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Acetylation regulates monopolar attachment at multiple levels during meiosis I in fission yeast

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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 16 March 2011

I have received the full set of referee reports regarding your study. As you will see, although referee 3 is more negative, referees 1 and 2 would support publication after substantial revision, and have very similar concerns. On further discussion with referee 3, s/he also agreed that if some crucial concerns could be addressed, the story would merit publication in EMBO reports (please see below).

A key issue would be to exclude that the effects you see are due to a general decrease in cohesion that simply sets centromeric cohesion below a threshold, and rather show a specific requirement of acetylation at the core centromere. All referees suggest how to address this experimentally, including measuring Psm3 acetylation during meiosis, its interaction with Rec8 or Rad21 and measuring cohesion. In addition, further support for Crl6 as the deacetylase is also requested by all three referees and would strengthen the study. As referees 1 and 2 provide constructive suggestions on how to make the work more conclusive, if their main concerns can be adequately addressed, we would be happy to accept your manuscript for publication.

Given that the first part of the study recapitulates recent results, it could be included in supplementary information in order to allow more space to incorporate the new data. We normally request that revised manuscripts are be submitted within three months of a request for revision. However, given the exceptional circumstances in Japan, we would agree to be somewhat flexible if you felt this would enable you to successfully revise the study. Please note that it is EMBO reports

policy to undergo one round of revision only and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review.

I look forward to seeing a revised form of your manuscript when it is ready. In the meantime, do not hesitate to get in touch with me if I can be of any assistance. Please let me know if you can make any estimates at this point of how much time you would be likely to need for revision.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

Reductional chromosome segregation during the first meiotic division requires that both sister-kinetochores attach to microtubules emanating from the same spindle pole, a process known as mono-orientation. Work in fission yeast has shown that mono-orientation requires sister-chromatid cohesion to be established within the central domain of centromeres as to join together the two kinetochores into a single functional unit. This regional cohesion is meiosis specific and its establishment / maintenance requires several factors, including the meiotic Rec8 cohesin sub-unit, the cohesin associated protein Pds5, components of the replication fork and a meiosis specific protein called Moa1. Recent work from several groups have demonstrated that the establishment of sister-chromatid cohesion during the vegetative cycle requires the acetylation of the cohesin core subunit Smc3 by a conserved acetyl-transferase called Eco1 in budding yeast, Esco1-2 in humans and Eso1 in S. pombe. In this study, Kagami et al. asked whether the acetyl-transferase and Smc3 acetylation play a role in the process of kinetochore mono-orientation during fission yeast meiosis.

In the first paragraph, they presented data that recapitulate the recently published observations made by Feytout et al. (Mol Cell Biol. 2011 Feb 7), namely that fission yeast Smc3 (called Psm3) is acetylated in an Eso1 dependent manner, that an acetyl-mimicking form of Psm3 can bypass Eso1 requirement and that - in contrast to budding yeast - Psm3 acetylation is largely dispensable for sister chromatid cohesion in the vegetative cycle. Like Feytout at al. they conclude that Eso1 must have other target(s) or function(s) besides Psm3 acetylation. They extend the current knowledge in S. pombe by providing evidence that Clr6 is likely the Psm3 deacetylase.
Next the authors provide strong experimental evidence that the acetylation of Psm3 by Eso1 is essential for mono-orientation during meiosis I. The eso1-H17 mutation or a non-acetylatable form of Psm3 abolishes cohesion within the central centromere domain and results in equational rather than reductional segregation during the first meiotic division. Conversely, a mutant form of Psm3 mimicking the acetylated state fully suppressed eso1-H17. They also provide evidence that Eso1 is counteracted by the Clr6 deacetylase since mono-orientation and core centromere cohesion defects in eso1-H17 are partially restored by the clr6-1 mutation.
Next the authors asked whether Moa1 would impinge on Psm3 acetylation. Core centromere cohesion and mono-orientation are abolished in moa1 deleted cells but partially restored in a clr6-1 mutant background. However the acetyl-mimicking form of Psm3 can not bypass moa1 requirement. This is an important observation because it shows that Psm3 acetylation is essential but not sufficient for mono-orientation and suggests that another Clr6 substrate must exist which when acetylated promotes core centromere cohesion in the absence of Moa1. The identity of this putative factor is currently unknown.

This study convincingly shows that Psm3 acetylation is required for kinetochore mono-orientation during meiosis I. This an important finding that contributes to our mechanistic understanding of a universal process at the heart of meiotic chromosome segregation. As such, this finding is of great interest to the field and of general interest to biologists. The relationship between Moa1 and acetylation is less clear but paves the way for future studies. The manuscript is clearly written and high quality data support the conclusions drawn by the authors. I think that some specific points deserve strengthening, as detailed below.

1- The data showing that Psm3 acetylation is required for mono-orientation are very clear-cut and to me, form the strongest part of the paper. I would therefore suggest to try to go a little bit farther in that direction. For instance, is Psm3 acetylation required to load Rec8 at centromeres? In psm3-KKRR or eso1-H17 cells, sister-chromatids segregate equationally rather than randomly (Fig 2A). The phenotype is similar to a rec8 deletion mutant in which mono-orientation is abolished as cohesion is lost at the central core but chromosome segregation is non-random because Rad21 ensures cohesion at peri-centromeres. This suggests that preventing Psm3 acetylation may result in a failure to load Rec8 at centromeres. A Rec8 ChiP assay would tell whether or not Rec8 is present at the central core and peri-centromere domains in psm3KK-RR and eso1-H17. Alternatively, if Rec8 is correctly loaded it would be important to check whether Moa1 is properly recruited to centromeres.

2- Is the sole function of Eso1 in mono-orientation achieved through the acetylation of Psm3? This can be addressed by analysing meiosis I chromosome segregation in a eso1 delete psm3KKQQ background.

3- Does the deletion of wpl1 suppress the eso1-H17, psm3-KK-RR or moa1 mono-orientation defects? It has been shown in several organisms that the Wapl protein is counteracted by the Eco1/Eso1/Esco1-2 acetyl-transferase. In fission yeast the deletion of wpl1 fully bypasses Eso1 requirement (Feytout et al. MCB). It would be interesting to know whether this holds true for the process of kinetochore mono-orientation.

4- The description of Psm3 acetylation during meiosis is lacking. It would be interesting to probe protein extracts from Figure 3 with anti-acetylated Psm3 antibodies.

5- The authors claim that Clr6 is the Psm3 deacetylase on the basis that Psm3 acetylation appears increased in hydroxyurea (HU) arrested clr6-1 cells (Figure 1F). A leak through the HU arrest may be responsible for the increase in acetylated Psm3. DNA content and septation index analyses are required to ensure that the clr6-1 mutant does arrest normally in HU. In addition, since de-acetylation is supposed to take place during anaphase it would be more convincing to show that the level of Psm3 acetylation fails to decrease at anaphase in the clr6-1 mutant, for instance in a cdc25 block and release experiment as in Figure S1B. Lastly, since clr6-1 partially restores mono-orientation in eso1-H17, it is expected that Psm3 acetylation is accordingly partially restored. This point should be addressed in meiosis and/or in the mitotic cycle. A partial restoration of Psm3 acetylation by clr6-1 would also strengthen the claim that Clr6 is indeed the Psm3 deacetylase.

Minor points:

1/ Figure 1A and C: the temperature at which eso1-H17 cells were grown should be indicated. Figure 1B: the indicated temperature is 32{degree sign}C in the figure but 30{degree sign}C in the legend.

