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Multivalent binding of PWWP2A to H2A.Z regulates mitosis and neural crest differentiation

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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 25 October 2016

Thank you for submitting your manuscript on H2A.Z-PWWP2A interaction and cooperation to our editorial office. We have now received reviews from three expert referees, copied below for your information. As you will see, all referees appreciate the interest of the topic as well as the potential importance of your results, and we would therefore be interested in pursuing this study further for publication in The EMBO Journal.

However, they also point out a number of serious criticisms with the study in its present form, including the important caveat that several key conclusions of the work (including those in the title) do not appear fully justified at this stage. Therefore, although we realize that the manuscript already contains a large body of data, I am afraid we feel that additional work addressing some of the critical issues would still be necessary to make this work a compelling and strong candidate for an EMBO Journal article.

Following in-depth discussions within the editorial team (including our Chief Editor Dr. Bernd Pulverer), we consider the following points arising from the reports essential for a successful revision:

- It needs to be clarified whether PWWP2A binding to H2A.Z is really causally involved in the developmental phenotypes observed upon PWWP2A morpholino knockdown (cf. ref 1 point 15), as claimed in the title.

- H2A.Z chromatin occupancy in PWWP2A loss- or gain-of-function conditions should be assessed in order to better define the molecular function of PWWP2A (cf. ref 2 point 2)

- The functional relevance of the bivalent nucleosome binding determinants in PWWP2A, possibly in relation to the nucleosome acidic patch, would need to be further dissected (cf. ref 3 point 2, and related point 4 of ref 2). In addition, some further data to separate PWWP2A promoter recruitment via H2A.Z and nucleosome-free DNA (ref 3 point 3, and related point 3 of ref 2) would clearly be helpful to further strengthen this part.

- Finally, there are also various more specific technical and presentational issues (especially in the report from referee 1) that will hopefully be straightforward to address. In particular, it will be important to clarify PWWP2A-H2A.Z interactions on the level of endogenous proteins and to rule out any confounding effects of the often problematic epitope-tagging of histones.

On the other hand, we feel it will not be essential to experimentally clarify whether PWWP2A affects cell division via gene expression or via a more direct chromosome segregation function as proposed for H2A.Z, although this possibility will need to be discussed in detail. Likewise, linking the morphogenesis defects further to altered gene regulation would appear somewhat beyond the scope of the present revision.

In summary, should you be able to adequately address the above main issues, we should be happy to consider a revised manuscript further for publication. I should however point out that we only allow for a single round of major revision, making it essential to diligently respond to all points raised by the referees and editors at the stage of resubmission. Therefore, should you have any specific questions/comments regarding the referee reports or your revision work, please do not hesitate to get in touch with me ahead of time for discussion. We might further arrange for an extension of the revision period beyond the regular three months, during which time the publication of any competing work elsewhere would as a matter of policy have no negative impact on our final assessment of your own study.

REFEREE REPORTS

Referee #1:

This is a very interesting manuscript that identifies a new H2A.Z interacting protein PWWP2A. It employs a multifaceted approach to investigate the function and genomic locations of PWWP2A, identified by a previously employed GFP-based mass spec approach. Interactions between PWWP2A and H2A.Z were supported by cellular and biochemical assays, and the region of PWWP2A required for this interaction was revealed. It genomic locations were identified, which was shown to co localise with H2A.Z at the TSS but not at non-promoter regions. Functionally, it was demonstrated to have a role in chromosome segregation, albeit, in a cancer cell line. Most interestingly, using Xenopus toads as a model organism, the knockdown of PWWP2A interfered with craniofacial morphogenesis.

To strengthen the manuscript, the following technical, informational and conceptual issues should be addressed or clarified:

1. Are GFP-H2A, H2A.Z.1 and H2A.Z.2 all expressed to a similar level?

2. Concerning the mass spec analysis, is it formally possibly that the presence of the GFP tag may prevent some H2A.Z-non-histone protein interactions?

3. The western blot in Fig.1D showing PWWP2A is unclear especially for HK cells. Three bands are observed, which one is PWWP2A? Plus all three bands can be seen for the H2A pull-down.

4. For nuclear staining experiments (Fig. 1SF), have you examined whether PWWP2A co localises with H2A.Z particularly the fraction that is stably bound to chromatin during metaphase?
5. In Fig 1E, H2A.Z IPs brings down H2A implying a fraction of H2A.Z nucleosomes are heterotypic. Following quantification and longer exposure times of PWWP2A IPs, can H2A also be

detected? This could be interesting as to whether PWWP2A binds to homotypic or heterotypic H2A.Z nucleosomes.

6. All of the experiments have been performed with GFP-tagged histones. Are either PWWP2A or H2A.Z antibodies good enough to see the endogenous interaction?

7. In Fig.2A it is stated that equal amounts of GST and PWWP2A-GST were used in pull-down assays yet in the figure shown there is dramatically more GST protein then PWWP2A-GST protein. Why is this the case? Also clearly the purity of PWWP2A-GST (and the deletion mutant constructs) differ significantly so how do you control for this? Similarly in Fig. 2C, PWWP2A and the two mutants all appear to bind to a similar amount of nucleosomes (H3) yet the amount of each protein appears to be dramatically different.

8. I am puzzled with Fig. 2B, as more H2A.Z is present in the PWWP2A pull downs of H2A nucleosomes then H2A.Z.2 nucleosomes.

9. In Fig. 2E, it appears that IC binds to H2A.Z but not nucleosomes because the other histones are depleted. How is this possible? Is Fig. 2F consistent with Fig. 2E because in Fig. 2F, IN now binds to H2A.Z.2?

10. On page 6 it is stated that the DNA binding activity of PWWP2A contributes to chromatin interactions but no experiments have been performed that actually demonstrates this. Indeed Fig. 2C shows that this is not the case (for nucleosomes). Unless a loss of chromatin binding is actually shown, it is better to remove this statement.

11. Again on page 6, it is stated that "In concordance with our in vitro data, mutant constructs containing either the internal or the PWWP domain featured much faster recovery kinetics than wild type" however the in vitro data presented shows the opposite. Fig. 2C demonstrates that both the PWWP mutant and this domain by itself binds to nucleosomes just as well as the full-length protein. This statement requires clarification.

12. There are over 10,000 total peaks and around 4,000 promoter peaks that contain PWWP2A and H2A.Z. What is the explanation as to why the expression of so few genes, in comparison (about 700), change when PWWP2A is knocked down. An important question is of this approx. 700, how many of these genes have PWWP2A directly associated with their TSS.

