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Greatwall maintains mitosis through regulation of PP2A

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

1st Editorial Decision 05 June 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. It was sent to three expert reviewers, and we have so far received the reports of two of them. A third referee has informed us that, for excusable personal circumstances, s/he may be delayed considerably further with providing a report. As the other two reviewers seem to be in fair agreement, I have chosen to contact you at this point with a preliminary decision on your manuscript, in order to save you from any unnecessary loss of time. As you will see from the comments copied below, both reviewers express some interest in your findings but also raise the same major issue concerning a demonstration that Greatwall kinase may indeed directly regulate PP2A activity. Both referees indicate that to make the study a sufficiently major advance worth of publishing in a general journal such as The EMBO Journal, more definitive and direct evidence for this notion would be essential, e.g. by showing interactions on the level of endogenous protein, or by demonstrating a direct effect of GW (or its depletion) PP2A activity.

Should you be able to obtain such crucial evidence (as well as to adequately address the more specific concerns of these reviewers), we should be able to consider a revised version for publication. I am thus inviting you to start preparing such a revision, but I nevertheless have to stress that this is a preliminary decision and thus still subject to change should the last, missing report bring up serious additional concerns. Once we will have heard back from the third, outstanding referee, we shall contact you as soon as possible to transmit his/her comments and finalize the decision.

I should add that it is EMBO Journal policy to allow a single round of revision only, and that it is thus essential that you completely answer the points raised if you wish the manuscript ultimately to be accepted. Please do not hesitate to get back to us should you need feedback on any issue regarding your revision.

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Yours sincerely,
Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

In this study the authors have investigate the role of the Greatwall kinase in mitosis. Previous studies had implicated Greatwall in opposing a phosphatase activity - most likely PP2A from its sensitivity to okadaic acid - in the amplification process of MPF. This study clarifies this further to show that Greatwall antagonises a phosphatase (most likely PP2A) in mitosis and that depleting Greatwall causes mitotic exit even when cyclin B-Cdk1 remains active. They further show that depleting Greatwall does not need to act via Wee1/Myt1 and Cdc25. The authors attempt to connect Greatwall directly to PP2A by showing that it co-immunoprecipitates with PP2A when co-transfected into mammalian cells.

This study has value in demonstrating that Greatwall may be acting on PP2A and PP2A in turn on some mitotic Cdk substrates, rather than to maintain Cdk activity via Wee1/Myt1 and Cdc25 as previously suggested. The authors also strengthen the connection between Greatwall and PP2A. These observations are of interest but of themselves do not go far enough to warrant publication in the EMBO Journal. To this referee it is important to strengthen the connection between Greatwall and PP2A. The current co-transfection experiments are too prone to artefacts of overexpression. Since the authors have reagents against both PP2A and Greatwall they should concentrate on showing an interaction between the endogenous proteins. Alternatively, if they do not form a stable complex, can they demonstrate that Greatwall alters the activity of PP2A? Without these data I cannot recommend publication in the EMBO Journal.

Specific points:
1) To this referee the experiments adding cyclin A to greatwall depleted extracts are somewhat tangential to the rest of the study. I think they can be removed without weakening the paper.
2) It is intriguing that it takes 30 min for microcystin to reverse the effect of depleting Greatwall in Fig 4A. This is a remarkably long time, possibly indicating that the effect of Greatwall on PP2A is indirect. Is protein synthesis required during this period? What happens if microcystin is added while depleting Greatwall?
3) The depletion of PP2A in Fig 6 does not look to me as if it is 90%. How did the authors estimate this?
4) Many of the blots are overloaded, making it difficult to see the changes in mobility of indicated proteins. This should be rectified and molecular weight markers included.
5) At present the data indicate that Greatwall is required to prevent some but not all Cdk substrates being dephosphorylated. I don't think this justifies including the word 'massive' in the title.

Referee #2 (Remarks to the Author):

This is an interesting paper to analyze the function of the Greatwall kinase in controlling mitotic exit. The authors contend that Greatwall controls the PP2A-mediated dephosphorylation of M phase substrates. Overall, I believe the authors are at least partially correct. It is not clear that there are not other things going on (eg. regulation of Cdc25 via effects on PP2A), but they probably have a nice piece of the puzzle here. They fall short, however, of demonstrating that Greatwall has a direct effect on PP2A activity. It would be helpful if they could either show a dephosphorylation assay using purified components or, minimally, if they could show that PP2A immunoprecipitates from Greatwall-depleted extracts have differential activity towards Cdc2 substrates in vitro. It would also be of interest to see if Greatwall depletion effects are modified by prior depletion of Cdc25.
Additional points:
1. Fig. 2C should show Greatwall depletion alone.