2/ Figure 1F. Cells are arrested by hydroxyurea and should be referred to early S phase and not G1/S as mentioned in the text.

2/ Fig. 3A shows that the Eso1 protein is present during S phase but most abundant at 5 hrs at the time of anaphase I. When eso1 is expressed under the moa1 promoter Eso1 expression during S phase appears abolished but the peak of Eso1 is still present at 5hrs. Strangely enough, mono-orientation is altered but not abolished. This suggests that Eso1 may have another function after S phase but this was not pointed out.

3/ Figure 3C clearly shows that the Moa1 protein first appears at 3hrs, that is after completion of DNA replication and not during S phase as mentioned in the text.

Referee #2:

Work performed so far mostly in budding yeast showed that the ability of the cohesin complex to

link together sister chromatids is regulated through cycles of acetylation and deacetylation of its Smc3 subunit by the Eco1 acetyltransferase and Hos1 deactetylase, respectively. In their current manuscript, the authors study the regulation of cohesion through acetylation in fission yeast during mitotic and meiotic cell divisions.

They find that during vegetative growth, cohesin's Psm3 subunit is acetylated between S-phase and mid M-phase at lysine residues homologous to the acetylated residues found in Smc3. The authors show that acetylation at these residues depends on the Eco1 homolog Eso1 and provide evidence that de-acteylation requires Clr6. Strikingly, mutation of the two lysines to non-acetylatable arginine residues has - in contrast to the situation in budding yeast - no apparent effect on cell viability or chromosome arm cohesion and only a moderate effect on centromere cohesion. Mutation to acetyl-mimicking residues meanwhile suppresses the requirement for Eso1. This suggests that acetylation by Eso1 of either Psm3 or a yet unknown target is sufficient for the establishment of cohesion during mitosis.

Mutation of both lysine residues in Psm3 to arginine, however, largely abolishes cohesion at core centromeres and thereby prevents co-segregation of sister centromeres during the first meiotic division. Inactivation or delayed/reduced expression of Eso1 during a meiotic cell cycle has similar consequences, suggesting Eso1 dependent acetylation of Psm3 during pre-meiotic S-phase is necessary for the establishment of centromeric cohesion. Inactivation of Clr6 can partially suppress the meiotic missegregation phenotype not only of eso1 mutants but also of cell deleted for Moa1, a meiosis-specific protein required for co-orientation of sister kinetochores, while the acetyl-mimicking form of Psm1 can only suppress the defects of eso1 mutants but not of moa1 mutants. These findings imply that acetylation not only of Psm3 but also of a yet unknown factor is essential for centromeric cohesion during meiosis.

Most of the experiments described on the mitotic regulation of fission yeast condensin by acetylation in the first part of the manuscript have been published very recently by the Javerzat group (Feytout A et al., Mol Cell Biol 2011). Nevertheless, the role of cohesin acetylation in the establishment of centromeric cohesion during meiotic divisions in the second, more substantial part of the manuscript is in my view an important novel contribution to the understanding of chromosome segregation, and as such merits publication in EMBO reports. While the majority of the data presented is of high quality as one would expect for a publication from the Watanabe lab, some of the data are in my opinion still incomplete and require further controls or additional experiments to support the authors' conclusions. I would therefore like to make the following suggestions:

1. Given that most part of the data on the mitotic Psm3 acetylation (Fig. 1A-E) has already been reported by another group, the most significant new finding in this part is the identification of Clr6 as the enzyme that deacetylates Psm3 (Fig. 1F and G). An increased level of Psm3 acetylation can only be observed in a single sample from a clr6-1 strain that was arrested by hydroxyurea (Fig. 1F, lane 5). To unequivocally demonstrate that Psm3 remains acetylated after exit from mitosis in clr6 mutants, it will be necessary to monitor Psm3 acetylation levels in a synchronized cell cycle similar to the experiment shown Supplementary Fig. 1B. Maybe it would be possible to synchronize the clr6-1 mutant by elutriation or by releasing cells from an HU arrest? The authors might also want to include the delta-hos2 strain in such a time course experiment to rule out that the Hos2 deacetylase may have a (minor) effect on the Psm3 acetylation status.

2. The paper could be strengthened if the authors could demonstrate that Psm3 is in fact acetylated during meiosis. Would it be possible to obtain sufficient material at different time points after cells are synchronously released into meiosis (e.g. using the pat1-114 mutant, see Fig. 3A and C) for detection of Psm3 acetylation by Western blotting? Since sister centromere co-orientation fails in the eso1-H17 mutant already at 26{degree sign}C (the permissive temperature for mitotic growth of this mutant), one would expect to see a strong reduction of meiotic Psm3 acetylation already at this temperature. Is this the case?

It would be even more informative if the authors could immunoprecipitate Rec8-containing cohesin (e.g. the cohesin at core centromeres) to test whether there is a preferential acetylation of Psm3 associated with Rec8 compared to Psm3 associated with Rad21. Since Psm3 acetylation is apparently essential for the centromeric cohesin, one might expect to detect higher levels of

acetylation in the Rec8 containing complexes.

3. Even though genetic evidence suggests that cohesion is impaired only at core centromeres and not at pericentomeric regions or chromosome arm regions in eso1-H17 or psm3-KKRR cells (otherwise one would expect random and not equational segregation in the first division, Fig. 2A), it would be important to test this directly. The authors could for example score cohesion at the cut3 locus as in Fig. 1C, or, more elegantly, loop out a pericentromeric region as they did in a recent paper (imr1-GFP, Sakuno et al., Nature 2009).

4. The authors claim that Eso1 expression during pre-meiotic G1-S phase is eliminated by placing the eso1 gene under the control of the moa1 promoter. Since the Eso1 levels in Pmoa1-eso1 cells never reach the Eso1 levels in Peso1-eso1 cells (compare 5 h samples in Fig. 3A), it is equally well possible that the segregation defect may be caused by insufficient Eso1 protein levels during metaphase or anaphase and not by an absence of Eso1 expression during pre-meiotic S-phase. Still, about half of the cells still co-segregate chromosomes in the first division, even when Eso1 expression is delayed/reduced. It is therefore difficult to conclude from these experiments that Eso1 function is required during pre-meiotic S phase. Would the results be clearer if eso1 gene were expressed under control of the spo6 promoter, as done for the moa1 gene in the next experiment?

5. If Psm3 acetylation were sufficient to restore centromeric cohesion, this should be clearly detectable in the centromere loop-out assay. The eso1-H17 psm3-KKQQ strain should therefore be included in Fig. 4B.

Minor points:

1. I think that it would be fair to downscale what sounds a bit like a priority claim in the last section of the introduction ("Here we establish the regulation of Psm acetylation in fission yeast") and also reference the Feytout et al. publication more appropriately than just in a note at the end of the paragraph.

2. Fig. 1F. It is not clear at which temperature the strains were grown in this experiment. It is mentioned in the methods sections that strains were grown at 30{degree sign}C, but given that the clr6-1 mutant doesn't show much of a phenotype at this temperature (Fig. 1G), shouldn't have the samples (from all strains) been taken instead at 37{degree sign}C?

3. In the last sentence of the section on the Clr6 deacetylase, the authors base the conclusion that Psm3 acetylation only plays a minor role in counteracting the function of Eso1 in cohesion establishment during mitosis on the finding that the clr6-1 mutation does not suppress eso1-H17 mutation. However, Clr6 may have an essential function that is completely different from Psm3 deacetylation (or even regulating sister chromatid cohesion). In my opinion this conclusion cannot be drawn so easily from a genetic experiment.

