THE EMBO JOURNAL

Manuscript EMBO-2013-85356

# Pds5B is required for cohesion establishment and Aurora B accumulation at centromeres

María Carretero, Miguel Ruiz-Torres, Miriam Rodríguez-Corsino, Isabel Barthelemy, Ana Losada

*Corresponding author: Ana Losada, Spanish National Cancer Research Centre CNIO*

**Review timeline:**

| | |
|---|---|
| Submission date: | 15 April 2013 |
| Editorial Decision: | 10 May 2013 |
| Revision received: | 17 September 2013 |
| Accepted: | 02 October 2013 |

*Editor: Hartmut Vodermaier*

## Transaction Report:

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

1st Editorial Decision 10 May 2013

Thank you again for submitting your manuscript on Pds5A/B roles in mammals for consideration by The EMBO Journal. We have now received the comments from three expert referees, copied below for your information. As you will see, the referees consider this work important in principle, but they also raise a considerable number of substantial, overlapping concerns with the analyses and their interpretations at the present stage. Nevertheless, all three reviewers feel that you should be given the opportunity to clarify these issues and respond to their criticisms by way of a revised manuscript. I would therefore like to invite you to prepare a revision of this study along the lines suggested by the referees, reminding you that our policy to allow only a single round of major revision makes it important that you carefully and comprehensively respond to all points raised at this stage. When preparing your letter of response to the referees' comments, please also remember that this will form part of the Review Process File, and will therefore be available online to the community.

We generally allow three months as standard revision time, and it is our policy that competing manuscripts published during this period will have no negative impact on our final assessment of your revised study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider this work, and please do not hesitate to contact me in case you should have any additional question regarding this decision or the reports. I look forward to your revision.

 

REFEREE REPORTS:

Referee #1:

Sister chromatid cohesion is mediated by the cohesin complex through entrapping sister DNAs inside the tripartite Smc3-Rad21-Smc1 ring. Cohesin has essential roles in chromosome segregation, DNA repair and transcriptional regulation. A number of proteins binds to cohesin and modulate its dynamic association with chromosomes. Wapl promotes cohesin (and cohesion) release presumably by weakening the Smc3-Rad21 interface. Cohesin-acetyl transferases (coATs) counteract Wapl during S phase and thereby promote cohesion by stabilizing cohesin interaction with chromosomes. The role of Pds5 proteins is still unclear. Pds5 forms a sub-complex with Wapl and is required for cohesin release in xenopus. In budding yeast, Pds5 appears essential for cohesion establishment and maintenance while in fission yeast, it is essential for Smc3 acetylation and stable cohesin-DNA interaction in G2. In vertebrates Pds5 may promote a stable cohesin-chromatin interaction by binding Sororin which may displace Wapl from Pds5-bound cohesin. The situation is further complicated in vertebrates by the existence of two versions of Pds5 (Pds5A and B), Scc3 (SA1, SA2) and two coATs, Esco1, Esco2.
This study addresses the functions of Pds5 proteins in the mouse. The authors generated mice KO for either Pds5A or Pds5B. As previously reported, Pds5A-/- or Pds5B-/- die perinatally with multiple organ malformations - emphasising Pds5 roles in regulating gene expression during development- but contrary to the previous studies, they discovered that Pds5 proteins have essential roles in sister-chromatid cohesion and chromosome segregation. By using MEFs from either Pds5A-/- or Pds5B-/- mice, they show that lack of a single Pds5 protein leads to a partial reduction in acetylated Smc3. When Pds5A was reduced by RNAi in Pds5B -/- MEFs, Smc3 acetylation and Sororin binding to chromatin were strongly reduced indicating that Pds5 proteins are required for Smc3 acetylation and Sororin recruitment. They clearly show that lack of Pds5B alone impaired Esco2 and Sororin recruitment to pericentromeric heterochromatin and caused cohesion defects at centromeres. Further, they provide strong evidence that Pds5B is required for Haspin-mediated phosphorylation of Histone 3 and Aurora B localization at the inner centromere. Consistently, they found that MEFs from Psd5B null embryos displayed a range of mitotic defects and an increased incidence of aneuploid cells.

This study is an important step towards understanding cohesin regulation and the consequences of cohesin dysfunction in metazoan. Overall the work is well conducted and good quality data support the major conclusions drawn by the authors. I have however specific concerns about some parts of the study, as detailed below.

Major points:

1- Effect of Pds5A and Pds5B depletion on the amount of chromatin-bound cohesin in interphase and prophase cells (Figure 2).
The authors address this question by treating Pds5A -/- MEFs with Pds5B siRNA and reciprocally, Pds5B-/- cells with Pds5A siRNA. Only a moderate increase in chromatin-bound Smc3 was observed in interphase cells when Pds5 proteins are co-depleted (Fig2BC) and no effect was seen on prophase cells (Fig2D). However, Western blot analyses (Fig.S3) revealed that Pds5A and Pds5B are not depleted by siRNA treatment in this particular experiment. The authors mention that the increase in chromatin-bound cohesin in interphase is consistent with a reduction in the presence of Wapl on chromatin as can be seen in Fig. 3B. This was however a WB from a distinct experiment in which the RNAi treatment appeared more efficient than in the Fig2 experiment (compare Fig.3B and Fig.S3).
Therefore, the modest effect seen on interphase cells (Fig2BC) and the lack of an effect in prophase (Fig2D) may well be a consequence of incomplete depletion and the conclusion that Pds5 proteins are not required for the efficient release of cohesin in prophase may not be valid.
The authors should either tune down their conclusions or repeat the experiment using conditions that give an apparent complete depletion of Pds5 proteins. It would be helpful to include a Wapl RNAi control as a reference for reduced unloading.

2- Both Pds5A and Pds5B contribute to telomere and arm cohesion in mouse cells (page 8 and

 

Fig.4).
I found this paragraph rather unclear. The authors used indirect assays to assess cohesion defects at telomeres and chromosomes arm sites. For telomeres, the rationale is that a cohesion defect should impair telomere replication which should increase the frequency of irregularly shaped telomeric FISH signals on metaphase plates. Using this assay, Pds5A-/- and Pds5B-/- MEFs behaved as the wild-type control (Fig. 4A). Surprisingly, they conclude that Pds5A and Pds5B contribute to telomere cohesion. I do not understand.
In Figure 4B, they treated cells with low dose of aphidicolin during a round of S phase and scored breaks on chromosome arms at metaphase, the rationale being that cohesin is required for homologous recombination mediated replication fork restart after aphidicolin induced replication fork stalling. It's a rather indirect assay. Pds5 proteins may well be involved in replication fork restart independently of their role in sister-chromatid cohesion. In addition, it is unclear to me how this experiment can be performed: cells were treated with aphidicolin for 24 hours so that a fraction of cells may replicate in the presence of the drug during this time frame. However, Pds5B-/- hardly proliferate in culture (~1 doubling in 6 days, Figure 1D). The authors should use a straightforward FISH assay on G2 cells to probe subtelomeric and arm cohesion as they did in a previous study (Remeseiro et al., 2012a).

