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9. My impression is that the cartoon of the model is not informative enough to justify a dedicated Figure.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The authors have addressed my comments in full and I support publication of this manuscript. I have a few minor suggestions:

Page 5. I am not sure what the sentence “Ubiquitination of the MEN scaffold Nud1 at SPBs prevents septin splitting and CAR contraction to silence these processes at the end of cytokinesis” means. Presumably at the end of cytokinesis there is no need to silence these processes as they’ve already occurred? Perhaps this can be rephrased.

Page 10. Related to Iqg1 being ‘slowly degraded’ (Fig. S4). I am not sure one can conclude it is degraded, in the absence of appropriate measurements. The only thing I can conclude from the presented time-lapse is that Iqg1 disappears from the ring.

Page 13. In a sentence starting with ‘In spite of their apparently normal cytokinesis, GAL1-DMA2 TEM1-Q79L cells could not complete cell division..’, I think a more appropriate way to state it would be ‘In spite of their apparently normal ring constriction’.

Reviewer #2 (Remarks to the Author):

In the revised version of their manuscript, Tamborrini et al. have made a significant effort to answer to the reviewer’s concerns. The authors have now included additional data that further support some of their claims, especially those regarding the role of Dma2 as an inhibitor of the role of the Mitotic Exit Network (MEN) in promoting cytokinesis. In this regard, the inhibition of cytokinesis by the constitutive targeting of Dma1/2 to the SPBs is a strong new argument favoring their hypothesis. However, in my opinion, the proposed mechanism by which Dma1/2 regulates the cytokinesis-promoting function of the MEN still falls somewhat short of strong experimental support. I am particularly concerned about whether the data in the manuscript satisfactorily proves that the Dma1/2-dependent inhibition of the MEN is mediated by direct interaction of Dma1/2 with Nud1 and ubiquitination of this SPB-component by the E3 ubiquitin ligase. Two main weak points related to this issue are the following:
1.- As previously raised to the consideration of the authors, one would expect that if Dma1/2 directly ubiquitinated Nud1 these proteins should interact with each other. Tamborrini et al. have made an effort to test this possibility by carrying out co-immunoprecipitation experiments with cells co-expressing Nud1-3PK and Dma2-3HA that, unfortunately, have been inconclusive due to unspecific binding binding of Dma2 to the beads. I believe, however, that this is an important issue that deserved a further effort. Not only the authors could have tried other tagged versions of the proteins, which they already have available, but there are additional ways to test this interaction that could even provide further information about it. An interesting option that the authors could explore is to use the Bimolecular Fluorescence Complementation Assay (Sung et al., 2007), which could not only confirm the interaction but also indicate where this interaction occurs within the cell (in this case, hopefully in the context of the SPB).
2.- With regards to the analysis of post-translational modifications, there is a general inconsistency in the levels of Nud1 protein both in the inputs and in the pulldowns that makes it difficult to draw solid conclusions from the results shown. A reliable quantification of the fraction of ubiquinated versus non-ubiquitinated Nud1, which the authors admit in their response to Reviewer #1 that is difficult to be estimated in their assays, would be required. One main problem seems to be the in vitro degradation of Nud1 in the conditions used for these assays, which is not however observed when protein extracts were prepared using TCA. If this were the case, they could simply use alternative extraction protocols in order to avoid this issue also in the pull-down experiments. As indicated in my original review, I believe that it would be critical to precisely determine both the

pattern of Nud1 ubiquitination throughout the cell cycle and how the changes in the expression of Dma1/2 modify this pattern. I do not coincide with the authors in that it should be tricky to evaluate this effect on a synchronized time course experiment (levels of Dma2 overexpression can be easily evaluated during the experiment for comparison), and the new data provided in Figure S9A is again difficult to interpret as a consequence of the differences in the levels of Nud1 protein among the different conditions and also among the inputs and the pull-downs.
Therefore, and despite still believing that the conclusions from this manuscript are potentially of interest in the field, I consider that, ideally, clarification of these two fundamental issues regarding the molecular mechanism proposed for Dma1/2-mediated inhibition of MEN signaling should be recommended before granting its final publication. Finally, some minor comments about the manuscript are:
1.- The authors claim to have fixed the problem regarding the black box that showed up in the background of Figure 3C from the original manuscript. However, the black box that obstructs the visibility of the graph shown in Figure 3C still shows up in the pdf file from the revised manuscript.
2.- In the text it is stated that introducing the TAB6-1 allele in GAL1-DMA2 cells accelerated mitotic exit (page 10 of the revised manuscript). However, no cell cycle progression analysis (or bibliographic reference) is provided.
3.- In page 13, the authors indicate that “deletion of both DMA1 and DMA2 […] did not affect the ubiquitination pattern of either Cdc11 or Shs1 (Fig. S8A, B)”. However, and as admitted in their reply to one of my concerns, ubiquitination of both septins is, in fact, heavily increased by the simultaneous lack of Dma1 and Dma2. The sentence should be thus corrected, and this observation commented in the manuscript.
4.- There are some overstatements in the description of the results. In this sense, in page15 it is said that “Localization of Bub2-Bfa1, Cdc15 and Mob1 at SPBs was markedly inhibited…”. Similarly, in page 16 it is stated that “[Total] Nud1 phosphorylation was markedly impaired upon DMA2-overexpression”. In both cases, changes are subtler than what these sentences imply.