4. Did the authors consider that the unknown Eso1 acetylation target may not necessarily be a yet unknown factor X but potentially another lysine residue on Psm3?

5. Since the conditions vary between the different experiments, it will be necessary to describe them more carefully in the figure legends. For example, it is essential that the authors state at which temperature the samples were taken in each experiment, given that they work with different ts strains (e.g. at which point was the temperature shifted back for the pat1-114 release, and to which temperature were the strains shifted?). There are also discrepancies between the conditions shown in the figure and listed in the figure legends (e.g. Fig. 1B, 32{degree sign}C in the figure but 30{degree sign}C in the figure legend).

6. Fig. 3A. A band running in close proximity to Eso1-FLAG is labelled as "unspecific band", yet the intensities of this band increase and decrease with the Eso1-FLAG intensities. Why is this the case? The authors need to show that this band is unspecific in a control lane (e.g. extract from a strain that doesn't express at FLAG tagged protein).

7. Fig. 4. It is not immediately obvious why the experiment with the eso1 ts mutants was performed

at 26{degree sign}C, while the experiment with the delta-moa1 strain was performed at 30{degree sign}C. Wouldn't it make more sense to also perform the experiment with the eso1 ts mutation at 30{degree sign}C to ensure that the protein is really inactivated?

8. Supplementary Fig. 1B. It is not clear why the authors used in this experiment the antibody raised against the acetylated lysine residues in S. cerevisiae Smc3 and not the specific antibody they generated against acetylated Psm3, which appears work rather nicely (see Fig. 1A).

Referee #3:

This manuscript investigates the role of the Eso1 acetyltransferase in core centromere cohesion in fission yeast. There are three main conclusions. First, Eso1 acetylates the cohesin subunit, Psm3, during S phase to establish cohesin. Second, the Moa1 protein maintains core centromere cohesion independently of Eso1 to promote monoorientation. Third, the gene encoding the deacetylase, Clr6, shows genetic interactions with Eso1 that suggest other targets for monoorientation.

The first conclusion (from Figure 1) has been well established by other recent studies in budding yeast, humans and also recently comprehensively reported in fission yeast (Feytout et al. MCB online). The second conclusion (from Figure 3) that Moa1 acts after cohesion establishment in S phase is expected from previous work from the Watanabe lab (Yokobayashi et al, Cell 2005). The new findings in this study relate to the Clr6 deacetylase. However, this is a variation on a theme because the budding yeast class I deacetylase, Hos1, has been should to be the relevant deacetylase antagonising Eso1 (Borges et al., 2010; Xiong et al., 2010).

Even the data presented on monoorientation in meiosis holds no surprises in. Several papers (mainly from the Watanabe lab) have shown that core centromere cohesion is required for monoorientation, so it is highly expected that mutations in eso1 or the acetylation-dead Psm3 versions which block cohesion would also prevent monoorientation (Figure 2).

The interactions between eso1 and clr6 and between moa1 and clr6 are intriguing but too preliminary. The simplest conclusion is that in clr6 mutants there is more cohesion in general and this rescues monoorientation. However, it does not offer any new mechanistic insight. Therefore in its present form it lacks significant new insight.

In addition, error bars are missing from graphs throughout this manuscript and the number of cells scored is very low in many cases (e.g. S2B, n>20; 4B and D, n>79). Considering the small changes presented a much greater statistical analysis is required.

Referee #3 (additional comments):

If the authors could show a specific requirement for acetylation at the core centromere, rather than just a specific requirement for lots of cohesion, then this would be interesting and novel. So far though there is no evidence to suggest this. The authors are also careful not to mention the difference between Rec8 and Rad21, but if they could show some meiosis-specific regulation, this would be a new finding. However, the phenotypes may be caused by a small weakening of cohesion all over.

The following experiments are critical in my opinion.

1. Determination of cohesin Rec8/Rad21 association with core centromeres, pericentromeres and chromosome arms in eco1 and non-acetylatable Psm3 mutants by ChIP.
2. Cohesion assays (loop out experiments) at pericentromeres, chromosomes arms in the above mutants.
3. Examination of cohesin acetylation during meiosis. Also ChIP assay to identify domains in which cohesin is acetylated.
4. Clear evidence that Clr6 is the deacetylase (as pointed out by both reviewers 1 and 2).

1st Revision - authors' response 15 June 2011

To the referees,

We thank the referees for supporting publication of our study and for their valuable comments. To address the referees' comments, we have carried out new experiments and incorporated the results in the revised manuscript. We addressed all comments raised by the referees and our responses are listed below.

(Bold letters are referees' comments)

Referee #1:

1- The data showing that Psm3 acetylation is required for mono-orientation are very clear-cut and to me, form the strongest part of the paper. I would therefore suggest to try to go a little bit farther in that direction. For instance, is Psm3 acetylation required to load Rec8 at centromeres? In psm3-KKRR or eso1-H17 cells, sister-chromatids segregate equationally rather than randomly (Fig 2A). The phenotype is similar to a rec8 deletion mutant in which mono-orientation is abolished as cohesion is lost at the central core but chromosome segregation is non-random because Rad21 ensures cohesion at peri-centromeres. This suggests that preventing Psm3 acetylation may result in a failure to load Rec8 at centromeres. A Rec8 ChiP assay would tell whether or not Rec8 is present at the central core and peri-centromere domains in psm3KK-RR and eso1-H17. Alternatively, if Rec8 is correctly loaded it would be important to check whether Moa1 is properly recruited to centromeres.

We performed ChIP assays in *psm3-KKRR* and *eso1-H17* cells, revealing that both Rec8 and Moa1 localized normally as in wild-type cells. We now present these data in Supplementary Fig S4 and mention this result in the text (p 3, line 30), suggesting that the establishment of cohesion itself is impaired in *psm3-KKRR* or *eso1-H17* cells.

2- Is the sole function of Eso1 in mono-orientation achieved through the acetylation of Psm3? This can be addressed by analysing meiosis I chromosome segregation in a eso1 delete psm3KKQQ background.

We performed this experiment. While co-segregation at meiosis I was observed in 93% of *psm3-KKQQ* cells, it was reduced to 67% in *eso1Δ psm3-KKQQ* cells, implying that most but not all Eso1 function is achieved through the acetylation of Psm3 (Supplementary S6 online). We now mention this result in the text (p 5, line 11), and made a slight change in the model in Fig 5. Because of this new result, we can now imply that Eso1may target non-Psm3-K105/K106 not only in mitosis but also in meiosis. We thank the referee for suggesting this point.

3- Does the deletion of wpl1 suppress the eso1-H17, psm3-KK-RR or moa1 mono-orientation defects? It has been shown in several organisms that the Wapl protein is counteracted by the Eco1/Eso1/Esco1-2 acetyl-transferase. In fission yeast the deletion of wpl1 fully bypasses Eso1 requirement (Feytout et al. MCB). It would be interesting to know whether this holds true for the process of kinetochore mono-orientation.

We performed this experiment (new Fig 4E) and mention the results in the text (p 5, line 16). Briefly, *wpl1Δ* suppressed *eso1-H17* and *psm3-KKRR* but not *moa1Δ*.

4- The description of Psm3 acetylation during meiosis is lacking. It would be interesting to probe protein extracts from Figure 3 with anti-acetylated Psm3 antibodies.