Minor points:

Figure 3C. The error bars should be defined

Page 8 top. "excess cohesin is acetylated by coATS during DNA replication relative to the acetylated complexes that are subsequently bound by Sororin". I don't think there is any good evidence that Sororin binds acetylated Smc3. The Nishiyama (Cell 143, 737-749, November 24, 2010) clearly shows that Sororin binding to chromatin is Esco1-2 dependent but no clear evidence that this is mediated by Smc3 acetylation.

Figure 4C. The legend is not clear. From the text (page 8, last lane), I understand that a metaphase was scored as "defective for centromeric cohesion" when more than 5 chromosomes displayed clearly separated centromeres. From the figure legend, "metaphase with single chromatids were excluded". The criteria should be clarified and moved in the figure legend.

The discussion recalls that the Zhang studies did not report any cohesion defect in Pds5 KO mice whereas the present work did find cohesion phenotypes. I believe the reader would expect the authors to discuss this point: why this difference?

Referee #2:

Chromosomes are held together and structurally organized by cohesin, which is in turn regulated by binding partners such as Pds5, Wapl, and sororin. Mice contain two Pds5-related genes, for which gene trap (Pds5a) or knockout (Pds5b) alleles were previously reported by Millbrandt and colleagues (Zhang et al. 2007 and Zhang et al. 2009). Surprisingly, those reports found no evidence of cohesion defects in either mutant, although developmental anomalies reminiscent of the 'cohesinopathy' Cornelia de Lange Syndrome (CdLS) were observed.

Here Carretero et al. have made independent Pds5a and Pds5b conditional knockout mice. They report quite different results, in that both Pds5a and Pds5b are embryonic lethal and important for proliferation and cohesion (to varying extents) at the cellular level. Focusing mainly on Pds5b, they show that this isoform affects levels of acetylated Smc3, sororin, and Wapl on chromatin, the localization of Esco2 (one of two Smc3 acetyltransferases in vertebrates) at pericentric heterochromatin (PCH) in late S phase cells, and the targeting of the Aurora B chromosome passenger kinase to centromeres during mitosis. Based on these data they argue that Pds5b controls cohesion establishment and maintenance specifically around centromeres, and via Aurora B regulation, contributes to error correction and enforcement of the spindle assembly checkpoint.

The mechanism of sister chromatid cohesion is intensively studied but still incompletely understood. The present study adds new and potentially interesting information on the effects of inactivating Pds5-related proteins in mouse cells. However, some analyses do not go far enough to draw

 

mechanistic insights, apart from corroborating ideas from other model organisms. These ambiguities arise in part from the fact that Pds5 has complex positive and negative effects on cohesin regulation, which are all superimposed on on another here. As a result I find some of the authors' conclusions to be preliminary and/or debatable based on the present dataset.

Specific points:

1. The authors reference the Zhang et al (2007, 2009) studies without addressing why their results are so different from these works. This is especially puzzling because the previous Pds5b knockout seemed to be a protein-null. An uniformed reader might not be aware of the controversy.
2. Figure 1 shows that Pds5a deletion has a more severe effect on cell growth, yet little effect on cohesion on its own. This surprising result is not commented on in the manuscript.
3. Figures 2 and 3: The authors use a combination of gene knockout and RNAi to codeplete both Pds5a and Pds5b. However, the strategy used here (transfection of four pooled siRNAs per gene) multiplies the risk of an off-target effect. To be convincing, the authors should show that the double-depletion phenotypes can be rescued by RNAi-resistant forms of Pds5a or Pds5b, or recapitulated by several singly-transfected siRNAs.
4. Figure 3: How does Pds5a and/or Pds5b inactivation affect the cell cycle profile? Could the changes in Smc3 acetylation and sororin binding to chromosomes be a consequence of having fewer S/G2 phase cells?
5. Based on the reduction in acetylation levels and sororin binding, the authors conclude that Pds5a/b have a role in cohesion establishment. If substantiated this would be a novel conclusion, since in other organisms Pds5 has been found to act positively in cohesion maintenance, yet negatively in cohesion establishment. Perhaps the clearest case for this comes from fission yeast, where pds5 deletion blocks Smc3 acetylation, yet bypasses the need for eso1 (the S. pombe acetyltransferase), which is essential. Likewise, the authors show that cohesin levels on interphase chromosomes are if anything increased after Pds5a/b downregulation, and that less Wapl is bound to chromosomes. Because all of the cohesion assays used by the authors are based on the analysis of metaphase chromosomes (when Pds5's role in stabilizing cohesin seems to disappear), one could argue that the main phenotype relates to a lack of cohesion maintenance after prolonged mitotic arrest, whereas cohesion establishment is normal or perhaps even enhanced (judging from the higher levels of cohesin binding in interphase, as well as the lack of spindle checkpoint activation during an unperturbed mitosis). In short, there are substantial ambiguities in when and how the defects in Pds5-deficient cells arise, since multiple pro- and anti-cohesive activities are simultaneously dysregulated. Ideally, a separation-of-function allele might be developed to resolve these ambiguities. Alternatively, the authors should be more precise in their statement of the results, and more carefully distinguish between mechanistic alternatives in the discussion section.
6. At the top of page 8, it is stated that "excess cohesin is acetylated by CoATs during DNA replication relative to the acetylated complexes that are subsequently bound by Sororin." I did not find data to support this in the manuscript. Please provide or delete.
7. Figure 4A: The main text states that Pds5a and Pds5b are important for telomere cohesion, but only the positive control seems elevated. What about cells codepleted of Pds5a and Pds5b?
8. Figure 4B: The authors show an increase in chromosome breaks in Pds5-deficient cells treated with aphidicolin. However, I am confused why they refer to this as an "arm cohesion" defect, since this term has a quite different meaning in the literature. To avoid confusion standard terminology ("fragile sites") should be used.
9. Figure 4C: I am not sure why the authors refer to this as a CEN cohesion defect, when it appears that the arms are also "open" (splayed apart) relative to the "no defect" control. Furthermore, it seems like most mitotic cells not subjected to spreading still have intact centromeric cohesion and normal interkinetochore distances (cf. Figure 5C, 7A). So does this phenotype develop only with prolonged mitotic arrest? Does it require hypotonic swelling and/or dropping onto slides?
10. Figure 5: Is Esco2 not chromatin-bound in Pds5-deficient cells? Or expressed at lower levels? This point should be addressed, either here or as part of Fig. 3B.
11. Figure 6B,C: Is there a statistically significant difference here?
12. Figure 7: Whelan et al. reported that Esco2 deficiency alters the localization of Bub1 and Sgo1. Are those proteins normally or abnormally localized in Pds5b-/- cells? Could those be responsible for the dysregulation of Aurora B (or vice versa)?
13. Figure 8: A key feature of the model is that Pds5b (but presumably not Pds5a) is selectively localized to PCH. Is this known? If not immunofluorescence and/or ChIP data should be provided. Otherwise, it is possible that both Pds5a and Pdsb are at PCH, but only Pds5b (via isoform-specific

 

motifs) recruits Esco2, Haspin, Aurora B, etc.