13. With regards to the experiments depicted in Fig. S6, in the PWWP2A knockdown cells, the chromosomes appear to be slightly fuzzier. Has any quantitative assessment been made with regards to the size of the chromosomes, which would fit with an increase in nuclear size. Also, is there any loss of cohesion i.e. does the distance between the centromeres at metaphase increase (a few chromosomes may show this)?

14. The following speculative sentence on page 8 of the results section should be left for the discussion, and thus removed from the results section "As many genes involved in cellular component assembly were deregulated (Fig. S5), it is likely that global rather than specific cellular changes affected nuclear".

15. In the literature, the following study used morpholino's to knockdown H2A.Z in Xenopus laevis (Ridgway, P., et al., J. Biol. Chem. 279, 43815-43820), and a different phenotype is observed compared to what is reported in this manuscript. Therefore, it is important to clarify that the observed impairment of craniofacial morphogenesis might be dependent or indeed independent of H2A.Z.

16. It is worth highlighting that while changes in gene expression due to the loss of PWWP2A may impact the segregation of chromosomes, other possibilities are not excluded by the data. For example, it has been shown that H2A.Z (and thus PWWP2A) can have a direct role in chromosome segregation (Sharma et al., Mol Cell Biol 33, 3473-81 (2013); Greaves et al. Proc Natl Acad Sci U S A 104, 525-30 (2007). While not seen here, a previous report also showed that INCENP is a direct H2A.Z interacting protein (Rangasamy et al., EMBO J. 22, 1599-1607(2003)) supporting the notion

that, in addition, protein interacting partners may also regulate H2A.Z function in chromosome segregation.

In conclusion, the finding that PWWP2A is a H2A.Z interacting protein is a significant finding.Whether this interaction is required for craniofacial morphogenesis remains to be determined.

Referee #2:

Pünzeler and co-authors employ a label-free quantitative mass spectrometry approach to identify a vertebrate-specific H2A.Z-nucleosome binder PWWP2A. Biochemical analyses show that PWWP2A interacts with H2A.Z-nucleosome through a PWWP domain and an internal region (I). Whereas the N-terminal internal region (IC) dictates the H2A.Z specificity, the PWWP domain and the N-terminal internal region (IN) contribute to nucleosome (or DNA) binding. Consistently, FRAP results suggest that the PWWP domain and internal region are required for maintaining recovery kinetics of PWWP2A. Further, the Chip-seq data reveal a PWWP2A chromatin occupancy profile which is largely determined by chromatin positioning of H2A.Z. The authors also demonstrate PWWP2A depletion causes mitotic delay in human cell and PWWP2A knockdown results in severe cranial facial defects in Xenopus organ development.

It is of general interest to understand the underlying mechanism by which H2A.Z affects various DNA-based processes of DNA transcription, replication and repair. There are several studies reporting how H2A.Z-H2B dimer specifically interact with H2A.Z chaperones, most notably YL1, Swr1, Anp32e, and others. The current studies provide new information into how proteins with important functions show preference for H2A.Z-nucleosome.

Collectively, this is a very interesting and important addition to the growing field of H2A.Z variant study. The work is well presented with good in vivo and in vitro data. Although the manuscript shows that the chromatin occupancy of PWWP2A largely overlapped with that of H2A.Z, the mechanism by which H2A.Z-nucleosome (and unknown elements) regulate PWWP2A occupancy remain mainly unrevealed, which limits overall enthusiasm.

Points to address:

• A early study based on mass spectrum approach has identified a number of H2A.Z-nucleosome binding proteins which include Brd2 and PWWP2A (Draker R et al, PloS genetics, 2012). It is demonstrated, in that study, that PWWP2A displays preference for H2A.Z-nucleosome over H2A-nucleosome. It is important that the authors should clarify the early result in this manuscript.

• It is unknown how H2A.Z-nucleosome regulates PWWP2A occupancy and how PWWP2A counteracts H2A.Z localization. To address this problem, it might be worth testing H2A.Z's chromatin occupancy in PWWP2A-knockdown or PWWP2A-over expression human cell. Analyses of H2A.Z's chromatin occupancy may give cues to of PWWP2A's functions

• In order to address effect of other elements like Post Transcription Modification (PTM) and Nuclear Depletion Region (NDR), it might be helpful if the authors could test binding of PWWP2A to recombinant mono-nucleosome containing H2A.Z or H2A.

• It is somewhat surprising that the C-terminal region of internal region (IC) fails to IP nucleosome while it shows substantial preference for H2A.Z (IP experiments in Figures 2E & 2F). In this regard, IC may serve as a H2A.Z chaperone and likely interact with H2A.Z-H2B dimer rather than H2A.Z-nucleosom. To test binding of IC to H2A.Z-H2B dimer would help to explore the biological functions of PWWP2A.

Minor points:
• Figure 2C, it is better to briefly describe PWWP2A domain/region in figure legends
• Figure 5A, the authors describe luci in figure legends. The result is shown in figure 5B.
• On page 12, 1st paragraph, the authors describe a "full rescue" of Xenopus PWWP2A depletion by human PWWP2A RNA. The result shows 70% normal head in Figure 7C.

• On page 15, 1st paragraph, citation format is incorrect.

Referee #3:

There's a relevant story to this study and a suitable succession of diverse experiments leading to consistent conclusions. The effective quantitative mass spectrometry approach allowed to identify novel H2A.Z interactors although some concerns can arise as for identifying weak and transient interactions. The previously uncharacterized protein PWWP2A has been identified as an H2A.Z-specific multivalent chromatin binder. The characterization of PWWP2A which is the focus of the study, did address the multivalent binding mode of PWWP2A to chromatin, the genome-wide localization of PWWP2A to the promoters of highly transcribed genes and the biological function of PWWP2A in developmental process regulation, cell morphogenesis, neural crest differentiation and migration during development. Although the characterization of PWWP2A is convincing, I do have some comments and suggestions for the authors.

- Introduction: The introduction is focused on the authors approach to identify and characterize PWWP2A and explains the logic and progression of this study. However, the introduction part on H2A.Z lacks some information in my opinion. I agree that the mechanisms by which H2A.Z contributes to DNA-based processes are still unclear, but we do have evidence that H2A.Z is involved in the recruitment of nuclear proteins at promoters. The H2A.Z-containing nucleosome possesses an uninterrupted acidic surface formed by amino acids from the H2A.Z C-terminal docking domain and one amino acid from H2B. This acidic patch on the surface of the H2A.Z-H2B dimer is thought to provide a binding platform for nuclear proteins including chromatin remodeling complexes and transcription factors (Suto et al., 2000).

- The authors hypothesize that H2A.Z would be a general but selective recruitment factor of chromatin-modifying proteins therefore it would be relevant to mention the acidic patch of H2A.Z-containing nucleosome in the introduction.