Reviewer #3 (Remarks to the Author):

Review on „Recruitment of the Mitotic Exit Network to the yeast centrosome couples septin displacement to actomyosin ring constriction“ by Davide Tamborrini et al.

I found the response of the authors to my suggestions and criticisms satisfying. I support the publication of the manuscript in Nature Communication.

Reviewer #1:

Page 5. I am not sure what the sentence "Ubiquitination of the MEN scaffold Nud1 at SPBs prevents septin splitting and CAR contraction to silence these processes at the end of cytokinesis" means. Presumably at the end of cytokinesis there is no need to silence these processes as they've already occurred? Perhaps this can be rephrased.

The sentence was rephrased accordingly.

Page 10. Related to Iqg1 being 'slowly degraded' (Fig. S4). I am not sure one can conclude it is degraded, in the absence of appropriate measurements. The only thing I can conclude from the presented time-lapse is that Iqg1 disappears from the ring.

We agree and rephrased the sentence.

Page 13. In a sentence starting with 'In spite of their apparently normal cytokinesis, GAL1-DMA2 TEM1-Q79L cells could not complete cell division..', I think a more appropriate way to state it would be 'In spite of their apparently normal ring constriction'.

We agree and rephrased the sentence.

Reviewer #2:

1.- As previously raised to the consideration of the authors, one would expect that if Dma1/2 directly ubiquitinated Nud1 these proteins should interact with each other. Tamborrini et al. have made an effort to test this possibility by carrying out co-immunoprecipitation experiments with cells co-expressing Nud1-3PK and Dma2-3HA that, unfortunately, have been inconclusive due to unspecific binding binding of Dma2 to the beads. I believe, however, that this is an important issue that deserved a further effort. Not only the authors could have tried other tagged versions of the proteins, which they already have available, but there are additional ways to test this interaction that could even provide further information about it. An interesting option that the authors could explore is to use the Bimolecular Fluorescence Complementation Assay (Sung et al., 2007), which could not only confirm the interaction but also indicate where this interaction occurs within the cell (in this case, hopefully in the context of the SPB).

We have made a huge effort to find good conditions to probe the Nud1-Dma2 interaction by co-immunoprecipitation because Dma2 is particularly sticky and binds aspecifically to resins. We obtained the best results by immunoprecipitation of 3Flag-tagged Nud1 and elution of immunoprecipitates with an excess of 3XFlag peptide. Using this strategy we find that a small fraction of Dma2-3HA associates to Nud1-3Flag in anaphase (new Fig. S9).
The BiFC complementation assay proposed by the Reviewer as an alternative to co-IPs would have been in principle a good suggestion, but is known to frequently generate false positives, prompting the need for a proper control where one of the two binding partners carries a mutation in the binding interface (which we obviously do not know). Furthermore, slow maturation of the chromophore is considered another limiting factor for the visualization by BiFC of transient or dynamic interactions in cells (reviewed in

Kodama and Hu, 2012, *BioTechniques* 53 : 285 ; Miller et al., 2015*, J. Mol. Biol.* 427 : 2039).
In this context, it is also worth noting that although the Dma1 ubiquitin ligase (paralogue of Dma2) was found at SPBs in late mitosis (Yau et al., 2014), we could not detect Dma1 or Dma2 localised at SPBs is our yeast strain background, using either the published constructs or GFP-tagged variants that we made in our lab.

2.- With regards to the analysis of post-translational modifications, there is a general inconsistency in the levels of Nud1 protein both in the inputs and in the pulldowns that makes it difficult to draw solid conclusions from the results shown. A reliable quantification of the fraction of ubiquinated versus non-ubiquitinated Nud1, which the authors admit in their response to Reviewer #1 that is difficult to be estimated in their assays, would be required. One main problem seems to be the in vitro degradation of Nud1 in the conditions used for these assays, which is not however observed when protein extracts were prepared using TCA. If this were the case, they could simply use alternative extraction protocols in order to avoid this issue also in the pull-down experiments. As indicated in my original review, I believe that it would be critical to precisely determine both the pattern of Nud1 ubiquitination throughout the cell cycle and how the changes in the expression of Dma1/2 modify this pattern. I do not coincide with the authors in that it should be tricky to evaluate this effect on a synchronized time course experiment (levels of Dma2 overexpression can be easily evaluated during the experiment for comparison), and the new data provided in Figure S9A is again difficult to interpret as a consequence of the differences in the levels of Nud1 protein among the different conditions and also among the inputs and the pull-downs.