Referee #3:

Pds5 is a cohesin regulatory protein present in all eukaryotes. In fission yeast, Pds5 is indispensable for acetylation and therefore for cohesion (Vaur et al, 2012). Two Pds5 proteins exist in vertebrates, named Pds5A and Pds5B; their functional specificity remains elusive. Additionally, there are inconsistencies on the function of Pds5 proteins among different model organisms. In HeLa and Xenopus cells, Pds5B is important for centromeric, Pds5A for arm cohesion (Losada et al, 2005). This could not be confirmed in mice, since Pds5A or Pds5B knockout mice do not show cohesion abnormalities (Zhang et al, 2007; 2009).
In this study, the authors have independently generated conditional knockout mice for Pds5A and B, respectively. The authors observe centromeric cohesion defects after Pds5B gene deletion in fibroblasts (MEFs) and hepatocytes, and arm and telomere cohesion defects upon deletion of either Pds5A or Pds5B. Consistent with the function of Pds5 in yeast, the authors show that both Pds5 proteins contribute to cohesin acetylation. Furthermore, it is evident that lack of centromeric cohesion in Pds5B knockout mice is linked to impaired localization of Esco2, an acetyltransferase important for cohesion at the centromere (Whelan et al, 2012). Consistently, recruitment of the mitotic kinase Aurora B to centromeres is also impaired in Pds5B knockout mice.
This study addresses an important topic and does so through loss-of-function genetics in mouse cells, which is more rigorous than previous RNAi approaches. The authors report a number of interesting observations, notably that Pds5B is required for centromeric cohesion and recruitment of Aurora B kinase to centromeres. This provides the first molecular insight into specific functions of Pds5B versus Pds5A proteins and is thus principally worthy of publication. However, there are also several findings in the manuscript that need to be clarified before publication, as is explained in more detail below.

Specific points:
1. Protein depletion in Pds5A and B knockout MEFs was shown by single antibodies. It is important to clarify if these antibodies are recognizing N or C terminal regions, and if it can be excluded that fragments of Pds5A or Pds5B are still expressed in the knockout cells.
2. For simultaneous Pds5A/B depletion, the authors are using combinations of knockout and RNAi. This is a useful, although not an ideal approach, because currently RNAi off targets effects cannot be excluded. The generation and analysis of double knockout mice would be ideal, or as an alternative the authors should compare the phenotypes of single Pds5A knockout cells with the phenotype of cells in which Pds5A was depleted by RNAi, as a way to assess the specificity of the Pds5A siRNAs used.
3. In figure 2, the authors use double depletion (combination of a knockout and RNAi) to claim that there is small but significant enrichment of cohesin on chromatin in interphase using immunofluorescence (IF) quantification. Since the difference in signal intensity is very small, the authors should provide another experimental approach to test if this effect exists. Analysis of MEFs generated from double knockout mice would again be useful here and might bring clearer result. Panel D is addressing if the prophase pathway of cohesin release is impaired, whereas the rest of the figure is addressing cohesin levels in interphase cells. I would suggest to place panel D into the supplementary information and to include a positive control where cohesin removal by the prophase pathway is affected, especially since the images provided depict mainly staining at centrosomes.
4. In figure 3, the authors show that lack of Pds5A or B proteins causes a reduction in cohesin acetylation; however, levels of the cohesion regulator sororin are not reduced. This is surprising since other reports indicate a correlation between these events (Lafont et al. 2010, Nishiyama et al. 2010, Whelan et al. 2012). However, the authors do observe a reduction in sororin signals if measured by IF (Figure 5, panel B and C). These inconsistencies need to be explained.
5. Throughout the main text, the authors argue that Pds5A and B are important for telomeric cohesion. However, this is not obvious from figure 4, panel A, where no difference between the knockout and wild-type cells is observed. In the same figure in panel B, the authors observe breaks in chromosome morphology after treating cells with aphidicholin and claim that Pds5A and B proteins are contributing to arm cohesion. Another interpretation could be that the proteins are important for DNA repair. The importance of Pds5A for arm cohesion is also not obvious from chromosome spreads (neither in MEFs nor in hepatocytes).
6. In figure 5, the authors claim that Pds5B is important for localization of Esco2 to pericentric

 

heterochromatin (PCH) regions. However, the graph in Figure 5A shows that also Pds5A knockout cells display a more than 10% decrease of Esco2 localization. On the other hand, chromosome spreads from Pds5B-/- MEFs show defects but not complete lack of centromeric cohesion. At the same time, chromosome arms seem to become more open after deletion of Pds5B (Figure 4C, Figure S4). Could it be that some functional redundancy exists between the two proteins, and that Pds5A is also contributing to centromeric cohesion? Also, why did the authors exclude metaphases showing single chromatids in figure 4C?
7. The authors observe defects in embryonic development after deletion of Pds5A or Pds5B. The latter deletion also increases the number of aneuploid cells. Could the authors comment if there is a causal link between these observations, and what the reason for the Pds5A knockout phenotype could be?
8. In Figure S6 it would be interesting to include Pds5A-/- as a comparison.

Minor points:
1. When using error bars please specify what kind of bars they are, especially when sample size is 2. [EDITOR's NOTE: for all statistical analyses, as stated in our author guidelines, please keep in mind that mean values and standard deviations are only meaningful for N equal to or greater than 3; for sample size of two therefore please display individual values instead]
2. In some IF images, scale bars are missing.
3. Figure 3 panel C is not mentioned in the main text.
4. The model in figure 8 needs some clarification, especially when during the cell cycle the depicted processes are expected to occur and if acetylation and sororin binding also contributes to Aurora B recruitment.

1st Revision - authors' response 17 September 2013

**Referee #1:**

*This study is an important step towards understanding cohesin regulation and the consequences of cohesin dysfunction in metazoan. Overall the work is well conducted and good quality data support the major conclusions drawn by the authors. I have however specific concerns about some parts of the study, as detailed below.*

*Major points:*

*1- Effect of Pds5A and Pds5B depletion on the amount of chromatin-bound cohesin in interphase and prophase cells (Figure 2).*
*The authors address this question by treating Pds5A -/- MEFs with Pds5B siRNA and reciprocally, Pds5B-/- cells with Pds5A siRNA. Only a moderate increase in chromatin-bound Smc3 was observed in interphase cells when Pds5 proteins are co-depleted (Fig2BC) and no effect was seen on prophase cells (Fig2D). However, Western blot analyses (Fig.S3) revealed that Pds5A and Pds5B are not depleted by siRNA treatment in this particular experiment. The authors mention that the increase in chromatin-bound cohesin in interphase is consistent with a reduction in the presence of Wapl on chromatin as can be seen in Fig. 3B. This was however a WB from a distinct experiment in which the RNAi treatment appeared more efficient than in the Fig2 experiment (compare Fig.3B and Fig.S3).*
*Therefore, the modest effect seen on interphase cells (Fig2BC) and the lack of an effect in prophase (Fig2D) may well be a consequence of incomplete depletion and the conclusion that Pds5 proteins are not required for the efficient release of cohesin in prophase may not be valid.*
*The authors should either tune down their conclusions or repeat the experiment using conditions that give an apparent complete depletion of Pds5 proteins. It would be helpful to include a Wapl RNAi control as a reference for reduced unloading.*

The reviewer is right in that depletion is not complete upon siRNA and around 20-25% of one Pds5 protein is left. We have now repeated this experiment using MEFs carrying KO conditional alleles for both Pds5 genes together with a Cre recombinase inducible by 4-hydroxy tamoxifen (4-OHT).