- Two separate internal region of PWWP2A confer nucleosome binding and H2A.Z-specificity: The pull-downs of the recombinant GST-PWWP2A PWWP domain deletions or PWWP2A internal deletions with the mononucleosomes show that two regions of PWWP2A seem to be involved in chromatin interaction. The first one is the PWWP domain which is involved in chromatin interaction. The second one is the Internal region (I) which can be further divided into the NI region, necessary for nucleosome binding, and the CI region, mediating H2A.Z-specificity.

- The authors hypothesized repeatedly that H2A.Z serves as a binding platform for distinct chromatin modifying complexes although they don't address the direct interaction between PWWP2A and the acidic patch of the H2A.Z-containing nucleosome which is thought to provide this binding platform. Mutations in the acidic patch of the H2A.Z-containing nucleosome would allow to verify if PWWP2A binds directly with the acidic surface on the nucleosome. Moreover, using the PWWP2A deletions, the authors would be able to identify which region of PWWP2A exactly binds the acidic patch. If PWWP2A interacts directly with the acidic patch of H2A.Z-containing nucleosomes, the authors could provide an explanation and a mechanism for PWWP2A recruitment at promoters.

- PWWP2 binds H2A.Z nucleosomes at TSS of actively transcribed genes: The ChIP-seq results suggest that H2A.Z is the main but not sole determinant for PWWP2A's site specificity. Moreover, PWWP2A accumulated at the nucleosome-depleted region (NDR), possibly recognizing free DNA via its PWWP domain. A major point of this study would be to separate the different ways by which PWWP2A is recruited to promoters, namely its interaction with H2A.Z and its recognition of free DNA at the NDR.

- Performing a ChIP-seq in a H2A.Z knockdown cell line would allow to verify the localization of PWWP2A in the absence of H2A.Z. It would be interesting to verify if the NDR free DNA alone is sufficient to recruit PWWP2A.

- Raisner et al. (2005) performed an experiment in which a 22 bp DNA fragment from the SNT1 gene promoter was introduce in a coding region. Surprisingly, this insertion was sufficient to form a

new nucleosome-depleted region (NDR) within the coding region and interestingly, H2A.Z was incorporated in the nucleosome flanking the new NDR. It would be interesting to verify if PWWP2A would be recruited to a new NDR in a coding region and to compare PWWP2A recruitment at the new NDR in a H2A.Z knockdown cell line.

- PWWP2A depletion results in a proliferation defect caused by a metaphase-anaphase block: Rangasamy et al. (2003) and Fan et al. (2004) showed that H2A.Z interacts directly with HP1α and INCENP in mice, which are essential proteins for chromosome segregation. H2A.Z co localizes with HP1α and INCENP at pericentric heterochromatin which plays a role in chromosome segregation. Moreover, it has been demonstrated that HP1α binds directly the C-terminal region of H2A.Z. Rangasamy et al. (2004) inhibited H2A.Z expression in Cos-7 and mouse L929 cells using an RNAi approach which resulted in a mislocalization of HP1α and a lagging in chromosome segregation. The lagging chromosome phenotype is also observed in fission yeast Swi6 (HP1) mutants (Ekwall et al., 1995).

- It has been demonstrated that H2A.Z has a global role in chromosome segregation. The authors could verify if a knockdown of H2A.Z in their experimental model results in proliferation defect caused by lagging chromosome as did their PWWP2A depletion.

- PWWP2A seems to regulate the expression of genes involved in developmental process regulation and cell morphogenesis. Maybe it would be interesting to investigate the effect of the expression of the PWWP2A mutants (ΔPWWP) or the PWWP2A deletions on the regulation of those genes. This could also investigate if a region of PWWP2A is essential for its role in regulation of gene expression. Also, this experiment would allow to identify the regions of PWWP2A which are essential for recruitment or interaction with target and downstream proteins.

- Figure 6F: the names PW#1 and PW#2 are partially overlapping the figure.

- Discussion: Although the discussion brings new elements and clarifications about the structure and function of PWWP2A, almost half of the discussion is focused on the quantitative mass spectrometry approach and the identification of H2A.Z interactors which led to the identification of PWWP2A. I understand that the identification of H2A.Z interactors is a really important part of the study but given that the identification of H2A.Z mononucleosome binders was part of previous work (Vardabasso et al., 2015) and that the majority of the experiments address the characterization of PWWP2A, this should reflect in the discussion.

References:

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Fan, J. Y., Rangasamy, D., Luger, K., and Tremethick, D. J. (2004). H2A.Z alters the nucleosome surface to promote HP1alpha-mediated chromatin fiber folding. Mol. Cell. 16:655-661.
Raisner, R. M., Hartley, P. D., Meneghini, M. D., Bao, M. Z., Liu, C. L., Schreiber, S. L., Rando, O. J., and Madhani, H.D. (2005). Histone variant H2A.Z marks the 5' ends of both active and inactive genes in euchromatin. Cell. 123:233-248.
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Rangasamy, D., Greaves, I., and Tremethick, D. J. (2004). RNA interference demonstrates a novel role for H2A.Z in chromosome segregation. Nat. Struct. Mol. Biol. 11: 650-655.
Suto. R. M., Clarkson, M. J., Tremethick, D. J., and Luger, K. (2000). Crystal structure of a nucleosome core particle containing the variant histone H2A.Z. Nat. Struct. Biol. 7:1121-1124.

1st Revision - authors' response 27 April 2017

In general, we have addressed the major concerns in the following ways:

1. *Is PWWP2A binding to H2A.Z causally involved in the developmental phenotypes upon PWWP2A morpholino knockdown*

This question is highly interesting, but also technically quite challenging to address as almost all feasible experiments will only provide correlative but not causative data. We chose to employ PWWP2A deletion rescue experiments in order to find out whether the region mediating H2A.Z-specificity (IC) or the PWWP domain needed for DNA binding (ΔPWWP) or the combination of these domains (I_S_PWWP) are sufficient for PWWP2A morpholino knockdown phenotype rescue. Hence, we had to generate one additional PWWP2A deletion protein (ΔIC) lacking the region directly responsible for H2A.Z-specificity (see Fig. 2F, G, new Fig. 7C and new Appendix Fig. S4C). While recues with human ΔPWWP or I_S_PWWP ameliorate the morphant phenotype, although at slightly lower efficiency than full length PWWP2A, the DIC variant, in contrast, cannot rescue this defect any more (new Fig. 8A and new Appendix Table S4). These new data sets suggest that PWWP2A's interaction with H2A.Z nucleosomes is important for its function in craniofacial development.