In order to detect the acetylation of Psm3, we have to immunoprecipitate Psm3 by FLAG tag (see Fig 1B). In Figure 3 *eso1* was tagged with FLAG; therefore, it was difficult to examine acetylation in this experiment. However, we now show that Psm3 acetylation is provoked during premeiotic S phase depending on Eso1 in a separate experiment (new Fig 2A).

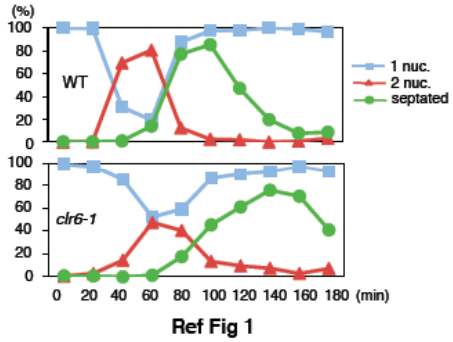
5- The authors claim that Clr6 is the Psm3 deacetylase on the basis that Psm3 acetylation appears increased in hydroxyurea (HU) arrested clr6-1 cells (Figure 1F). A leak through the HU arrest may be responsible for the increase in acetylated Psm3. DNA content and septation index analyses are required to ensure that the clr6-1 mutant does arrest normally in HU.

We now show the cell cycle profile of these samples (new Fig1B), indicating that overall *clr6-1* cells arrest with G1 DNA content as in wild-type cells.

In addition, since de-acetylation is supposed to take place during anaphase it would be more convincing to show that the level of Psm3 acetylation fails to decrease at anaphase in the clr6-1 mutant, for instance in a cdc25 block and release experiment as in Figure S1B.

We found that *clr6-1* cells are not suitable for the *cdc25* block and release experiment because they show less synchronicity after release (Ref Fig 1). Another experiment using HU block-release did not work even in wild-type cells because of ill-synchronicity of entry into anaphase (data not shown).

Lastly, since clr6-1 partially restores mono-orientation in eso1-H17, it is expected that Psm3 acetylation is accordingly



Ref Fig 1

partially restored. This point should be addressed in meiosis and/or in the mitotic cycle. A partial restoration of Psm3 acetylation by clr6-1 would also strengthen the claim that Clr6 is indeed the Psm3 deacetylase.

We tried acetylation recovery in *eso1-H17* cells by *clr6-1* but could not detect it. We assume that that is because the temperature-sensitive nature of both mutations makes it difficult to optimize Psm3 acetylation (please note that Psm3 acetylation in *eso1-H17* cells is undetectable even at the permissive temperature). However, we now demonstrate that over-expression of Clr6 impairs Psm3 acetylation as well as sister chromatid cohesion in G2 arrested cells (Fig 1C), thus providing a strong evidence that Clr6 acts as a deacetylase of Psm3.

Minor points:
1/ Figure 1A and C: the temperature at which eso1-H17 cells were grown should be indicated. Figure 1B: the indicated temperature is 32{degree sign}C in the figure but 30{degree sign}C in the legend.

These figures were moved to Supplementary Fig S1 and the incorrectly described figure legends were corrected.

2/ Figure 1F. Cells are arrested by hydroxyurea and should be referred to early S phase and not G1/S as mentioned in the text.

We have taken this suggestion.

2/ Fig. 3A shows that the Eso1 protein is present during S phase but most abundant at 5 hrs at the time of anaphase I. When eso1 is expressed under the moa1 promoter Eso1 expression during S phase appears abolished but the peak of Eso1 is still present at 5hrs. Strangely enough, mono-orientation is altered but not abolished. This suggests that Eso1 may have another function after S phase but this was not pointed out.

We now mention this point briefly (p 4, line 11) and change the model in Fig 5, suggesting that Eso1 might acetylate non-Psm3-K105/K106 substrate that acts after S phase.

3/ Figure 3C clearly shows that the Moa1 protein first appears at 3hrs, that is after completion of DNA replication and not during S phase as mentioned in the text.

Because Moa1 is absent just before S phase (at 2 hr) but fully expressed just after S phase (at 3 hr), we reason that Moa1 is expressed during S phase. Indeed, Moa1 is largely expressed in HU (S phase) arrested cells, supporting our interpretation (Yokobayashi & Watanabe, Cell 2005).

Referee #2:
1. Given that most part of the data on the mitotic Psm3 acetylation (Fig. 1A-E) has already been reported by another group, the most significant new finding in this part is the identification of Clr6 as the enzyme that deacetylates Psm3 (Fig. 1F and G). An increased level of Psm3 acetylation can only be observed in a single sample from a clr6-1 strain that was arrested by hydroxyurea (Fig. 1F, lane 5). To unequivocally demonstrate that Psm3 remains acetylated after exit from mitosis in clr6 mutants, it will be necessary to monitor Psm3 acetylation levels in a synchronized cell cycle similar to the experiment shown Supplementary Fig. 1B. Maybe it would be possible to synchronize the clr6-1 mutant by elutriation or by releasing cells from an HU arrest? The authors might also want to include the delta-hos2 strain in such a time course experiment to rule out that the Hos2 deacetylase may have a (minor) effect on the Psm3 acetylation status.

We address this comment in comment 5 by referee #1.

2. The paper could be strengthened if the authors could demonstrate that Psm3 is in fact acetylated during meiosis. Would it be possible to obtain sufficient material at different time points after cells are synchronously released into meiosis (e.g. using the pat1-114 mutant, see Fig. 3A and C) for detection of Psm3 acetylation by Western blotting? Since sister centromere co-orientation fails in the eso1-H17 mutant already at 26{degree sign}C (the permissive temperature for mitotic growth of this mutant), one would expect to see a strong reduction of meiotic Psm3 acetylation already at this temperature. Is this the case?

We performed this experiment (new Fig 2A). Yes, it is the case.

It would be even more informative if the authors could immunoprecipitate Rec8-containing cohesin (e.g. the cohesin at core centromeres) to test whether there is a preferential acetylation of Psm3 associated with Rec8 compared to Psm3 associated with Rad21. Since Psm3 acetylation is apparently essential for the centromeric cohesin, one might expect to detect higher levels of acetylation in the Rec8 containing complexes.

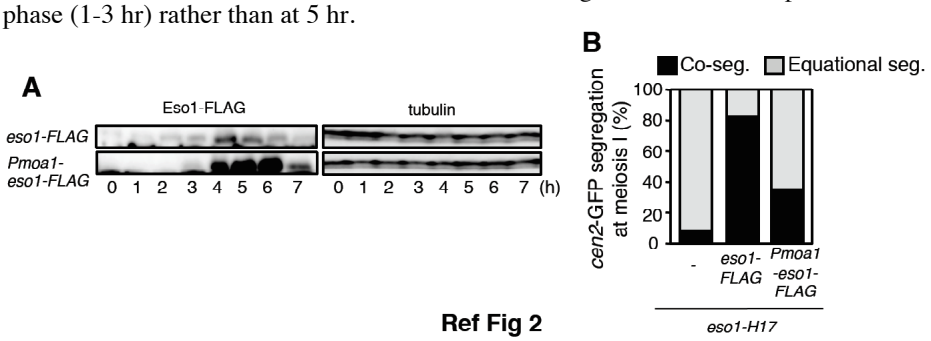
Because Rad21 is largely excluded from chromatin by the presence of Rec8 in meiosis (Yokobayashi et al. Mol Cell Biol 2003), we think that the proposed experiment would not allow any firm conclusion even if the result is as expected.