2. In that the authors are using gel-shifted bands as their major indicator of activities, they need to provide molecular weights on their gels-since depletions and controls are clearly run on different gels.
3. A longer timecourse is required for Fig. 3B. Phosphorylation looks like it is beginning to decrease following depletion at 60 min; it would not be uncommon for everything to move more slowly in a depleted (ie slightly diluted) extract.
4. I find the results in Fig. 4A a little odd. Why does dephosphorylation occur initially even in the presence of microcystin?
5. The authors have not provided a positive control in their I2 experiments. This is important in that the okadaic acid dose response also has no control and okadaic acid titrations are notoriously variable depending on the extract, okadaic acid supplier, and batch of okadaic acid.
6. Given the recent implication of PP1 in M phase exit, as the authors cite, it would be nice to see some negative data for PP1-Greatwall interactions.

1st Revision - authors' response 19 June 2009

Enclosed you will find a revised version of our manuscript EMBOJ-2009-71343 by Vigneron et al. Despite the fact that we have not the report of the third reviewer, we decided to send you our revised version since all the concerns of the two reviewers have been addressed. In this regard, two different experiments answering to the two major points of the reviewers have been added to the Figures. The first one shows an association of endogenous GW and PP2A in Xenopus egg extracts. The second one demonstrate that PP2A activity is higher in GW-depleted CSF than in control CSF extracts indicating that GW modulates PP2A. The minor points raised by the two referees have also been addressed as you can see in the attached point per point response.

REVIEWER 1

Major points

(1) Results showing an association of endogenous GW with endogenous PP2A has been added in Figure 4D. As we suggest in the part of the text describing this figure, the fact that a high amount of GW is present in PP2A IP whereas a small quantity of PP2A is visualized in the GW IP could indicate that only a specific sub-complex of PP2A is associated with GW.
(2) Results showing an increased of the phosphatase activity of PP2A from GW-depleted CSF extracts have been added in Figure 5B. As depicted in this figure dephosphorylation activity of PP2A from GW-depleted CSF extract was three-fold the dephosphorylation activity of the PP2A obtained from control CSF extracts. Thus, these results indicate that GW is required to promote the inhibition of PP2A during mitosis.

Minor points

(1) As suggested by this referee, all the results showing the effect of the addition of cyclin A to GW-depleted CSF extracts have been removed.
(2) As shown in Figure1A Referee 1, the addition of cycloheximide to CSF extracts did not affect the microcystin-dependent reversion of the GW phenotype.
(3) As depicted in Figure 1B Referee 1, the addition of microcystin before GW depletion prevented mitotic exit.
(4) The levels of PP2A/A and PP2A/C left after depletion in Figure 6 (Figure 5 of the revised version) have been analysed again by using GelEval software. PP2A/A decreased 61% of and PP2A/C levels decreased 83% from the depleted CSF extract. Accordingly, these two values have been modified in the text.
(5) Blots that were overexposed have been changed and the molecular weights were indicated in all the gels in which a gel-shifted band was present.
(6) The word "massive" has been removed from the title and from the rest of the text.

REVIEWER 2

Major points

(1) As described above, results showing a higher activity of PP2A from GW-depleted CSF extracts compared to non-depleted CSF extracts have been shown in Figure 5B (revised version).
(2) The effect of Cdc25 on GW depletion cannot be tested by a prior depletion of Cdc25 from the extracts since the depletion of this phosphatase by itself induces mitotic exit. However, the lost of the mitotic state induced by Cdc25 depletion in these extracts is dependent on Myt1/ Wee1 activity since mitotic exit has not take place after Cdc25 depletion if a prior removal of Myt1/Wee1 is performed (manuscript in preparation).