2. *Does H2A.Z occupancy change upon PWWP2A loss- or gain-of-function conditions to better define the molecular function of PWWP2A*

We agree that this is an important point to address and we have performed several independent experiments to examine whether H2A.Z occupancy changes upon PWWP2A depletion or overexpression. Interestingly, reduction of PWWP2A by RNAi does not influence H2A.Z chromatin binding ability as determined by FRAP (new Fig. 7A and new Appendix Fig. S4A) and does not significantly change H2A.Z chromatin occupancy as shown by ChIP-seq (new Fig. 7B and new Fig. EV4A, B) or global H2A.Z levels (new Fig. EV4C). Likewise, overexpression of PWWP2A (new Appendix Fig. S4B) did not significantly influence H2A.Z occupancy, as we revealed by ChIP-qPCR (new Fig. EV4D). Both approaches indicate that PWWP2A is not needed for H2A.Z deposition, ejection or nucleosome stability. In agreement with this data set is our new observation that PWWP2A interact with H2A.Z's C-terminal tail (new Fig. 7E and new Fig. EV4E), but not the acidic patch needed for chaperone binding and H2A.Z deposition. Additionally, as we and others have never observed PWWP2A to be part of one of the distinct H2A.Z chaperone complexes (Bönisch et al., 2012, NAR), we do not expect it to be involved in H2A.Z deposition or ejection.

3. *What is the functional relevance of the bivalent binding determinants in PWWP2A, possibly in relation to the nucleosome acidic patch? Additionally, separate PWWP2A promoter recruitment via H2A.Z and nucleosome-free DNA.*

The referees and the editor raise an interesting point, which we tried to address experimentally. We have tried the following to address the question about a possible H2A.Z acidic-patch binding-requirement. We have tested cell-derived mononucleosomes containing Flag-tagged H2A, H2A.Z and H2A.Z C-terminus deletion proteins for their ability to bind recombinant PWWP2A. Interestingly, deletion of the last nine amino acids of the C-terminal tail of H2A.Z (the extended acidic patch is still present and the mutant incorporated into nucleosomes) significantly reduced PWWP2A interaction down to the level observed with H2A-containing nucleosomes (new Fig. 7E and new Fig. EV4E). This indicates that H2A.Z's C-terminus and not the acidic patch is crucial for PWWP2A interaction. We also tested whether H2A.Z is needed for PWWP2A chromatin recruitment *in vitro* (new Fig. 2C) and *in vivo* (new Fig. 7C, D and new Appendix Fig. S4C). Indeed, cellular loss of H2A.Z reduces but does not abolish PWWP2A chromatin binding due to its multivalent binding abilities. We tried several approaches to look whether H2A.Z loss affects PWWP2A recruitment to promoter regions, but we did not succeed in knocking down both H2A.Z isoforms in human cells (see also Vardabasso et al., Mol Cell, 2015), most likely due to H2A.Z' crucial function. Unfortunately, the PWWP2A antibody used in this study does not seem to work in IP and therefore also not in ChIP-seq experiments,. Hence, any *in vivo* statement on PWWP2A's recruitment to promoters via H2A.Z and nucleosome-free DNA can presently not be made.

4. *Clarify PWWP2A-H2A.Z interactions on the level of endogenous proteins and solve specific technical and presentational issues.*

We apologize for any technical and presentational mistakes, which we have now corrected. Additionally, we now show the interaction of endogenous H2A.Z nucleosomes with endogenous PWWP2A (new Fig. EV1H). Please notice that we had to move some detailed descriptions from the materials and method part into the appendix in order to meet the 55,000 character restriction criteria. Additionally, due to the vast amount of new data sets we also included EV and Appendix Figures.

All changes made to the manuscript are highlighted in red and described point-by-point in our response below.

Responses to Referee #1:

This is a very interesting manuscript that identifies a new H2A.Z interacting protein PWWP2A. It employs a multifaceted approach to investigate the function and genomic locations of PWWP2A, identified by a previously employed GFP-based mass spec approach. Interactions between PWWP2A and H2A.Z were supported by cellular and biochemical assays, and the region of PWWP2A required for this interaction was revealed. It genomic locations were identified, which was shown to co localise with H2A.Z at the TSS but not at non-promoter regions. Functionally, it was demonstrated to have a role in chromosome segregation, albeit, in a cancer cell line. Most interestingly, using Xenopus toads as a model organism, the knockdown of PWWP2A interfered with craniofacial morphogenesis. To strengthen the manuscript, the following technical, informational and conceptual issues should be addressed or clarified.

1. Are GFP-H2A, H2A.Z.1 and H2A.Z.2 all expressed to a similar level?

Using flow cytometry and immunoblotting we could show that all GFP-tagged histones are expressed to equal amounts. This new data set is found in new Figure EV1A, B.

2. Concerning the mass spec analysis, is it formally possibly that the presence of the GFP tag may prevent some H2A.Z-non-histone protein interactions?

While we cannot formally exclude this possibility, we find it highly unlikely. The GFP-tag is located at the flexible N-terminus and allows recognition of all H2A.Z-specific chaperone complex members via the C-terminus, as we have demonstrated previously (Bönisch et al, NAR, 2012). Additionally, we recently compared GFP-H2A.Z.1 / GFP-H2A.Z.2 ChIP-seq profiles with ChIP-seq data sets acquired using an antibody against endogenous H2A.Z and did not observe any differences in chromatin occupancy (Vardabasso et al., Mol Cell, 2015). All of these data support the notion that the N-terminal GFP-tag does not have any structural or functional influence on H2A.Z.

3. The western blot in Fig.1D showing PWWP2A is unclear especially for HK cells. Three bands are observed, which one is PWWP2A? Plus all three bands can be seen for the H2A pull-down.

We agree with the reviewer and are puzzled by this observation as well. In all experiments, regardless of whether we use GFP-tagged PWWP2A or detect endogenous PWWP2A with the specific Novus/Acris antibody, we always observe multiple bands (ranging from the predicted ~80 kDa to 120 kDa, see also new Fig. 1D and Fig. EV4C). Although, PWWP2A has been predicted to be alternatively spliced giving rise to at least three different isoforms (isoform 1 (canonical): 82 kD; isoform 2: 61 kD and isoform 3: 56 kD; http://www.uniprot.org/uniprot/Q96N64) the masses do not match the detected band heights. Also, since our GFP-PWWP2A protein shows a similar running behavior as the endogenous protein (see Fig. 1E), alternative splicing cannot be the cause for the detection of these many bands. We speculate that PWWP2A is differentially modified and possibly degraded, a hypothesis we plan to address in the future.