3. Even though genetic evidence suggests that cohesion is impaired only at core centromeres and not at pericentomeric regions or chromosome arm regions in eso1-H17 or psm3-KKRR cells (otherwise one would expect random and not equational segregation in the first division, Fig. 2A), it would be important to test this directly. The authors could for example score cohesion at the cut3 locus as in Fig. 1C, or, more elegantly, loop out a pericentromeric region as they did in a recent paper (imr1-GFP, Sakuno et al., Nature 2009).

We performed the suggested experiments (new Fig 2D), and describe the results in the text (p 3, line 32). Please note that looping out a pericentromeric region (a more elegant experiment) is technically very difficult during meiosis I unlike during mitosis. We do not know the reason but guess that the stiffness of chromatin around the centromeres might be different.

4. The authors claim that Eso1 expression during pre-meiotic G1-S phase is eliminated by placing the eso1 gene under the control of the moa1 promoter. Since the Eso1 levels in Pmoa1-eso1 cells never reach the Eso1 levels in Peso1-eso1 cells (compare 5 h samples in Fig. 3A), it is equally well possible that the segregation defect may be caused by insufficient Eso1 protein levels during metaphase or anaphase and not by an absence of Eso1 expression during pre-meiotic S-phase. Still, about half of the cells still co-segregate chromosomes in the first division, even when Eso1 expression is delayed/reduced. It is therefore difficult to conclude from these experiments that Eso1 function is required during pre-meiotic S phase. Would the results be clearer if eso1 gene were expressed under control of the spo6 promoter, as done for the moa1 gene in the next experiment?

In *Peso1-eso1-FLAG* and *Pmoa1-eso1-FLAG* cells used in Fig 3A, *eso1-FLAG* is expressed from an ectopic chromosome locus because the endogenous locus must be *eso1-H17*. In the same experiment, we examined *eso1-FLAG* cells, in which endogenous *eso1*$^{+}$ is tagged with FLAG. As shown below (Ref Fig 2), *eso1-FLAG* cells express much less Eso1 at 5 hr than *Pmoa1-eso1-FLAG* cells do, while mono-orientation is higher. Thus, the minimum requirement of Eso1 expression for mono-orientation is relatively low as seen in *eso1-FLAG* cells. These results clearly indicate that the defect in *Pmoa1-eso1-FLAG* cells originates form the expression level during S phase (1-3 hr) rather than at 5 hr.



Ref Fig 2

5. If Psm3 acetylation were sufficient to restore centromeric cohesion, this should be clearly detectable in the centromere loop-out assay. The eso1-H17 psm3-KKQQ strain should therefore be included in Fig. 4B.

We included this experiment in Fig 4B.

Minor points:

1. I think that it would be fair to downscale what sounds a bit like a priority claim in the last section of the introduction ("Here we establish the regulation of Psm acetylation in fission yeast") and also reference the Feytout et al. publication more appropriately than just in a note at the end of the paragraph.

We rewrote the introduction substantially to cite data recapitulating the Feytout et al. publication, and these data, which were described in the first section of the last manuscript, are now moved to Supplementary Fig S1.

2. Fig. 1F. It is not clear at which temperature the strains were grown in this experiment. It is mentioned in the methods sections that strains were grown at 30{degree sign}C, but given that the clr6-1 mutant doesn't show much of a phenotype at this temperature (Fig. 1G), shouldn't have the samples (from all strains) been taken instead at 37{degree sign}C?

At 37°C, HU arrest does not work well in *clr6-1* cells presumably because *clr6-1* cells have problems in other cell cycle stages (Grewal et al. Genetics 1998).

3. In the last sentence of the section on the Clr6 deacetylase, the authors base the conclusion that Psm3 acetylation only plays a minor role in counteracting the function of Eso1 in cohesion establishment during mitosis on the finding that the clr6-1 mutation does not suppress eso1-H17 mutation. However, Clr6 may have an essential function that is completely different from Psm3 deacetylation (or even regulating sister chromatid cohesion). In my opinion this conclusion cannot be drawn so easily from a genetic experiment.

We have taken this suggestion. We now describe that 'although the *clr6-1* mutation did not suppress the growth defect in *eso1-H17* cells (Supplementary Fig S1F), this might be tenable because *clr6-1* itself causes growth defect in a different context from Psm3 deacetylation. (p 3, line 3)'

4. Did the authors consider that the unknown Eso1 acetylation target may not necessarily be a yet unknown factor X but potentially another lysine residue on Psm3?

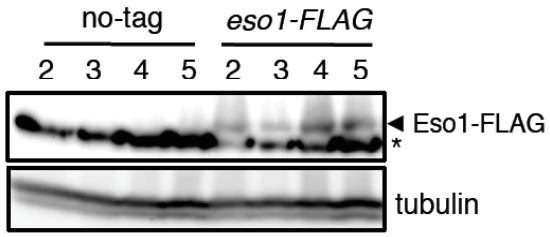
We have taken this suggestion and describe that 'this second acetylation target might be another site(s) of Psm3 or completely distinct protein (p2, line 26)'.

5. Since the conditions vary between the different experiments, it will be necessary to describe them more carefully in the figure legends. For example, it is essential that the authors state at which temperature the samples were taken in each experiment, given that they work with different ts strains (e.g. at which point was the temperature shifted back for the pat1-114 release, and to which temperature were the strains shifted?). There are also discrepancies between the conditions shown in the figure and listed in the figure legends (e.g. Fig. 1B, 32{degree sign}C in the figure but 30{degree sign}C in the figure legend).

We have taken this suggestion. We corrected the legend to Fig 1B (new Supplementary Fig S1).

6. Fig. 3A. A band running in close proximity to Eso1-FLAG is labelled as "unspecific band", yet the intensities of this band increase and decrease with the Eso1-FLAG intensities. Why is this the case? The authors need to show that this band is unspecific in a control lane (e.g. extract from a strain that doesn't express at FLAG tagged protein).

As shown below (Ref Fig 3), this band is indeed an unspecific band.



Ref Fig 3

7. Fig. 4. It is not immediately obvious why the experiment with the eso1 ts mutants was performed at 26{degree sign}C, while the experiment with the delta-moa1 strain was performed at 30{degree sign}C. Wouldn't it make more sense to also perform the experiment with the eso1 ts mutation at 30{degree sign}C to ensure that the protein is really inactivated?

Please note that even at 26˚C, *eso1-H17* is sufficiently inactive since most cells show equational segregation. We intended here that *eso1-H17* can be suppressed by *clr6-1*.

8. Supplementary Fig. 1B. It is not clear why the authors used in this experiment the antibody raised against the acetylated lysine residues in S. cerevisiae Smc3 and not the specific antibody they generated against acetylated Psm3, which appears work rather nicely (see Fig. 1A).

We replaced the data with those using anti-Psm3.

Referee#3:

1. The interactions between eso1 and clr6 and between moa1 and clr6 are intriguing but too preliminary. The simplest conclusion is that in clr6 mutants there is more cohesion in general and this rescues monoorientation. However, it does not offer any new mechanistic insight. Therefore in its present form it lacks significant new insight.

Our genetic evidences sufficiently support that Clr6 antagonizes Eso1 function in core centromere cohesion and Psc3 acetylation. We now show new evidence that Clr6 acts as a deacetylase of Psm3 (Fig 1C). We admit that the link between Moa1 and Cl6 is relatively week, although our current data reveal that Moa1 is required for maintaining cohesion after S phase rather than establishing it during S phase, a process requiring Eso1 (Fig 5).