Minor points

(1) It is not clear for us whether this referee is asking for the immunoblotting of all the proteins shown in Figure 2C after GW depletion alone or whether it refers to exclusively to Cdc25, since in the text we claim that this phosphatase is dephosphorylated 5 min after GW depletion and it is not shown in this Figure (although it is shown in Figure 1A and Figure 3A). Taking into account that the big dimensions of this figure and that the dephosphorylation pattern of the majority of the proteins after GW depletion alone has already been depicted in Figure 2B left panel, we decided to add exclusively the dephosphorylation pattern of Cdc25 after depletion of GW alone. However, the phosphorylation pattern of the rest of the proteins can be added if required.
(2) Molecular weights are provided in all the gels in which a band-shift is present.
(3) As suggested by Referee 1, Figure 3 has been removed from the manuscript.
(4) In Figure 4A (Figure 3A of the revised version), CSF extracts were first depleted of GW and a sample (point indicated in the figure as GW) was taken in the absence of microcystin. Microcystin was then added and just after addition a sample corresponding to time 0 was removed. The other two samples were taken 30 and 60 min after microcystin addition. Thus, points corresponding to GW and 0 min are in the absence of microcystin and just after the addition of this drug respectivelly, this is the reason why the proteins are initially dephosphorylated in these two samples. The text of the manuscript has been modified to clarify the way in which this experiment has been performed.
(5) Supplementary Figure S4 has been modified and the positive control of the I2 inhibitor has been added.

2nd Editorial Decision 14 July 2009

Your revised manuscript has now been seen once more by referees 1 and 2. Both of them consider the study generally improved and would thus in principle support publication pending the satisfactory revision of some remaining issues. Some of these concerns pertain to a more cautious interpretation of the results, also in light of divergent recent reports implicating phosphatases other than PP2A (referee 2 has therefore indicated to us that in their opinion, a somewhat more generic title such as 'Greatwall maintains mitosis through regulation of PP2A' might be a safer option). At the same time, there are however also more serious concerns with regard to the newly added data - referee 1 is not convinced that the blots presently shown in Figure 4D are sufficient to conclusively judge the validity of the PP2A-GW co-IP data, given the apparent close cropping of band(s) in the control lane and the absence of molecular markers.
As I am hoping you will be able to address these remaining issues, I am herewith returning the manuscript once more for a final round of revision. In this extra round, I would kindly like to ask you to provide data panels that can be more thoroughly assessed, as requested by this reviewer (and possible explanatory statements for bands that may not fit the expectations of the experiment). Should you be able to satisfactorily address this issue, as well as the various other points brought up by the reviewers, we should be happy to proceed with publication of the manuscript. However, I do have to stress that this will have to be the final round of revision for this manuscript for The EMBO Journal. I thus hope you will be able to get a adequately re-revised version back to us as soon as possible.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This paper is a revised version of one I have previously refereed. The authors have provided data to show that endogenous GW and PP2A to co-immunoprecipitate but these data need to be improved. The authors should include molecular weight markers and show more of the blot since it appears that a large band has been cropped out in the control IP.
The authors have also provided data to show that GW alters the activity of PP2A. These data should be repeated at least twice more and the results presented as mean and variation. Ideally the authors should assay activity as a time-course not as an end-point assay.

Other points:
1) The authors should be careful not to refer to their experiments as demonstrating a role for Greatwall in mitosis. CSF extracts are meiotic not mitotic.
2) The discussion of the antephase checkpoint is incorrectly referenced. Scolnick and Halazonetis described the Chfr as regulating a checkpoint preventing entry to metaphase after the full activation of cyclin B-Cdk1. This was corrected by Matsusaka and Pines (2004) to a role in the antephase checkpoint, a checkpoint previously described by Rieder and Cole, 1998.

3) There are two remaining instances where the authors refer to 'massive dephosphorylation'.

2nd Revision - authors' response 20 July 2009

Enclosed you will find a second revised version of our manuscript EMBOJ-2009-71343 by Vigneron et al. in which the concerns of the two referees have been addressed. In this regard, the gels corresponding to Greatwall and PP2A immunoprecipitations of Figure 4D have been completely shown (we do not dispose of the part of the gel inferior in GW IP and superior in PP2A IP to 45 Kda since they have been proved against other antibodies). The chopped band that was partially evident in the previous version of the manuscript corresponds to the top part of the smeared bands of the immunoglobulins used in the CT IP that unfortunately were not correctly dissociated after boiling.

We have also added a new part (C) in Figure 5 where we show the results as mean values, error bars and statistical differences obtained from three different experiments in which a time-course analysis of the dephosphorylation of c-Mos by PP2A obtained from CSF and GW-depleted CSF extracts were performed.

The reference concerning the G2-prophase or antephase checkpoint in the Discussion section has been corrected and the two different cases where "massive dephosphorylation" was present have been corrected to "dephosphorylation". However, we were a little puzzled concerning the words "mitotic versus meiotic" since while referee 1 recommends us to change to meiotic, referee 2 recommends to include mitosis in the title. Thus we decided to maintain mitotic in the text, however, these statements can be changed if the editor considers that are not correct.

Finally, we have changed the title as suggested by referee 2.