4. For nuclear staining experiments (Fig. 1SF), have you examined whether PWWP2A co localises with H2A.Z particularly the fraction that is stably bound to chromatin during metaphase?

Unfortunately, both H2A.Z and PWWP2A antibodies are of rabbit origin and co-stainings of the endogenous proteins were therefore extremely difficult to perform. Using GFP-tagged cell lines the signals were either too strong (in case of GFP-H2A.Z) or too low (in case of GFP-PWWP2A) and we therefore did not manage to obtain any meaningful chromosome spreads by now.

5. In Fig 1E, H2A.Z IPs brings down H2A implying a fraction of H2A.Z nucleosomes are heterotypic. Following quantification and longer exposure times of PWWP2A IPs, can H2A also be detected? This could be interesting as to whether PWWP2A binds to homotypic or heterotypic H2A.Z nucleosomes.

Indeed, we see some few H2A precipitated with PWWP2A in different pull-down experiments. This observation can be explained in two different ways: 1. PWWP2A binds to heterotypic nucleosomes, and/or 2. PWWP2A also binds, to a lesser degree, to H2A nucleosomes. As the N-terminal part of PWWP2A's internal region mediates nucleosome binding independent of histone variant content (Fig. 2F, G) and the PWWP domain is able to interact with naked DNA (Fig. 3B), the second possibility is likely to be true as well. Novel *in vitro* nucleosome binding assays support this alternative (without excluding the first possibility), as PWWP2A is able to directly bind both H2A.Z- as well as H2A-containg recombinant nucleosomes, albeit with a higher affinity for H2A.Z nucleosomes (new Fig. 2C). In conclusion, our data suggest that due to its multivalent binding mode PWWP2A is able to bind to nucleosomes and DNA, with a preference for, but not exclusively to, H2A.Z thereby being also able to interact with heterotypic nucleosomes.

6. All of the experiments have been performed with GFP-tagged histones. Are either PWWP2A or H2A.Z antibodies good enough to see the endogenous interaction?

We have used GFP-H2A.Z to detect endogenous PWWP2A binding (Fig. 1D) and GFP-PWWP2A to demonstrate endogenous H2A.Z interaction (Fig. 1E). Now, we have also tried to pull-down endogenous H2A.Z or H2A with respective antibodies and could detect endogenous PWWP2A enrichment in the H2A.Z and not the H2A precipitated sample (see new Fig. EV1H) further confirming this interaction.

7. In Fig.2A it is stated that equal amounts of GST and PWWP2A-GST were used in pull-down assays yet in the figure shown there is dramatically more GST protein then PWWP2A-GST protein. Why is this the case? Also clearly the purity of PWWP2A-GST (and the deletion mutant constructs) differ significantly so how do you control for this? Similarly in Fig. 2C, PWWP2A and the two mutants all appear to bind to a similar amount of nucleosomes (H3) yet the amount of each protein appears to be dramatically different.

We apologize for this confusion and have removed the sentence. Due to different degrees of degradation we could not provide exactly equal amounts of the different constructs for pull-down assays. Nevertheless, although we are not able to quantitatively measure any interaction strengths using these assays, our conclusion on PWWP2A's multivalent binding properties have been confirmed in many biologically repeated experiments and diverse assays.

8. I am puzzled with Fig. 2B, as more H2A.Z is present in the PWWP2A pull downs of H2A nucleosomes then H2A.Z.2 nucleosomes.

In this experiment, we have used recombinant GST-PWWP2A and incubated it with mononucleosomes derived from HeLaK cells expressing GFP-H2A, GFP-H2A.Z.1 or GFP-H2A.Z.2. Indeed, when using an α-GFP antibody for detection of the presence of GFP-H2A or GFP-H2A.Z variant, we find a strong enrichment of both GFP-H2A.Z isoforms compared to GFP-H2A. Using the antibody against endogenous H2A.Z, it is obvious that we pull-down endogenous H2A.Z in all three experiments with GFP-PWWP2A. The small difference in endogenous H2A.Z band intensity between the lanes where GFP-H2A or GFP-H2A.Z.2 mononucleosomes were used as prey does not appear to be biological meaningful. Overall, this experiment, together with many others, clearly depicts PWWP2A's specificity for H2A.Z over H2A.

9. In Fig. 2E, it appears that IC binds to H2A.Z but not nucleosomes because the other histones are depleted. How is this possible? Is Fig. 2F consistent with Fig. 2E because in Fig. 2F, IN now binds to H2A.Z.2?

We apologize for the bad quality of the image, which we have replaced with another new experiment showing the same (binding of some few nucleosomes to IC) but in higher resolution (new Fig. 2F). We hope that it is now easier to see that GST-IC also pulls down some few nucleosomes (in contrast to the negative control GST), suggesting that these few nucleosomes all contain H2A.Z, which is also confirmed in Fig. 2G. Concerning IN, this particular region does bind nucleosomes without any specificity towards the H2A variant content, meaning it binds H2A, as well as H2A.Z (Fig. 2G).

10. On page 6 it is stated that the DNA binding activity of PWWP2A contributes to chromatin interactions but no experiments have been performed that actually demonstrates this. Indeed Fig. 2C shows that this is not the case (for nucleosomes). Unless a loss of chromatin binding is actually shown, it is better to remove this statement.

To address this comment, we have now tested the ΔPWWP construct that is still able to bind nucleosomes (Fig. 2D) in FRAP assays. Indeed, loss of the PWWP domain leads to a strong increase in protein mobility (new Fig. 3C), but never reaches the high mobility of GFP or PWWP, S_PWWP constructs. These data confirm our suggestion that the PWWP domain contributes to some extent to PWWP2A's strong chromatin interaction.

11. Again on page 6, it is stated that "In concordance with our in vitro data, mutant constructs containing either the internal or the PWWP domain featured much faster recovery kinetics than wild type" however the in vitro data presented shows the opposite. Fig. 2C demonstrates that both the PWWP mutant and this domain by itself binds to nucleosomes just as well as the full-length protein. This statement requires clarification.

We thank the reviewer for this clarification. We have now removed the beginning of the sentence "In concordance with our in vitro data…" from the text.

12. There are over 10,000 total peaks and around 4,000 promoter peaks that contain PWWP2A and H2A.Z. What is the explanation as to why the expression of so few genes, in comparison (about 700), change when PWWP2A is knocked down. An important question is of this approx. 700, how many of these genes have PWWP2A directly associated with their TSS.