2. In addition, error bars are missing from graphs throughout this manuscript and the number of cells scored is very low in many cases (e.g. S2B, n>20; 4B and D, n>79). Considering the small changes presented a much greater statistical analysis is required.

We confirmed that all our data are statistically significant. In Figure S2B (new Supplementary Fig S1E), *psm3-KKRR* cells shows a significant cohesion defect if compared with either *psm3*$^{+}$ or *psm3-KKQQ* cells ($p < 0.001$; unpaired two-tailed *t*-tests). Moreover, the suppression of *eso1-H17* or *moa1Δ* by *clr6-1* in Fig 4B, D is also significant ($p < 0.01$; Chi-squared tests).

3. Determination of cohesin Rec8/Rad21 association with core centromeres, pericentromeres and chromosome arms in eco1 and non-acetylatable Psm3 mutants by ChIP.

We address this comment in comment 1 by referee#1.

4. Cohesion assays (loop out experiments) at pericentromeres, chromosomes arms in the above mutants.

We address this comment in comment 3 by referee#2.

5. Examination of cohesin acetylation during meiosis. Also ChIP assay to identify domains in which cohesin is acetylated.

We tried to see cohesin acetylation by ChIP assay but found that our anti-AcPsm3 antibodies do not work for ChIP. However, we now show cohesin acetylation during meiosis, which depends on Eso1 (new Fig 2A).

6. Clear evidence that Clr6 is the deacetylase (as pointed out by both reviewers 1 and 2).

We now demonstrate that over-expression of Clr6 impairs Psm3 acetylation as well as sister chromatid cohesion in G2 arrested cells (Fig 1C), thus providing a strong evidence that Clr6 acts as a deacetylase of Psm3.

Finally, as suggested by the editor, we moved the first part recapitulating the published results to Supplementary information (Supplementary Fig S1).

We hope that these changes are satisfactory and that the revised manuscript is now acceptable.

2nd Editorial Decision 04 July 2011

Please accept my apologies for the time it has taken me to contact you with a decision on your revised manuscript. I sent your study to referees 1 and 2, and referee 2 had some unforeseen problems that led to his/her submitting the report just this weekend. As you will see, both consider the study much improved and are very supportive of publication. However, both have asked me to give you an extraordinary chance to further revise your study, as some issues are still pending and both feel that addressing them would considerably strengthen the study (please find their reports below). After these issues are clarified, I will be happy to accept your study for publication.

As you will see, several of their concerns can be dealt with by modifying the text. However, referee 1's point 2 and referee 2's point 1, regarding the demonstration of Crl6 as the Psm3 deacetylase, are central to the message of the study and I believe should be addressed experimentally. On the other hand, referee 1's point 3, although clearly of interest, I feel is further-reaching and would not be absolutely required for publication. Please also respond to referee 2's concern regarding the variability of the experiment that places the eso1 gene under the control of the moa1 promoter.

I look forward to receiving a final version of your study when it is ready.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

The revised version of the manuscript has taken into consideration all the points I raised following the inital submission. This is a very nice work that clearly demonstrate the importance of Psm3 acetylation for core-centromere cohesion and reductional chromosome segregation during meiosis I. The manuscript also uncovers a complex relationship between acetylation, deacetylation and the monopolin MoaI. Although the molecular mechamisms remain to be clarified, the data presented deepen our understanding and pave the way for future studies. The new data in the revised version raise some additional comments or suggestions which could be addressed to further improve the manuscript:

I- Page 3. Figure 1C presents new data in which the Clr6 deacetylase is overexpressed in the vegetative cell cycle. The authors found that Psm3 K106 acetylation is reduced, consistent with the notion that Clr6 antagonizes the Eso1 acetyl-transferase. The authors also observed an elevated rate of sister-centromere separation and they conclude that "Clr6 has the potential to antagonize Eso1 by removing acetylation even in G2". I disagree with this statement. Clr6 over-expression was driven by the nmt promoter. Cells were cultured for 24 hours wihout thiamine to induce Clr6 overexpression and then shifted to 36{degree sign}C for 4 hours to arrest cells in G2 by the cdc25-22 mutation. It is very likely that cells overexpressed Clr6 before the G2 arrest. Hence Clr6 may have antagonized Psm3 acetylation already during S phase, leading to the observed elevated rate of sister-centromere separation in the following G2 arrest. I suggest the authors modify this sentence and conclude simply that this experiment agrees with the idea that Clr6 de-acetylates Psm3. Also, since Psm3 acetylation was monitored on K106 only, it would be more rigorous to say that Clr6 is the Psm3K106 de-acetylase (rather than Psm3-K105-K106 as seen page 2).

II-Page 4: The Clr6 deacetylase antagonizes Eso1-dependent acetylation of Psm3 and mono-orientation
I'm not sure of this conclusion because the authors mention in the rebuttal letter that Psm3 acetylation remains undetectable in a eso1-H17 clr6-1 strain. It is thus possible that the partial suppression of the mono-orientation defect of eso1-H17 by clr6-1 does not transit through increased Psm3 K106 acetylation. One way to address this question would be to look at mono-orientation in eso1-H17 clr6-1 versus eso1-H17 clr6 psm3-KKRR. If indeed clr6-1 restores mono-orientation through increased Psm3 acetylation, then the suppressing effect of clr6-1 should not be observed in a psm3-KKRR background.

III-The relationship between Moa1, Eso1 and Clr6.
The authors show that clr6-1 partially suppresses the moa1D mono-orientation defect independently of the acetylation status of Psm3, suggesting that "an acetylation target distinct from Psm3K105K106 contributes to cohesion at the central core domain" (page 4). The next sentence suggests that this other acetylation may be performed by Eso1 since mono-orientation is not fully restored in a eso1 deleted strain expressing psm3-KKQQ. This indeed suggests that Eso1 may have another target or function. Whether this putative other Eso1 substrate is the one targeted by Clr6 can only be speculated (Clr6 may de-acetylate a non-Eso1 substrate). One way to address this question would be to compare mono-orientation frequency in eso1+ psm3-KKQQ moa1D clr6-1 versus eso1D psm3-KKQQ moa1D clr6-1. In the eso1+ background, clr6-1 partially restores mono-orientation (Fig. S5). If mono-orientation is similarly restored in a eso1D background, this would strongly suggest that Clr6 acts on a non-Eso1 substrate. Reciprocally, if mono-orientation is not restored at all, this would suggest that Clr6 counteracts the acetylation of an Eso1 target.

IV- Fig. S4: the legend is not correct (Cnd2-HA). Error bars are not defined in the legend.

Referee #2:

Watanabe and colleagues have significantly re-written their manuscript and have added a number of additional experiments that address many of the points raised by the reviewers. In my view, the new focus on the role of cohesin acetylation during meiosis now clearly distinguishes the work from

previous publications. I also find the new manuscript well structured and concise, and therefore - in principle - recommend its publication in EMBO reports. However, I would like to invite the authors to address the following two remaining concerns before publication.