 

The depletion of Pds5A and Pds5B achieved after 4-5 days incubation with 4-OHT is better (although not complete for any of the two Pds5 proteins). We again see an increase in the amount of cohesin in interphase and a reduction of Wapl on chromatin. As suggested by the reviewer, we have also performed Wapl siRNA and measured the effects on cohesin for comparison. The reduction of Wapl levels with siRNA leaves 25% of Wapl, and leads to a similar increase of cohesin on chromatin. This result supports the conclusion that Pds5 proteins work with Wapl to promote cohesin release from chromatin. We have included these data in Figure 2C (quantification of cohesin intensity by immunofluorescence) and Figure S3 (remaining levels of Pds5A and Pds5B; levels of Wapl after siRNA; cohesin staining in interphase cells without Pds5 and without Wapl). We have also included in Figure S3E a chromatin fractionation of the double KO MEFs.

Regarding the prophase dissociation, we now show in Figure 2D that it is defective in the absence of both Pds5 proteins (in the double KO MEFs) as well as in MEFs with reduced levels of Wapl. Thus, our conclusion has changed:
"We therefore conclude that Pds5 contributes to cohesin unloading both in interphase and mitosis".

*2- Both Pds5A and Pds5B contribute to telomere and arm cohesion in mouse cells (page 8 and Fig.4).*
*I found this paragraph rather unclear. The authors used indirect assays to assess cohesion defects at telomeres and chromosomes arm sites. For telomeres, the rationale is that a cohesion defect should impair telomere replication which should increase the frequency of irregularly shaped telomeric FISH signals on metaphase plates. Using this assay, Pds5A-/- and Pds5B-/- MEFs behaved as the wild-type control (Fig. 4A). Surprisingly, they conclude that Pds5A and Pds5B contribute to telomere cohesion. I do not understand.*

We had previously shown that Sororin, and therefore cohesion, is required to prevent telomere fragility (Remeseiro et al 2012). Since we did not see telomere fragility in either Pds5A null or Pds5B null MEFs, we concluded that they can both accomplish telomere cohesion, i.e. there is no specific requirement for one or the other. We have now performed a telomere fragility assay using three conditions of downregulation of both Pds5 proteins (Pds5A-/- siPds5B, Pds5B-/-siPds5A and the double KO MEFs). In all three conditions we observe a similar increase in telomere fragility above the levels in wildtype cells, although it is not as pronounced as in the SA1 KO MEFs. These new data have been added to Figure 4A.

*In Figure 4B, they treated cells with low dose of aphidicolin during a round of S phase and scored breaks on chromosome arms at metaphase, the rationale being that cohesin is required for homologous recombination mediated replication fork restart after aphidicolin induced replication fork stalling. It's a rather indirect assay. Pds5 proteins may well be involved in replication fork restart independently of their role in sister-chromatid cohesion. In addition, it is unclear to me how this experiment can be performed: cells were treated with aphidicolin for 24 hours so that a fraction of cells may replicate in the presence of the drug during this time frame. However, Pds5B-/- hardly proliferate in culture (~1 doubling in 6 days, Figure 1D). The authors should use a straightforward FISH assay on G2 cells to probe subtelomeric and arm cohesion as they did in a previous study (Remeseiro et al., 2012a).*

The drawback of the FISH assay is that one is looking at cohesion in just one or few sites whereas the other assays (telomere fragility and breaks) provide a comprehensive view of the whole genome. In Remeseiro et al (2012), arm cohesion measured by FISH with two probes appeared to depend more on cohesin-SA2 than cohesin-SA1. However, the break assay indicates that both contribute to arm cohesion. We acknowledge the criticism that this is an "indirect" way to measure cohesion. However, it is unlikely that Pds5 proteins contribute to fork restart independently of cohesion. In Remeseiro et al (2012) we found that depletion of cohesin (through siRNA of both SA1 and SA2) and depletion of Sororin led to very similar effects on breaks upon aphidicolin treatment.
We have performed FISH, as requested, using two subtelomere and arm probes from chromosome 8 and chromosome 10. We do not find defects in Pds5A or Pds5B null cells above the levels of wildtype cells. In the double KO MEFs, the number of doublets increases only for one of the probes (located in the arm of chromosome 8). We have included these data as new Supplementary Figure S4. It is possible that in the other chromosomal locations examined the positive and negative effects of Pds5 proteins in cohesion are balanced and make it difficult to observe a clear cohesion defect.

 

*Minor points:*

*Figure 3C. The error bars should be defined*
The error bars indicate the standard error of the mean (s.e.m) from quantification of three different experiments, as it is now explained in the Figure legend

*Page 8 top. "excess cohesin is acetylated by coATS during DNA replication relative to the acetylated complexes that are subsequently bound by Sororin". I don't think there is any good evidence that Sororin binds acetylated Smc3. The Nishiyama (Cell 143, 737-749, November 24, 2010) clearly shows that Sororin binding to chromatin is Esco1-2 dependent but no clear evidence that this is mediated by Smc3 acetylation.*

The Nishiyama 2010 paper shows an immunoprecipitation of cohesin Smc3 from control and Esco1/2 siRNA treated cell extracts (in Figure S3E). In the immunoprecipitates there is acetylated Smc3 and Sororin in control cells and both are much reduced in Esco1/2 depleted cells. This suggests that Sororin is bound to acetylated cohesin. In turn, our result that decreased acetylation of cohesion is not paralleled by a similar decrease in Sororin in the absence of a single Pds5 protein suggests that only a fraction of acetylated cohesin is stabilized by Sororin binding. Nevertheless, we have deleted the sentence and now just say:
"These results suggest that Pds5 proteins are required for cohesin acetylation in mammalian cells, as recently found in yeast (Vaur et al, 2012; Chan et al, 2013), as well as for recruitment of Sororin and Wapl."

*Figure 4C. The legend is not clear. From the text (page 8, last lane), I understand that a metaphase was scored as "defective for centromeric cohesion" when more than 5 chromosomes displayed clearly separated centromeres. From the figure legend, "metaphase with single chromatids were excluded". The criteria should be clarified and moved in the figure legend.*

We have modified the Figure legend:
The graph below represents the percentage of metaphases showing more than five chromosomes with centromere cohesion defects among n≥100 cells of each condition. We did not count metaphases showing only single chromatids, i.e. in which pairs of sisters cannot be recognized.