This is indeed an interesting observation, for which we currently do not have an explanation. We have reanalyzed the data to determine the overlap between all PWWP2A promoter peaks (6468) and genes that are deregulated upon PWWP2A depletion (247 down- and 342 up-regulated) (Rebuttal Fig. R1). It is obvious that no direct correlation between presence of PWWP2A at the TSS and up- or down-regulation of a particular gene is observed. We are therefore not able to tell what are possible direct targets of PWWP2A and what are indirect effects we observe.

[Data not included in peer review process file.]

13. With regards to the experiments depicted in Fig. S6, in the PWWP2A knockdown cells, the chromosomes appear to be slightly fuzzier. Has any quantitative assessment been made with regards to the size of the chromosomes, which would fit with an increase in nuclear size. Also, is there any loss of cohesion i.e. does the distance between the centromeres at metaphase increase (a few chromosomes may show this)?

Indeed, we sometimes observed differences in fuzziness of chromosomes. But, these differences were not consistent between biological replicates implying that chromosome thickness depends rather on experimental conditions than on biological effects. Nevertheless, we have looked at several markers involved in chromosome movement (Aurora B, INCENP, etc) to see whether PWWP2A loss affects their localization. In all cases, PWWP2A depletion did not show any effect (see below response to point 16).

14. The following speculative sentence on page 8 of the results section should be left for the discussion, and thus removed from the results section "As many genes involved in cellular component assembly were deregulated (Fig. S5), it is likely that global rather than specific cellular changes affected nuclear".

We have removed the sentence.

15. In the literature, the following study used morpholino's to knockdown H2A.Z in Xenopus laevis (Ridgway, P., et al., J. Biol. Chem. 279, 43815-43820), and a different phenotype is observed compared to what is reported in this manuscript. Therefore, it is important to clarify that the observed impairment of craniofacial morphogenesis might be dependent or indeed independent of H2A.Z.

We do not see a discrepancy between this study and our data. Ridgway et al. reported that H2A.Z mRNA expression is developmentally regulated and increases over maternal levels from gastrula stages onwards, with mRNA peaks in both mesodermal (Notochord) and ectodermal placode (Otic vesicle) derived tissues. Together with other studies, demonstrating broad expression of H2A.Z in Xenopus (Iouzalen et al, NAR, 1996), we conclude that this histone variant and pwwp2a are coexpressed from at least gastrula stages onwards, the most important prerequisite for a potential biochemical interaction. The RNAi mediated knockdown of H2A.Z mRNA was reported to cause predominantly gastrulation defects, and the consequences on subsequent development were assessed at a developmental stage, before discrete landmarks of head differentiation such as retinal pigment and branchial arch formation are overtly visible. It is possible that defects in head formation are masked by the prominent gastrulation defect and /or have been overlooked, because embryos were analysed earlier than in our study. Clearly, Ridgway et al. have neither investigated *twi* mRNA expression nor cartilage formation. Nevertheless, the question whether the observed impairment of craniofacial morphogenesis depends or not depends on H2A.Z is valid. We have addressed this issue by testing, which domains in human PWWPA protein are required to restore craniofacial structures in pwMO morphants. In the updated Figure 8 and new Appendix Table S4 we now show that both I_S_PWWP and DPWWP proteins ameliorate the pwMO phenotype, although at slightly lower efficiency than full length PWWP2A, in contrast to the DIC variant, which cannot rescue this defect any more. This result strongly suggests that Xenopus pwwp2a protein exerts its developmental function in craniofacial development through interaction with H2A.Z. This conclusion is in full agreement with other data in our manuscript, and applies to regions of maximal *pwwp2a* mRNA expression in the frog.

16. It is worth highlighting that while changes in gene expression due to the loss of PWWP2A may impact the segregation of chromosomes, other possibilities are not excluded by the data. For example, it has been shown that H2A.Z (and thus PWWP2A) can have a direct role in chromosome segregation (Sharma et al., Mol Cell Biol 33, 3473-81 (2013); Greaves et al. Proc Natl Acad Sci U S A 104, 525-30 (2007). While not seen here, a previous report also showed that INCENP is a direct H2A.Z interacting protein (Rangasamy et al., EMBO J. 22, 1599-1607(2003)) supporting the notion that, in addition, protein interacting partners may also regulate H2A.Z function in chromosome segregation.
We agree with the reviewer that it is possible that INCENP or other proteins may impact chromosome segregation via H2A.Z-PWWP2A. We tested some of the most likely candidate proteins (INCENP, CTCF) on chromosome spreads (Rebuttal Figure R2) and Aurora-B, as well as INCENP by immunofluorescence microscopy of mitotic cells (Rebuttal Figure R3) upon PWWP2A knockdown.

[Data not included in peer review process file.]

Although some cells showed differences in localization of the analyzed proteins when using one siRNA, they did not show any aberrant behavior when using a second independent siRNA. These data suggest that at least these proteins are not likely the cause of the observed mitotic phenotype.

Responses to Referee #2:

Punzeler and co-authors employ a label-free quantitative mass spectrometry approach to identify a vertebrate-specific H2A.Z-nucleosome binder PWWP2A. Biochemical analyses show that PWWP2A interacts with H2A.Z-nucleosome through a PWWP domain and an internal region (I). Whereas the N-terminal internal region (IC) dictates the H2A.Z specificity, the PWWP domain and the N-terminal internal region (IN) contribute to nucleosome (or DNA) binding. Consistently, FRAP results suggest that the PWWP domain and internal region are required for maintaining recovery kinetics of PWWP2A. Further, the Chip-seq data reveal a PWWP2A chromatin occupancy profile which is largely determined by chromatin positioning of H2A.Z. The authors also demonstrate PWWP2A depletion causes mitotic delay in human cell and PWWP2A knockdown results in severe cranial facial defects in Xenopus organ development.
It is of general interest to understand the underlying mechanism by which H2A.Z affects various DNA-based processes of DNA transcription, replication and repair. There are several studies reporting how H2A.Z-H2B dimer specifically interact with H2A.Z chaperones, most notably YL1, Swr1, Anp32e, and others. The current studies provide new information into how proteins with

important functions show preference for H2A.Z-nucleosome. Collectively, this is a very interesting and important addition to the growing field of H2A.Z variant study. The work is well presented with good in vivo and in vitro data. Although the manuscript shows that the chromatin occupancy of PWWP2A largely overlapped with that of H2A.Z, the mechanism by which H2A.Z-nucleosome (and unknown elements) regulate PWWP2A occupancy remain mainly unrevealed, which limits overall enthusiasm.

Points to address:

1. A early study based on mass spectrum approach has identified a number of H2A.Z-nucleosome binding proteins which include Brd2 and PWWP2A (Draker R et al, PloS genetics, 2012). It is demonstrated, in that study, that PWWP2A displays preference for H2A.Z-nucleosome over H2A-nucleosome. It is important that the authors should clarify the early result in this manuscript.