1) The first concern relates to the role of Clr6 as the Psm3 deacetylase. While the genetic proof for a link between Clr6 and Psm3 was quite obvious from the initial data, all three reviewers requested additional biochemical evidence for deacetylation of Psm3 by Clr6 (reviewer 1, point 5; reviewer 2, point 1; reviewer 3, point 1). The authors have tried to address this as follows:

- In Fig. 1B, the authors now show FACS profiles of the HU-arrested strains. It seems that the clr6-1 mutant has further progressed into S-phase compared to the control strains (compare the width of the peaks). Given that the authors have difficulties with arresting and releasing the clr6-1 strain with HU (see reply to reviewer 1 point 5 and reviewer 2 point 1), the concern that a clr6-1 mutant shows more Psm3 acetylation because it hasn't properly arrested (reviewer 1) is therefore a valid one and needs to be ruled out.

- The authors now added new data showing decreased Psm3 acetylation after Clr6 overexpression (Fig. 1C). In this experiment, the authors induce Clr6 overexpression for 24 hours before shifting the strain for the cdc25 arrest. Given that Clr6 has roles at other cell cycle stages (see reply to reviewer 2, minor point 2), it is equally well possible that the observed reduction in Psm3 acetylation and the slight increase in centromere splitting are due to indirect effects. A better experiment might be to arrest the strains first in G2 phase and then induce Clr6 overexpression (if shorter overexpression times are possible; the authors should also monitor Clr6 levels in this experiment).

In my view, the current data fail to unambiguously show that Clr6 is the enzyme that deacetylates Psm3, and I encourage the authors to add further proof for this claim. If it is not feasible to perform the requested experiments with the clr6-1 mutant, maybe it is possible to generate a degron version of Clr6?

2) The second concern relates to the timing of Psm3 acetylation by Eso1 during meiosis. The authors now demonstrate Psm3 acetylation during meiosis using western blotting in Fig. 2A. This is an important addition to the paper, one that was obviously lacking in the previous version. It now seems that AcPsm3 is only detectable 3-4 h after the start of the experiment, e.g. after premeiotic S phase is largely complete (see FACS profile). Yet the authors write once that cohesin is acetylated "during S phase (Fig. 2A)" (pg. 3, second paragraph), once that "acetylation of cohesin is detected during prophase I (Fig. 2A ..." (pg. 4, first paragraph), and once again that "acetylation ... occurs mainly during S phase (Fig. 2A)" (pg. 4, first paragraph). Maybe at least a more accurate description (e.g. that cohesin acetylation is detectable at late S phase and persists until prophase) would be required.

I'm also still worried about the experiment that tries to delay expression of Eso1 by placing the eso1 gene under control of the moa1 promoter (Fig. 3A). In Ref. Fig. 2, the authors claim that Eso1 levels after expression from the moa1 promoter are higher than expression levels of Eso1 from its endogenous promoter. The authors cannot compare the intensities on two different western blots! In the same western blot, Eso1 is only detectable at the 4 h time point or later, e.g. again after S phase has long been completed. In the identical experiment shown in Fig. 3A, Eso1 can already be detected at the 3 h time point. It therefore seems to me that there is too much variation in this experiment to make any firm conclusions about the timing of Eso1 requirement.

Minor comments:

The legend to Supplementary Figure S4 lacks a description of the error bars (standard deviation?). Also the number of experimental repeats (immunoprecipitations and qPCR reactions) should be mentioned in the figure legend.

I suggest rephrasing the last subtitle ("A model of Moa1-dependent ..."), since the reader would expect a discussion section and not another results section following this title.

2nd Revision - authors' response 13 August 2011

To the referees,

We thank the referees for supporting publication of our study and for their valuable comments. To address the referees' comments, we have carried out new experiments and incorporated the results in the revised manuscript. We addressed all comments raised by the referees and our responses are listed below.

(Bold letters are referees' comments)

Referee #1:

1) Page 3. Figure 1C presents new data in which the Clr6 deacetylase is overexpressed in the vegetative cell cycle. The authors found that Psm3 K106 acetylation is reduced, consistent with the notion that Clr6 antagonizes the Eso1 acetyl-transferase. The authors also observed an elevated rate of sister-centromere separation and they conclude that "Clr6 has the potential to antagonize Eso1 by removing acetylation even in G2". I disagree with this statement. Clr6 over-expression was driven by the nmt promoter. Cells were cultured for 24 hours wihout thiamine to induce Clr6 overexpression and then shifted to 36{degree sign}C for 4 hours to arrest cells in G2 by the cdc25-22 mutation. It is very likely that cells overexpressed Clr6 before the G2 arrest. Hence Clr6 may have antagonized Psm3 acetylation already during S phase, leading to the observed elevated rate of sister-centromere separation in the following G2 arrest. I suggest the authors modify this sentence and conclude simply that this experiment agrees with the idea that Clr6 de-acetylates Psm3. Also, since Psm3 acetylation was monitored on K106 only, it would be more rigorous to say that Clr6 is the Psm3K106 de-acetylase (rather than Psm3-K105-K106 as seen page 2).

We corrected the sentences describing the Figure 1C experiments as the referee suggested. Moreover, we changed the subtitle to 'Clr6 is the deacetylase of Psm3-K106 acetylation' (p2).

2) Page 4: The Clr6 deacetylase antagonizes Eso1-dependent acetylation of Psm3 and mono-orientation. I'm not sure of this conclusion because the authors mention in the rebuttal letter that Psm3 acetylation remains undetectable in a eso1-H17 clr6-1 strain. It is thus possible that the partial suppression of the mono-orientation defect of eso1-H17 by clr6-1 does not transit through increased Psm3 K106 acetylation. One way to address this question would be to look at mono-orientation in eso1-H17 clr6-1 versus eso1-H17 clr6 psm3-KKRR. If indeed clr6-1 restores mono-orientation through increased Psm3 acetylation, then the suppressing effect of clr6-1 should not be observed in a psm3-KKRR background.

We performed the suggested experiments. Consequently, *clr6-1* suppresses *eso1-H17* even in *psm3-KKRR* cells albeit less efficiently than in *psm3*$^{+}$ cells (new Fig 4A). This result indicate that Clr6 antagonizes Eso1 function in establishing mono-orientation by deacetylating not only Psm3 but also an unknown Eso1 target, consistent with previous result that *eso1*Δ is not completely suppressed by *psm3-KKQQ* (Supplementary Fig S7). We described these results in the text (p4, last 3 sentences).

3) The relationship between Moa1, Eso1 and Clr6. The authors show that clr6-1 partially suppresses the moa1D mono-orientation defect independently of the acetylation status of Psm3, suggesting that "an acetylation target distinct from Psm3K105K106 contributes to cohesion at the central core domain" (page 4). The next sentence suggests that this other acetylation may be performed by Eso1 since mono-orientation is not fully restored in a eso1 deleted strain expressing psm3-KKQQ. This indeed suggests that Eso1 may have another target or function. Whether this putative other Eso1 substrate is the one targeted by Clr6 can only be speculated (Clr6 may de-acetylate a non-Eso1 substrate). One way to address this question would be to compare mono-orientation frequency in eso1+ psm3-KKQQ moa1D clr6-1 versus eso1D psm3-KKQQ moa1D clr6-1. In the eso1+ background, clr6-1 partially restores mono-orientation (Fig. S5). If mono-orientation is similarly restored in a eso1D background, this would strongly suggest that Clr6 acts on a non-Eso1 substrate. Reciprocally, if mono-

orientation is not restored at all, this would suggest that Clr6 counteracts the acetylation of an Eso1 target.