*The discussion recalls that the Zhang studies did not report any cohesion defect in Pds5 KO mice whereas the present work did find cohesion phenotypes. I believe the reader would expect the authors to discuss this point: why this difference?*

At present we have no explanation for the discrepancy. Ours is a positive result, i.e., we do see a defect, whereas Zhang et al present a negative result. The may have chosen mostly prometaphase-like chromosomes in which the defect could be less evident. We have added this sentence in the Discussion:

"We do not know the reason for the discrepancy with the abovementioned studies regarding detection of centromere cohesion defects in metaphase spreads. In our hands, this phenotype is observed not only in Pds5B knock out MEFs but also in hepatocytes from Pds5B knock out embryos. Also in HeLa cells, downregulation of Pds5B levels results in centromeric cohesion defects (Losada et al, 2005)".

**Referee #2 :**

*The mechanism of sister chromatid cohesion is intensively studied but still incompletely understood. The present study adds new and potentially interesting information on the effects of inactivating Pds5-related proteins in mouse cells. However, some analyses do not go far enough to draw mechanistic insights, apart from corroborating ideas from other model organisms. These ambiguities arise in part from the fact that Pds5 has complex positive and negative effects on cohesin regulation, which are all superimposed on one another here. As a result I find some of the authors' conclusions to be preliminary and/or debatable based on the present dataset.*

 

Specific points:

*1. The authors reference the Zhang et al (2007, 2009) studies without addressing why their results are so different from these works. This is especially puzzling because the previous Pds5b knockout seemed to be a protein-null. An uniformed reader might not be aware of the controversy.*

I must say that looking at cohesion defects in metaphase spreads is not trivial and there is a lot of cell-to cell variation. We have looked at hundreds of cells from different MEF clones and different conditions (e.g., plus or minus colcemid), and in fetal liver cells. As we answered to referee #1, who raised the same point, we have no explanation for the discrepancy. Our result is a positive result, i.e., we do see a defect. Zhang et al present a negative result. The may have chosen mostly prometaphase-like chromosomes in which the defect could be less evident. On the other hand, these studies are focused on phenotypic analyses of the knock out embryos in relation with CdLS, whereas ours is focused on cohesion in Pds5 deficient cells.

We have added this sentence in the Discussion:

"We do not know the reason for the discrepancy with the abovementioned studies regarding detection of centromere cohesion defects in metaphase spreads. In our hands, this phenotype is observed not only in Pds5B knock out MEFs but also in hepatocytes from Pds5B knock out embryos. Also in HeLa cells, downregulation of Pds5B levels results in centromeric cohesion defects (Losada et al, 2005)".

*2. Figure 1 shows that Pds5a deletion has a more severe effect on cell growth, yet little effect on cohesion on its own. This surprising result is not commented on in the manuscript.*

We do not know the reason for the proliferation defects observed in the Pds5A null cells. We speculate that it has to do with transcriptional alteration of proliferation genes. We plan to address the role of Pds5 proteins in transcription but this is not the focus of the current manuscript. We have added a final paragraph to the Discussion:

"Decreased proliferation of Pds5B null cells could be explained by mitotic cell death and aneuploidy. Cells lacking Pds5A have a stronger proliferation defect and Pds5A null embryos present an earlier lethality, but in this case cells display correct ploidy and no mitotic defects. Previous results indicate that Pds5 regulates transcription in Drosophila (Dorsett et al, 2005; Gause et al, 2010). We speculate that the Pds5A null phenotypes may be related to altered transcription. Future experiments will have to address the genome wide distribution of Pds5A and Pds5B and the effects of their ablation in gene expression during development."

*3. Figures 2 and 3: The authors use a combination of gene knockout and RNAi to codeplete both Pds5a and Pds5b. However, the strategy used here (transfection of four pooled siRNAs per gene) multiplies the risk of an off-target effect. To be convincing, the authors should show that the double-depletion phenotypes can be rescued by RNAi-resistant forms of Pds5a or Pds5b, or recapitulated by several singly-transfected siRNAs.*

In Figure 2C and Figure 4B (and new Figure 4A), the effects of using Pds5A siRNAs in Pds5B null MEFs and, conversely, Pds5B siRNAs in Pds5A null MEFs are very similar, arguing against off-target effects. In Figure 4C, the transfection of Pds5A siRNA in Pds5B null MEFs does not have a phenotype that can be rescued.

In the case of Figure 3B, however, we find important to address this criticism. We have now repeated the chromatin fractionation experiment using MEFs carrying knock out conditional alleles for both Pds5 genes together with an inducible Cre recombinase inducible by 4-hydroxy tamoxifen (4-OHT). After 5 days in media with 4-OHT, a dramatic reduction in the protein levels of both Pds5A and Pds5B can be observed. Supporting the results obtained in Figure 3B with the siRNA, we find that acetylation of Smc3 and binding of Sororin are abolished in the double KO MEFs (data added to new Figure S3E). One caveat of this experiment is that there is a larger fraction of unreplicated cells in the asynchronous cultures of the double knock out MEFs (new Figure S3E). However, by immunostaining we can observe that G2 cells in these cultures do not have chromatin-

 

bound Sororin (new Figure 3D).

*4. Figure 3: How does Pds5a and/or Pds5b inactivation affect the cell cycle profile? Could the changes in Smc3 acetylation and sororin binding to chromosomes be a consequence of having fewer S/G2 phase cells?*

We now include in Figure 1E the results from FACS analyses in the single KO cells. There are no major alterations in the cell cycle although Pds5A null cells usually have a slightly larger fraction of cells in G1 and less in S phase. However, there is no difference in Sororin recruitment (which occurs in S phase) and thus it is unlikely that the decrease in Smc3 acetylation observed is due to having fewer cells traversing S phase. As just mentioned in the previous paragraph, we have also performed FACS and EdU incorporation analyses in the double KO MEFs and observed an increase of cells in G1 and a decrease in S phase (new Figure S3E). However, if we look specifically at the G2 cells in these cultures by staining wit Aurora B, we can see that most cells do not have chromatin-bound Sororin (new Figure 3D). The few positive cells are likely to be those in which Cre deletion of Pds5A and/or Pds5B has not occurred.

*5. Based on the reduction in acetylation levels and sororin binding, the authors conclude that Pds5a/b have a role in cohesion establishment. If substantiated this would be a novel conclusion, since in other organisms Pds5 has been found to act positively in cohesion maintenance, yet negatively in cohesion establishment. Perhaps the clearest case for this comes from fission yeast, where pds5 deletion blocks Smc3 acetylation, yet bypasses the need for eso1 (the S. pombe acetyltransferase), which is essential. Likewise, the authors show that cohesin levels on interphase chromosomes are if anything increased after Pds5a/b downregulation, and that less Wapl is bound to chromosomes. Because all of the cohesion assays used by the authors are based on the analysis of metaphase chromosomes (when Pds5's role in stabilizing cohesin seems to disappear), one could argue that the main phenotype relates to a lack of cohesion maintenance after prolonged mitotic arrest, whereas cohesion establishment is normal or perhaps even enhanced (judging from the higher levels of cohesin binding in interphase, as well as the lack of spindle checkpoint activation during an unperturbed mitosis). In short, there are substantial ambiguities in when and how the defects in Pds5-deficient cells arise, since multiple pro- and anti-cohesive activities are simultaneously dysregulated. Ideally, a separation-of-function allele might be developed to resolve these ambiguities. Alternatively, the authors should be more precise in their statement of the results, and more carefully distinguish between mechanistic alternatives in the* discussion section.

