containing 562 unique consensus sequences.
Gene Ontology of the PP2A-Cdc55 potential substrates. The gene ontology terms of proteins displaying enhanced phosphorylation in our dataset are summarized in the Non-Clustered sheet and the functional clustering of the GO terms are summarized in the Clustered sheet.

String Network analysis of the hyperphosphorylated proteins identified in our dataset. Interactions found for each protein was plotted. A magnification of the Cdc28 and Cla4 nodes is shown.

Detail list of the proteins nodes described in Figure 3 and Additional file 7. Proteins for each node and the interaction score are shown.

Proteins identified in two TAP-Cdc55 purification assays. List of proteins identified in the two TAP-Cdc55 pull-downs that are not found in the negative control purification. A strain without the TAP epitope was used as negative control.

Proteins identified in the HA-Cdc55 purifications. Proteins and peptides identified after HA-Cdc55 purification using HA-affinity columns. The eluted fractions were subjected to
TiO$_2$ enrichment to search for proteins that are undergoing phosphorylation modifications among the newly identified Cdc55 associated proteins. Peptide and protein modifications were obtained using the Mascot search engine.

Additional file 13.xlsx

Common elements between PP2A$^{Cdc55}$ targets identified in Godfrey et al (61) and our study. A list of common proteins for each cell cycle stages is presented.

Additional file 14.xlsx

Proteins and peptides identified containing a Cdc5 consensus site. List of proteins from our PP2A-Cdc55 phosphoproteome dataset containing the D/E/N-x-S/T Cdc5 polo-like kinase consensus sites. We identified 161 phosphopeptides corresponding to 140 unique proteins.

Additional file 15.doc

Detailed R Scripts used for the quantification of the SILAC experiments.
Table 1. Kinases and their substrates found in our phosphoproteomic study.

<table>
<thead>
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<th>Kinase found</th>
<th>Type of kinase</th>
<th>consensus motif</th>
<th>biological process</th>
<th>substrates found</th>
</tr>
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<tr>
<td>ATG1</td>
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<td>H-X-R-R-X-s</td>
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<td>CDC28</td>
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<td>Shs1, Nap1, cd3</td>
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<tr>
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<td>S/T - Calmodulin</td>
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<td>stress response</td>
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<td>cell wall integrity pathway, lipid metabolism</td>
<td>Gpd1</td>
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Table 2. Major Gene Ontology categories of all hyperphosphorylated proteins identified in the three approaches.
Figure 1

wt  cdc55Δ

Heavy Arg13C Lys13C

Light Arg Lys

Cell Lysate
Mix Lysate 1:1
In solution digestion (Trypsin)
Phosphopeptide Enrichment

Method 1 (M1): SIMAC
Method 2 (M2): TiO2
Method 3 (M3): TisH

LC-MS/MS
Quantification Proteome Discoverer

Peptides

Proteins

Volcano Plot

Common Proteins

Figure 1
A Serine  

Serine

Threonine

% of phosphosites in every motif

S*XXE  

S*XXXXP  

S*XS  

SD  

T*P  

SXT*

Not Matched

B

<table>
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Common elements in Cdk1 and Cdc55

- ABP1  
- GFD1  
- PIN4  
- SSD1  
- ACE2  
- HER1  
- POL1  
- SSK2  
- ACF4  
- INP53  
- PSP2  
- SSN2  
- ASH1  
- ISW2  
- PTK2  
- STB1  
- ASK10  
- KEL1  
- REG1  
- STE20  
- AVO2  
- KIN2  
- RIM15  
- SWI4  
- BAP2  
- LEO1  
- RSC2  
- SWI5  
- BEM3  
- MDS3  
- RTS1  
- TCB3  
- BNI4  
- MLF3  
- SAC3  
- TCO89  
- BOI1  
- MSC3  
- SAC7  
- TIF4632  
- BRL1  
- MLS5  
- SDS24  
- TOP2  
- CDC3  
- NET1  
- SEC10  
- TSL1  
- CLA4  
- NOT5  
- SEC31  
- VRP1  
- ECM21  
- NTE1  
- SHS1  
- WHI5  
- EDE1  
- NUP159  
- SIR4  
- YER079W  
- FAB1  
- NUP60  
- SIS2  
- YMR196W  
- FLC1  
- ORC4  
- SLA1  
- YPR091C  
- FUN19  
- PAL1  
- SPA2  
- GCS1  
- PAR32  
- SRC1

Figure 2
### Interactions observed in the network for every node (protein)

**Protein Node** | **Proteins** |
--- | --- |
Cdc28 | Ace2, Ash1, Bni1, Bud3, Bud6, Cdc13, Cdc14, Cdc28, Cdc37, Fir1, Gin4, Grr1, Hho1, Hsl1, Kip2, Lie1, Mbp1, Net1, Pah1, Pcl6, Pcl7, Pol1, Pkl2, Pxl1, Rad9, Rim15, Sic1, Skg3, Sli5, Snf1, Srt3, Stb1, Ste20, Swi4, Swi5, Swi6, Tfb3, Tfb6, Ubp3, Whi5, Yox1 |
Cla4 | Bck1, Bem3, Bni1, Bud6, Cdc12, Cdc14, Cdc3, Gin4, Hsl1, Km4, Myo2, Nbp2, Rga1, Rho5, Rts1, Rtt107, Sli9, Sny1, Spa2, Ste20, Swi4, Vac14 |
PKC1 | Bck1, Bni1, Cyr1, Mbp1, Mig1, Pah1, Pkh1, Rho5, Smi1, Spa2, Spo14, Ssd1, Stb1, Swi4, Tsc11, Ypk1 |
SNF1 | Acc1, Cdc28, Ctk3, Cyc8, Ena1, Mig1, Man2, Reg1, Rod1, Sip1, Ssn2, Swi6, Tfb3, Tfb6, Tup1, Ypk2 |
STB1 | Ash1, Cdc28, Mbp1, Slt3, Snt1, Swi4, Swi6, Taf12, Taf5, Tfa2, Tfb3, Tfb6, Tfg1, Ume6, Whi5 |
SWI4 | Bck1, Cdc28, Chd1, Cla4, Mbp1, Paf1, Pdr1, Pkc1, Ssd1, Stb1, Swi6, Taf12, Taf5, Tfb3, Tfb6, Tfg1, Whi5 |

---

**Figure 3**
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D

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Figure 4
Figure 5

Mob1/Cdc55 Centroids

PP2A-A

PP2A-B

PP2A-C

Cdc55

Mob1

Interface Propensity

Less

More

90°

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