We have now included a sentence in the beginning of the manuscript.

2. It is unknown how H2A.Z-nucleosome regulates PWWP2A occupancy and how PWWP2A counteracts H2A.Z localization. To address this problem, it might be worth testing H2A.Z's chromatin occupancy in PWWP2A-knockdown or PWWP2A-over expression human cell. Analyses of H2A.Z's chromatin occupancy may give cues to of PWWP2A's functions.

We thank the reviewer for his/her insightful suggestion. We have now performed FRAP, as well as H2A.Z ChIP-seq analyses upon PWWP2A RNAi knockdown and ChIP-qPCR analyses upon PWWP2A overexpression. Interestingly, neither H2A.Z mobility (new Fig. 7A and new Appendix Fig. S4A) nor H2A.Z chromatin occupancy or global expression levels (new Fig. 7B and new Fig. EV4A-C) did change when comparing control knockdown versus PWWP2A-reduced cells. Additionally, ChIP-qPCR experiments after transient overexpression of Cherry-tagged PWWP2A in GFP-H2A.Z.1 cells and FACS sorting of those cells expressing low or high Cherry-PWWP2A levels (new Appendix Fig. S4B) revealed that H2A.Z occupancy is not influenced by the amount of PWWP2A protein (new Fig. EV4D). In conclusion, these results strongly suggest that PWWP2A does not regulate H2A.Z deposition or chromatin occupancy, a result that is in accordance to our previous finding that PWWP2A is not part of an H2A.Z-specific chaperone/remodeling complex (Bönisch et al., NAR, 2012). In agreement, PWWP2A recognizes the C-terminal tail of H2A.Z but not the extended acidic patch that mediates chaperone interaction (please see response to referee #3, point 2 and new Fig. 7E and new Fig. EV4E).

3. In order to address effect of other elements like Post Transcription Modification (PTM) and Nuclear Depletion Region (NDR), it might be helpful if the authors could test binding of PWWP2A to recombinant mono-nucleosome containing H2A.Z or H2A.

This is a very good suggestion. We have now reconstituted recombinant nucleosomes containing either H2A or H2A.Z and have preformed competitive EMSA experiments with recombinant GST-PWWP2A (new Fig. 2C). Interestingly, GST-PWWP2A is able to bind to recombinant nucleosomes, showing that this interaction is direct and independent of PTMs or additional factors. Using this assay, GST-PWWP2A slightly prefers H2A.Z- over H2A-containing nucleosomes suggesting that variant-specificity is directly conferred but H2A can also be recognized by PWWP2A.

4. It is somewhat surprising that the C-terminal region of internal region (IC) fails to IP nucleosome while it shows substantial preference for H2A.Z (IP experiments in Figures 2E & 2F). In this regard, IC may serve as a H2A.Z chaperone and likely interact with H2A.Z-H2B dimer rather than H2A.Z-nucleosom. To test binding of IC to H2A.Z-H2B dimer would help to explore the biological functions of PWWP2A.

Please see also our comment to point 9 of referee #1. In order to improve image quality showing that IC is also able to bind nucleosomes, albeit at a much lower level than IN, we have replaced the previous blot and Coomassie gel with another own showing the same result (binding of some few nucleosomes to IC) but in higher resolution (new Fig. 2F). We hope that it is now easier to see that GST-IC also pulls down some few nucleosomes (in contrast to the negative control GST), suggesting that these few nucleosomes bound by IC all contain H2A.Z, which is in agreement with

results depicted in Fig. 2G. Concerning the second comment: from all experiments performed, it is not very likely that PWWP2A's function relates to a possible H2A.Z-H2B dimer binding ability. There are several reasons: 1. PWWP2A was not identified in any H2A.Z-specific chaperone complex that binds to H2A.Z-H2B dimers (see Bönisch et al., NAR, 2012 and unpublished data), it was only identified in H2A.Z-mononucleosome IPs. 2. IC is able to bind nucleosomes (does not exclude the possibility of recognizing dimers). 3. PWWP2A depletion does not change H2A.Z chromatin binding and occupancy (new Fig. 7A, B and new Fig. EV4A-C) not does PWWP2A overexpression affect H2A.Z genomic localization (new Fig. EV4D and new Appendix Fig. S4B).

Minor points:

• *Figure 2C, it is better to briefly describe PWWP2A domain/region in figure legends*

We have changed the figure legend accordingly (notice that Fig. 2C is now Fig. 2D).

• *Figure 5A, the authors describe luci in figure legends. The result is shown in figure 5B.*

We have normalized to PWWP2A expression in luciferase control transfectant cells. Therefore, we also mention luci in the figure legend of panel A.

• *On page 12, 1st paragraph, the authors describe a "full rescue" of Xenopus PWWP2A depletion by human PWWP2A RNA. The result shows 70% normal head in Figure 7C.*

We apologize for our mistake and have corrected the text accordingly.

• *On page 15, 1st paragraph, citation format is incorrect.*

We thank the referee for his/her attentiveness and have corrected this mistake.

Responses to Referee #3:

There's a relevant story to this study and a suitable succession of diverse experiments leading to consistent conclusions. The effective quantitative mass spectrometry approach allowed to identify novel H2A.Z interactors although some concerns can arise as for identifying weak and transient interactions. The previously uncharacterized protein PWWP2A has been identified as an H2A.Z-specific multivalent chromatin binder. The characterization of PWWP2A which is the focus of the study, did address the multivalent binding mode of PWWP2A to chromatin, the genome-wide localization of PWWP2A to the promoters of highly transcribed genes and the biological function of PWWP2A in developmental process regulation, cell morphogenesis, neural crest differentiation and migration during development. Although the characterization of PWWP2A is convincing, I do have some comments and suggestions for the authors.

1. Introduction: The introduction is focused on the authors approach to identify and characterize PWWP2A and explains the logic and progression of this study. However, the introduction part on H2A.Z lacks some information in my opinion. I agree that the mechanisms by which H2A.Z contributes to DNA-based processes are still unclear, but we do have evidence that H2A.Z is involved in the recruitment of nuclear proteins at promoters. The H2A.Z-containing nucleosome possesses an uninterrupted acidic surface formed by amino acids from the H2A.Z C-terminal docking domain and one amino acid from H2B. This acidic patch on the surface of the H2A.Z-H2B dimer is thought to provide a binding platform for nuclear proteins including chromatin remodeling complexes and transcription factors (Suto et al., 2000).