We certainly agree with the referee that the positive and negative effects of Pds5 proteins in cohesion make their study very complex and that separation-of-function alleles for Pds5 would be extremely useful. However, they are out of the scope of the current study.

Previous studies have shown that cohesin acetylation and Sororin recruitment are essential steps for cohesion establishment in vertebrate cells. Thus, I think it is correct to say (in Duscussion):
"Our analysis indicates that Pds5A and Pds5B are indeed critical for cohesion establishment and maintenance in vertebrates, as these proteins are required for full Smc3 acetylation and Sororin binding."

In fission yeast (Vaur 2012) and more recently also in budding yeast (Chan 2012), it has been shown that Pds5 is required fro Smc3 acetylation, suggesting a conserved role of Pds5 in cohesion establishment.

We also would like to clarify the following points:
*"Likewise, the authors show that cohesin levels on interphase chromosomes are if anything increased after Pds5a/b downregulation, and that less Wapl is bound to chromosomes".*
Cohesin levels are indeed increased but this cohesin is not cohesive since it is not acetylated and bound to Sororin.

*"Because all of the cohesion assays used by the authors are based on the analysis of metaphase chromosomes (when Pds5's role in stabilizing cohesin seems to disappear), one could argue that the main phenotype relates to a lack of cohesion maintenance after prolonged mitotic arrest, whereas cohesion establishment is normal or perhaps even enhanced (judging from the higher levels of cohesin binding in interphase, as well as the lack of spindle checkpoint activation during an*

 

*unperturbed mitosis).”*
There is no prolonged mitotic arrest in Pds5B null cells (as shown by videomicroscopy, Figure 6A) and the centromeric cohesion defects can be observed in cells not treated with colcemid (new Figure S5A). Finally, the telomere fragility and fragile site assays (Figure 4) also indicate a lack of cohesion during S phase in the absence of both Pds5 proteins.

*6. At the top of page 8, it is stated that "excess cohesin is acetylated by CoATs during DNA replication relative to the acetylated complexes that are subsequently bound by Sororin." I did not find data to support this in the manuscript. Please provide or delete.*

This sentence has been deleted. However, we show that acetylation can be decreased in the single KO MEFs without decreasing Sororin binding. An explanation for this result is that only a fraction of acetylated cohesin is bound by Sororin so that reduction of acetylated complexes by half does not affect the fraction of complexes bound by Sororin.

*7. Figure 4A: The main text states that Pds5a and Pds5b are important for telomere cohesion, but only the positive control seems elevated. What about cells codepleted of Pds5a and Pds5b?*

We have performed a telomere fragility assay using three conditions of downregulation of both Pds5 proteins (Pds5A-/- siPds5B, Pds5B-/-siPds5A and the double KO MEFs). In all three conditions we observe a similar increase in telomere fragility, although it is not as pronounced as in the SA1 KO MEFs. These new data have been added to Figure 4A.

*8. Figure 4B: The authors show an increase in chromosome breaks in Pds5-deficient cells treated with aphidicolin. However, I am confused why they refer to this as an "arm cohesion" defect, since this term has a quite different meaning in the literature. To avoid confusion standard terminology ("fragile sites") should be used.*

We have rewritten this paragraph to clarify the assays that we use.

*9. Figure 4C: I am not sure why the authors refer to this as a CEN cohesion defect, when it appears that the arms are also "open" (splayed apart) relative to the "no defect" control. Furthermore, it seems like most mitotic cells not subjected to spreading still have intact centromeric cohesion and normal interkinetochore distances (cf. Figure 5C, 7A). So does this phenotype develop only with prolonged mitotic arrest? Does it require hypotonic swelling and/or dropping onto slides?*

In the spreads one can find a lot of variability regarding arm cohesion, especially in colcemid arrested cells. This is why the only information that we choose to take from the spreads is the status of centromere cohesion.
We agree with the reviewer that the centromeric cohesion defect in Pds5B null cells (and also in cohesin depleted cells for that matter) is more evident in the spreads. However, it does not require mitotic arrest: it can also be observed in Pds5B null cells not treated with colcemid (see new Figure S5A).

*10. Figure 5: Is Esco2 not chromatin-bound in Pds5-deficient cells? Or expressed at lower levels? This point should be addressed, either here or as part of Fig. 3B.*

To address this question we have analyzed total cell extracts and chromatin fractions from wildtype and Pds5A and Pds5B null MEFs with a rabbit polyclonal antibody from G. Eichele’s lab against mouse Esco2 (different form the one raised in guinea pig, from the same lab, that we used for immunofluorescence). Although in some cases there is a reduction in the amount of Esco2 on chromatin in Pds5B deficient cells, it is not always the case (see new Figure S3G). Importantly, when we analyzed the chromatin of double knock out MEFs, we saw no major reduction in Esco2 (Figure S3E). This suggests that Esco2 recruitment to chromatin does not depend on Pds5 proteins. However, by immunofluorescence we see a clear reduction in the number of cells in which we detect Esco2 in heterochromatin foci whose borders are labeled by PCNA. We have now added these data and modified the title (*Pds5B recruits Sororin to PCH*) and the text of this section:

“Chromatin-bound levels of Esco2 are not consistently different between wild type and Pds5B null

cells (Figure S3G) or cells depleted from both Pds5A and Pds5B (Figure S3F), at least by immunoblot. Thus, it is unlikely that Pds5 proteins recruit Esco2 to chromatin. During late S phase, wild type MEFs show a characteristic horseshoe-shaped PCNA staining around DAPI-dense heterochromatin foci, with Esco2 staining inside (Figure 5A). In contrast, Esco2 was not visible in almost 50% of Pds5B null cells showing this PCNA staining pattern. This result suggests that Pds5B promoteS Esco2 action on cohesin present at PCH in concert with DNA replication".

*11. Figure 6B,C: Is there a statistically significant difference here?*

Yes. We have included the statistical analysis of the euploidy data in this Figure.

*12. Figure 7: Whelan et al. reported that Esco2 deficiency alters the localization of Bub1 and Sgo1. Are those proteins normally or abnormally localized in Pds5b-/- cells? Could those be responsible for the dysregulation of Aurora B (or vice versa)?*

We have performed Bub1 staining in wildtype, Pds5A null and Pds5B null cells with an antibody from S. Taylor lab and found no delocalization of this protein. He also sent us a Sgo1 antibody but it does not work in our hands. We have prepared a new Supplementary Figure 9 with these data.

1*3. Figure 8: A key feature of the model is that Pds5b (but presumably not Pds5a) is selectively localized to PCH. Is this known? If not immunofluorescence and/or ChIP data should be provided. Otherwise, it is possible that both Pds5a and Pdsb are at PCH, but only Pds5b (via isoform-specific motifs) recruits Esco2, Haspin, Aurora B,* etc.