We performed the suggested experiment, although this is not always requested in editorial comment. In fact, *clr6-1* did not restore mono-orientation in *eso1*Δ *psm3-KKQQ moa1*Δ cells, while it suppressed *eso1*$^{+}$ *psm3-KKQQ moa1*Δ cells. This result suggests that the acetylation of the non-Psm3-K105/K106 substrate counteracted by *clr6-1* in the *moa1*Δ background is executed also by Eso1. This implication is consistent with the schematic model depicted in Figure 5 and now mentioned in the text (p, line). Because this and other results strengthen the notion that Eso1 and Clr6 share the non-Psm3 substrate (X'), we made clear this point in Fig 5 by changing the grey arrow (lined from Eso1 to X') into black one.

4) Fig. S4: the legend is not correct (Cnd2-HA). Error bars are not defined in the legend.

We corrected the legend.

Referee #2:

1) In Fig. 1B, the authors now show FACS profiles of the HU-arrested strains. It seems that the clr6-1 mutant has further progressed into S-phase compared to the control strains (compare the width of the peaks). Given that the authors have difficulties with arresting and releasing the clr6-1 strain with HU (see reply to reviewer 1 point 5 and reviewer 2 point 1), the concern that a clr6-1 mutant shows more Psm3 acetylation because it hasn't properly arrested (reviewer 1) is therefore a valid one and needs to be ruled out.

We think that the slight broadness of the G1 peak in HU-arrested *clr6-1* cells cannot account for the obviously elevated acetylation at G1/S in this mutant.

2) The authors now added new data showing decreased Psm3 acetylation after Clr6 overexpression (Fig. 1C). In this experiment, the authors induce Clr6 overexpression for 24 hours before shifting the strain for the cdc25 arrest. Given that Clr6 has roles at other cell cycle stages (see reply to reviewer 2, minor point 2), it is equally well possible that the observed reduction in Psm3 acetylation and the slight increase in centromere splitting are due to indirect effects. A better experiment might be to arrest the strains first in G2 phase and then induce Clr6 overexpression (if shorter overexpression times are possible; the authors should also monitor Clr6 levels in this experiment).

In the Fig 1C experiment, we intended to arrest cells at G2 phase simply in order to monitor the cohesion defect at this cell cycle stage. We believe that the previous result in Figure 1C can itself lead to a firm conclusion that the overexpression of Clr6 decreases the acetylation of Psm3 and impairs sister chromatid cohesion, as indicated by referee 1. Therefore, this issue would be resolved only by rephrasing the text (see comment 1 by referee 1). Nevertheless, we performed the requested experiment by transiently expressing Clr6 only after G2 arrest. We obtained consistent results, although the deacetylation of Psm3 and cohesion defect is less impressive when compared with the previous result (new Supplementary Figure 3). Overall, these results support the notion that Clr6 counteract Eso1-dependent acetylation of Psm3 *in vivo.*

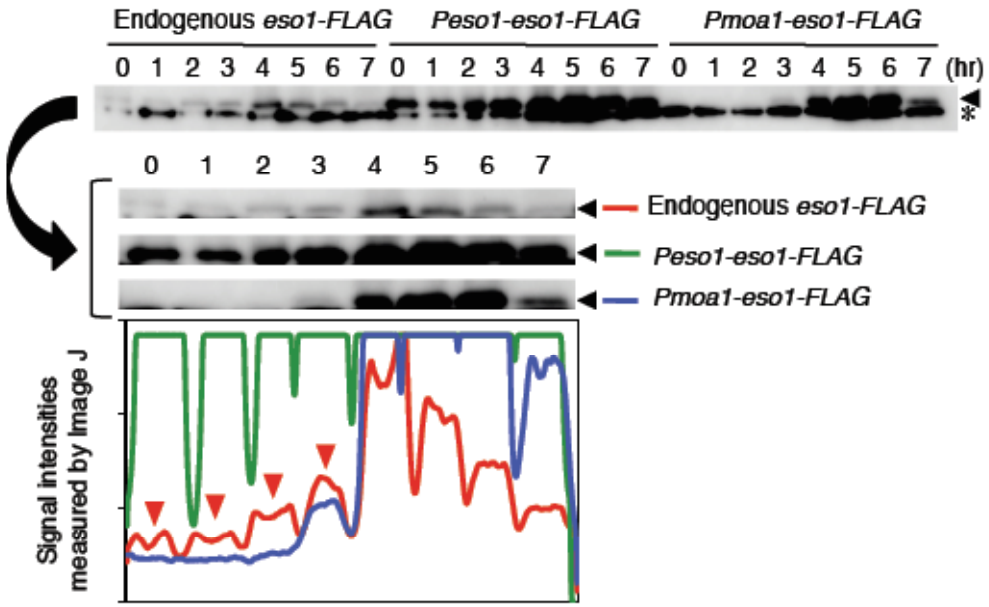
3) The second concern relates to the timing of Psm3 acetylation by Eso1 during meiosis. The authors now demonstrate Psm3 acetylation during meiosis using western blotting in Fig. 2A. This is an important addition to the paper, one that was obviously lacking in the previous version. It now seems that AcPsm3 is only detectable 3-4 h after the start of the experiment, e.g. after premeiotic S phase is largely complete (see FACS profile). Yet the authors write once that cohesin is acetylated "during S phase (Fig. 2A)" (pg. 3, second paragraph), once that "acetylation of cohesin is detected during prophase I (Fig. 2A ..." (pg. 4, first paragraph), and once again that "acetylation ... occurs mainly during S phase (Fig. 2A)" (pg. 4, first paragraph). Maybe at least a more accurate description (e.g. that cohesin acetylation is detectable at late S phase and persists until prophase) would be required.

We accept the referee's suggestion and have corrected the corresponding texts.

4) I'm also still worried about the experiment that tries to delay expression of Eso1 by placing the eso1 gene under control of the moa1 promoter (Fig. 3A). In Ref. Fig. 2, the authors claim that Eso1 levels after expression from the moa1 promoter are higher than expression levels of Eso1 from its endogenous promoter. The authors cannot compare the intensities on two

different western blots! In the same western blot, Eso1 is only detectable at the 4 h time point or later, e.g. again after S phase has long been completed. In the identical experiment shown in Fig. 3A, Eso1 can already be detected at the 3 h time point. It therefore seems to me that there is too much variation in this experiment to make any firm conclusions about the timing of Eso1 requirement.

In contrast to the referee's misgiving, we indeed compared the intensities of Eso1 signals in the same western blot (see the following figure). Moreover, we now show the precise quantification of the Eso1 bands. The data clearly indicate that endogenous Eso1 is detectable throughout prophase and that Eso1 expressed by the *moa1* promoter is hardly expressed before 3 hr but quite abundantly expressed after 4 hr.



Note that the expression level of endogenous Eso1 is higher than that of *Pmoa1* driven Eso1 between 0 – 3 hr, while Pmoa1 driven Eso1 much exceed after 4 hr.

5) Minor comments:
The legend to Supplementary Figure S4 lacks a description of the error bars (standard deviation?). Also the number of experimental repeats (immunoprecipitations and qPCR reactions) should be mentioned in the figure legend. I suggest rephrasing the last subtitle ("A model of Moa1-dependent ..."), since the reader would expect a discussion section and not another results section following this title.

We corrected these errors and rephrased the last subtitle as 'Acetylation is involved in the regulation of Moa1-dependent mono-orientation'.

We hope that these changes are satisfactory and that the revised manuscript is now acceptable.

3rd Editorial Decision 26 August 2011

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Editor
EMBO Reports