Following the Reviewer's advice, we have spent a considerable amount of time to set up the conditions to preserve Nud1 protein stability in vitro in our ubiquitination assays. We have succeeded by lysing cells directly in TCA, as detailed in Materials and Methods. We have repeated our analysis of Nud1 ubiquitination throughout the cell cycle and our new data (Fig. 5c) show that Nud1 is ubiquitinated in late mitosis and in the following G1 phase.

Using these new conditions we have also performed Nud1 ubiquitination assays during a synchronous release of wild type versus *GAL1-DMA2* cells in the presence of galactose. These data show that *DMA2* overexpression can stimulate Nud1 ubiquitination throughout the cell cycle but most markedly in late mitosis and in G1, i.e. during the cell cycle phases when Nud1 ubiquitination reaches its maximal levels in wild type cells (new Fig. S10a). It should be noticed, however, that persistent *DMA2* overexpression combined with ubiquitin overexpression led unexpectedly to abnormal Nud1 destabilisation and accumulation of cells in mitosis. This is not what we see upon *DMA2* overexpression in cells expressing endogenous levels of ubiquitin (see for instance Fig. 5f and S11c).

Quantifying the fraction of ubiquitinated versus non-ubiquitinated Nud1 is technically impossible at this stage because it would require detection of upshifted ubiquitinated forms of Nud1 in the inputs, something that we have never detected so far. This argues that either only a small fraction of Nud1 is ubiquitinated (but biologically relevant!) or a fraction of total Nud1 goes to SPBs where it is ubiquitinated. Another major shortcoming of these measurements is that other post-translational modifications could upshift the

electrophoretic mobility of Nud1 indistinguishably from ubiquitination, thereby interfering with reliable measurements.

Minor points:

1.- The authors claim to have fixed the problem regarding the black box that showed up in the background of Figure 3C from the original manuscript. However, the black box that obstructs the visibility of the graph shown in Figure 3C still shows up in the pdf file from the revised manuscript.

We have tried to remake this figure with Inkscape and on some, but not all computers, the black box mentioned by the Reviewer remains apparent. We do not understand why this is the case, but it is likely due to the conversion from .svg to .pdf. We can provide the figure in .svg if required.

2.- In the text it is stated that introducing the TAB6-1 allele in GAL1-DMA2 cells accelerated mitotic exit (page 10 of the revised manuscript). However, no cell cycle progression analysis (or bibliographic reference) is provided.

Since the *TAB6-1* allele increases the fraction of *GAL1-DMA2* cells with more than one septin ring during a 2hr time frame, presumably it facilitates (« accelerates ») mitotic exit of *GAL1-DMA2* cells. We have rephrased this sentence for the sake of clarity.

3.- In page 13, the authors indicate that "deletion of both DMA1 and DMA2 […] did not affect the ubiquitination pattern of either Cdc11 or Shs1 (Fig. S8A, B)". However, and as admitted in their reply to one of my concerns, ubiquitination of both septins is, in fact, heavily increased by the simultaneous lack of Dma1 and Dma2. The sentence should be thus corrected, and this observation commented in the manuscript.

We have corrected this sentence, but at the moment we have no data to support any additional comment.

4.- There are some overstatements in the description of the results. In this sense, in page15 it is said that "Localization of Bub2-Bfa1, Cdc15 and Mob1 at SPBs was markedly inhibited…". Similarly, in page 16 it is stated that "[Total] Nud1 phosphorylation was markedly impaired upon DMA2-overexpression". In both cases, changes are subtler than what these sentences imply.

We have corrected these sentences accordingly.

REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

No further comments

Reviewer #2 (Remarks to the Author):

The new results incorporated by Tamborrini et al. in their last revised version significantly strengthen the main weak points that I raised in my comments, adding further experimental support to the conclusions drawn in their manuscript. Hence, I support its final acceptance in Nature Communications.
Note: Please, check whether reference to figure S10A in line 356 from page 15 is correct, since I believe it is a typo.

Reviewer #3 (Remarks to the Author):

No further comments

REVIEWERS' COMMENTS:

Reviewer #2 (Remarks to the Author):

The new results incorporated by Tamborrini et al. in their last revised version significantly strengthen the main weak points that I raised in my comments, adding further experimental support to the conclusions drawn in their manuscript. Hence, I support its final acceptance in Nature Communications.
Note: Please, check whether reference to figure S10A in line 356 from page 15 is correct, since I believe it is a typo.

Thank you for noticing this typo. We have now corrected it.

Sincerely

Simonetta Piatti