Our current data do not exclude the possibility mentioned by the reviewer. By immunofluorescence it is not possible to see specific localization of the two proteins. We have performed ChIP, as suggested, with a double-cross linking protocol used by other researchers for heterochromatin regions. As a control, and in order to test the specificity of our antibodies, we assessed binding to the myc gene, a well-known cohesin binding site. We find binding of both Pds5A and Pds5B to the myc gene that is much reduced in the corresponding knock out cells. We conclude that the antibodies work fine. When we use primers against the major satellite, we find only a little bit of Pds5B and less Pds5A. This could indicate a certain enrichment of Pds5B in pericentromeric regions. Unfortunately, we have not been able to reproduce these results (in heterochromatin, in the myc binding site everything is fine). Thus, further work is needed to clarify this important issue. We have rewritten the Discussion to avoid referring to "localization" of specific complexes at different locations and instead refer to functionality or action. Also in the legend of Figure 8 we have included a sentence: "Whether this functional distribution reflects a specific localization of the corresponding complexes is not known".

**Referee #3:**

*This study addresses an important topic and does so through loss-of-function genetics in mouse cells, which is more rigorous than previous RNAi approaches. The authors report a number of interesting observations, notably that Pds5B is required for centromeric cohesion and recruitment of Aurora B kinase to centromeres. This provides the first molecular insight into specific functions of Pds5B versus Pds5A proteins and is thus principally worthy of publication. However, there are also several findings in the manuscript that need to be clarified before publication, as is explained in more detail below.*

*Specific points:*
*1. Protein depletion in Pds5A and B knockout MEFs was shown by single antibodies. It is important to clarify if these antibodies are recognizing N or C terminal regions, and if it can be excluded that fragments of Pds5A or Pds5B are still expressed in the knockout cells.*

The antibodies recognize the C-termini of Pds5A and Pds5B. We have now included this information in the Figure Legend of Figure 1C. Upon removal of exon 6 the locus produces a Pds5A truncated protein is 176 aa (full length is1332 aa). Pds5B truncated protein is 124 aa (full length is

1442). These fragments lack HEAT repeats and are unlikely to be functional. We now mention this information in the first paragraph of Results.

*2. For simultaneous Pds5A/B depletion, the authors are using combinations of knockout and RNAi. This is a useful, although not an ideal approach, because currently RNAi off targets effects cannot be excluded. The generation and analysis of double knockout mice would be ideal, or as an alternative the authors should compare the phenotypes of single Pds5A knockout cells with the phenotype of cells in which Pds5A was depleted by RNAi, as a way to assess the specificity of the Pds5A siRNAs used.*

The possible off-target effects of the siRNAs was also a concern for referee#2. Since double knock out embryos are not viable, we had to generate a strain carrying the conditional alleles together with a Cre recombinase inducible by 4-hydroxy tamoxifen (4-OHT). We have been able to repeat some critical experiments with these double KO MEFs. In the experiments with siRNAs shown in Figure 2B-C and Figure 4B, the effects of using Pds5A siRNAs in Pds5B knock out MEFs and, conversely, Pds5B siRNAs in Pds5A null MEFs are very similar, arguing against off target effects. In Figure 4C, the transfection of Pds5A siRNA in Pds5B null MEFs does not have a phenotype. For confirmation of chromatin bound levels of Sororin, Wapl, acSMC3 (Figure 3B) we have now repeated the chromatin fractionation with the new double knock out MEFS. After 5 days in media with 4-OHT, a dramatic reduction in the protein levels of both Pds5A and Pds5B can be observed. Supporting the results obtained in Figure 3B with the siRNA, we find that acetylation of Smc3 and binding of Sororin are abolished in the double knock out MEFs (data added to new Figure S3E). One caveat of this experiment is that there is a larger fraction of unreplicated cells in the asynchronous cultures of the double knock out MEFs (new Figure S3E). However, by immunostaining we can observe that G2 cells in these cultures do not have chromatin-bound Sororin (new Figure 3D).

*3. In figure 2, the authors use double depletion (combination of a knockout and RNAi) to claim that there is small but significant enrichment of cohesin on chromatin in interphase using immunofluorescence (IF) quantification. Since the difference in signal intensity is very small, the authors should provide another experimental approach to test if this effect exists. Analysis of MEFs generated from double knockout mice would again be useful here and might bring clearer result.*
*Panel D is addressing if the prophase pathway of cohesin release is impaired, whereas the rest of the figure is addressing cohesin levels in interphase cells. I would suggest to place panel D into the supplementary information and to include a positive control where cohesin removal by the prophase pathway is affected, especially since the images provided depict mainly staining at centrosomes.*

Reviewer 1 and 2 had a similar criticism regarding incomplete depletion and possible off target effects with the RNAi approach. Indeed, depletion is not complete upon siRNA and between 10-25% of one Pds5 protein is left. The depletion of Pds5A and Pds5B achieved in the double knock out MEFs after 4-5 days incubation with 4-OHT is better (although not complete for any of the two Pds5 proteins). We again see an increase in the amount of cohesin in interphase and a reduction of Wapl on chromatin. Moreover, regarding the prophase dissociation, we now show in Figure 2D that it is defective in the absence of both Pds5 proteins as well as in MEFs with reduced levels of Wapl (used as positive control).

*4. In figure 3, the authors show that lack of Pds5A or B proteins causes a reduction in cohesin acetylation; however, levels of the cohesion regulator sororin are not reduced. This is surprising since other reports indicate a correlation between these events (Lafont et al. 2010, Nishiyama et al. 2010, Whelan et al. 2012). However, the authors do observe a reduction in sororin signals if measured by IF (Figure 5, panel B and C). These inconsistencies need to be explained.*

Our favourite hypothesis to explain this result is that all cohesin present on chromatin becomes acetylated but only a fraction of it is stabilized by Sororin binding whereas the rest will eventually dissociate and become deacetylated. Thus, acetylation happens "in excess" relative to Sororin binding, so even if a clear two-fold reduction in acSmc3 is observed in Pds5A or Pds5B null cells, Sororin levels are not changed in a manner that can be detected by immunoblot. These results are not at odds with those in the studies mentioned by the reviewer.

- Lafont 2010 show that Sororin cannot be found on chromatin assembled in extracts depleted of Esco2 in Xenopus egg extracts (a condition that eliminates completely Smc3 acetylation in this system according to the Takahasi lab). We also find that in the absence of both Pds5 proteins there

is no acetylation of Smc3 and no Sororin binding.
- In Nishiyama 2010, single depletion of Esco1 or Esco2 does not reduce the amount of acSmc3 on chromatin while binding of Sororin in these conditions is not reported. Double depletion of Esco1/2 eliminates most acSmc3 and also Sororin binding. This is again in line with our results, no acetylation equals no Sororin.

- Whelan 2012 is the only report with a partial reduction of acSmc3. In this case, a two-fold reduction in acSmc3 in Esco2 null cells is accompanied by a similar reduction in Sororin. Maybe cohesin acetylated by Esco2 is the one preferentially bound by Sororin. We are depleting Pds5A or Pds5B, which is different from depleting Esco1 or Esco2. We find that Esco2 and Pds5B work together at PCH but Esco2 most likely works with Pds5A at other regions.

While we do not find a clear reduction of Sororin in chromatin by immunoblot in Pds5B null cells, we do observe a reduction of Sororin at PCH of G2 cells by immunofluorescence. It is difficult to compare both results since one looks at all chromatin in asynchronous cell population and the other at specific regions (PCH) in G2 cells (marked by Aurora B staining). As indicated in the bar graph, 35% of Pds5B KO cells still have detectable Sororin at PCH foci. It is possible that Pds5A is also at PCH and partially contributes to Sororin binding there, at least in Pds5B KO cells. However, it is also clear that there is defect in accumulation of Sororin in these regions that translates into cohesion defects at the centromeres.

*5. Throughout the main text, the authors argue that Pds5A and B are important for telomeric cohesion. However, this is not obvious from figure 4, panel A, where no difference between the knockout and wild-type cells is observed. In the same figure in panel B, the authors observe breaks in chromosome morphology after treating cells with aphidicholin and claim that Pds5A and B proteins are contributing to arm cohesion. Another interpretation could be that the proteins are important for DNA repair. The importance of Pds5A for arm cohesion is also not obvious from chromosome spreads (neither in MEFs nor in hepatocytes).*

We had previously shown that Sororin, and therefore cohesion, is required to prevent telomere fragility (Remeseiro et al 2012). Since we did not see telomere fragility in either Pds5A null or Pds5B null MEFs, we concluded that they can both accomplish telomere cohesion, i.e. there is no specific requirement for one or the other. If this is correct, the double KO cells should present telomere cohesion defects. We have performed a telomere fragility assay using three conditions of downregulation of both Pds5 proteins (Pds5A-/- siPds5B, Pds5B-/-siPds5A and the double KO MEFs). In all three conditions we observe a similar increase in telomere fragility, although it is not as pronounced as in the SA1 KO MEFs. These new data have been added to Figure 4A.

A similar logic applies to our conclusion in term of arm cohesion. If we do not see an effect in single KO cells is because neither Pds5 protein has a specific role in arm cohesion. Upon referee#1 request, we have performed FISH analysis in interphase cells with two arm and two subtelomere probes and again did not observe cohesion defects (new Figure S4). Regarding the use of chromosome breaks after low aphidicolin treatment as a readout for arm cohesion, we think that it is unlikely that Pds5 proteins contribute to DNA repair through a mechanism other than ensuring cohesion. In a previous study [Remeseiro et al (2012)] we showed that depletion of cohesin (through siRNA of both SA1 and SA2) and depletion of Sororin led to very similar effects on chromosome breaks break upon aphidicolin treatment.

*6. In figure 5, the authors claim that Pds5B is important for localization of Esco2 to pericentric heterochromatin (PCH) regions. However, the graph in Figure 5A shows that also Pds5A knockout cells display a more than 10% decrease of Esco2 localization. On the other hand, chromosome spreads from Pds5B-/- MEFs show defects but not complete lack of centromeric cohesion. At the same time, chromosome arms seem to become more open after deletion of Pds5B (Figure 4C, Figure S4). Could it be that some functional redundancy exists between the two proteins, and that Pds5A is also contributing to centromeric cohesion? Also, why did the authors exclude metaphases showing single chromatids in figure 4C?*

We cannot discard a certain level of redundancy in Pds5A and Pds5B, although our results clearly indicate a preferential role of Pds5B in this region. It is possible that in the absence of Pds5A, Pds5B

 

is the only Pds5 to work with Esco2 and this may alter the normal distribution/dynamics of Esco2. It is also important to note that Pds5 is not equivalent of cohesin and the fact that this protein both promotes release and stabilization of cohesin on chromatin when working with Wapl and Sororin, respectively, complicates its analysis. There are not many single chromatid chromosomes among the spreads of Pds5B KO cells so including them in our analyses does not make much of a difference. Loss of centromeric cohesion in Pds5B null cells is not 100% penetrant, i.e. it is not observed in every chromosome of every cell despite the fact that we have complete elimination of the protein. This could be because cohesion depends not only on cohesin but also in catenation and the balance between the two can be different between chromosomes within a cell and may vary depending on the time the cell has spent in mitosis before the spread is performed. As indicated above, a small contribution to Pds5A to centromeric cohesion cannot be ruled out. When both proteins are depleted (although not completely), the centromeric cohesion defects are not significantly increased (Figure 4C). Potential explanations for this result are:
- despite the fact that little cohesin is actually cohesive (there is little Sororin) the prophase pathway is also less efficient and more cohesin remains on chromatin and hinders decatenation;
- the population of cells that gets to mitosis after Pds5AsiRNA knock down in Pds5B KO cells corresponds to cells in which silencing has been less efficient and therefore these cells have higher levels of Pds5A.

*7. The authors observe defects in embryonic development after deletion of Pds5A or Pds5B. The latter deletion also increases the number of aneuploid cells. Could the authors comment if there is a causal link between these observations, and what the reason for the Pds5A knockout phenotype could be?*

We have added a final paragraph to the Discussion:

"Decreased proliferation of Pds5B null cells could be explained by mitotic cell death and aneuploidy. Cells lacking Pds5A have a stronger proliferation defect and Pds5A null embryos present an earlier lethality, but in this case cells display correct ploidy and no mitotic defects. Previous results indicate that Pds5 regulates transcription in Drosophila (Dorsett et al, 2005; Gause et al, 2010). We speculate that the Pds5A null phenotypes may be related to altered transcription. Future experiments will have to address the genome wide distribution of Pds5A and Pds5B and the effects of their ablation in gene expression during development."

*8. In Figure S6 it would be interesting to include Pds5A-/- as a comparison.*

We have included the data requested.

*Minor points:*
*1. When using error bars please specify what kind of bars they are, especially when sample size is 2. [EDITOR's NOTE: for all statistical analyses, as stated in our author guidelines, please keep in mind that mean values and standard deviations are only meaningful for N equal to or greater than 3; for sample size of two therefore please display individual values instead]*
Figure legends now indicate the statistical analyses used. Bars represent mean+s.e.m when N≥3. We have removed error bars when N<3.

*2. In some IF images, scale bars are missing.*
Corrected

*3. Figure 3 panel C is not mentioned in the main text.*
Corrected

*4. The model in figure 8 needs some clarification, especially when during the cell cycle the depicted processes are expected to occur and if acetylation and sororin binding also contributes to Aurora B recruitment.*
We have extended the Figure legend to address these issues.

 

Acceptance letter 01 October 2013

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by one of the original referees (see comments below), and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

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

---

Referee #2

The revised manuscript from Losada and colleagues responds fully to the issues I had with the previous version. I believe the paper provides valuable novel insight into the role of the cohesin accessory factors Pds5a/b in mammalian cells and should be published in the EMBO Journal.

 