➢ *The authors hypothesize that H2A.Z would be a general but selective recruitment factor of chromatin-modifying proteins therefore it would be relevant to mention the acidic patch of H2A.Z-containing nucleosome in the introduction.*

We thank the referee for his/her suggestion and have now changed the introduction part accordingly.

2. Two separate internal region of PWWP2A confer nucleosome binding and H2A.Z-specificity: The pull-downs of the recombinant GST-PWWP2A PWWP domain deletions or PWWP2A internal

deletions with the mononucleosomes show that two regions of PWWP2A seem to be involved in chromatin interaction. The first one is the PWWP domain which is involved in chromatin interaction. The second one is the Internal region (I) which can be further divided into the NI region, necessary for nucleosome binding, and the CI region, mediating H2A.Z-specificity.

➢ *The authors hypothesized repeatedly that H2A.Z serves as a binding platform for distinct chromatin modifying complexes although they don't address the direct interaction between PWWP2A and the acidic patch of the H2A.Z-containing nucleosome which is thought to provide this binding platform. Mutations in the acidic patch of the H2A.Z-containing nucleosome would allow to verify if PWWP2A binds directly with the acidic surface on the nucleosome. Moreover, using the PWWP2A deletions, the authors would be able to identify which region of PWWP2A exactly binds the acidic patch. If PWWP2A interacts directly with the acidic patch of H2A.Z-containing nucleosomes, the authors could provide an explanation and a mechanism for PWWP2A recruitment at promoters.*

This is a very interesting point. Using a construct with a 9 aa deletion of the C-terminus of H2A.Z (the extended acidic patch is still present) (new Fig. EV4E) we were able to show that PWWP2A binding to these nucleosomes is strongly reduced (new Fig. 7E), demonstrating the independence of the acidic patch for PWWP2A nucleosome interaction and the necessity of the last 9 amino acids of the flexible C-terminal tail.

3. PWWP2 binds H2A.Z nucleosomes at TSS of actively transcribed genes: The ChIP-seq results suggest that H2A.Z is the main but not sole determinant for PWWP2A's site specificity. Moreover, PWWP2A accumulated at the nucleosome-depleted region (NDR), possibly recognizing free DNA via its PWWP domain. A major point of this study would be to separate the different ways by which PWWP2A is recruited to promoters, namely its interaction with H2A.Z and its recognition of free DNA at the NDR.

➢ *Performing a ChIP-seq in a H2A.Z knockdown cell line would allow to verify the localization of PWWP2A in the absence of H2A.Z. It would be interesting to verify if the NDR free DNA alone is sufficient to recruit PWWP2A.*

These are all highly interesting ideas, but technically extremely challenging. We never obtained a complete reduction of H2A.Z protein using siRNAs or shRNAs, see also our recent paper Vardabasso et al., Mol Cell, 2015. H2A.Z is an essential variant in higher eukaryotes, therefore only an inducible system with the removal of both H2A.Z alleles (*H2AFZ* and *H2AFV*) will work. We have therefore teamed up with the group of Masahiko Harata (Tohoku University), who together with his group members Daisuke Takahashi and Masayuki Kusakabe are now coauthors on this manuscript. His group has developed a tetracycline-inducible H2A.Z double knockout (DKO) system in DT40 chicken cells (Kusakabe et al., Genes Cells, 2016). While birds also contain PWWP2A, the antibody epitope is only partially conserved (see alignment in Rebuttal Fig. R4). Unfortunately, antibody IP tests did not show any promising results (data not shown), we therefore suspect that the epitope is too divergent (especially N-terminally). Additionally, the antibody does not seem to work in pull-down experiments, as we are unable to perform IP experiments with HeLaK extracts. Hence, we could not perform ChIP-seq experiments with endogenous PWWP2A in DKO DT40 cells. In order to address the referee's question to the best of our abilities, we isolated mononucleosomes from WT and DKO DT40 cells and incubated those with recombinant GST-PWWP2A protein. As suspected, an enrichment of wt nucleosomes in comparison to H2A.Z-deleted nucleosomes was observed in pull-down assays (new Fig. 7D). Our result strongly supports the notion that H2A.Z is needed for a strong chromatin interaction of PWWP2A but it not the sole mediator. Again, this result underlines the finding of multivalent binding of PWWP2A. This hypothesis is also supported by our new frog rescue experiments using ΔIC, a PWWP2A construct lacking the H2A.Z-binding region that showed some slightly higher mobility in FRAP than wildtype protein (new Fig. 7C and new Appendix Fig. S4C). We demonstrate that PWWP2A lacking IC is not able to fully rescue the observed craniofacial developmental defect (new Fig. 8A and new Appendix Table S4).

[Data not included in peer review process file.]

➢ *Raisner et al. (2005) performed an experiment in which a 22 bp DNA fragment from the SNT1 gene promoter was introduce in a coding region. Surprisingly, this insertion was sufficient to form a new nucleosome-depleted region (NDR) within the coding region and interestingly, H2A.Z was incorporated in the nucleosome flanking the new NDR. It would be interesting to verify if PWWP2A would be recruited to a new NDR in a coding region and to compare PWWP2A recruitment at the new NDR in a H2A.Z knockdown cell line.*

Although we find this a highly interesting idea, it will most likely not work in vertebrate/mammalian cells. The here mentioned experiments by Raisner et al. were performed in yeast. Unfortunately, *S. cerevisiae* does not contain PWWP2A, which is vertebrate-specific. In addition, Htz1 (the yeast homolog of H2A.Z) is greatly divergent in its C-terminal tail (see Rebuttal Fig. R5), the region recognized by PWWP2A (new Fig. 7E). Therefore, it is not feasible to use this model system to test H2A.Z incorporation into a newly generated NDR region and subsequent PWWP2A recruitment.

[Data not included in peer review process file.]

4. PWWP2A depletion results in a proliferation defect caused by a metaphase-anaphase block: Rangasamy et al. (2003) and Fan et al. (2004) showed that H2A.Z interacts directly with HP1α and INCENP in mice, which are essential proteins for chromosome segregation. H2A.Z co localizes with HP1α and INCENP at pericentric heterochromatin which plays a role in chromosome segregation. Moreover, it has been demonstrated that HP1α binds directly the C-terminal region of H2A.Z. Rangasamy et al. (2004) inhibited H2A.Z expression in Cos-7 and mouse L929 cells using an RNAi approach which resulted in a mislocalization of HP1α and a lagging in chromosome segregation. The lagging chromosome phenotype is also observed in fission yeast Swi6 (HP1) mutants (Ekwall et al., 1995).

➢ *It has been demonstrated that H2A.Z has a global role in chromosome segregation. The authors could verify if a knockdown of H2A.Z in their experimental model results in proliferation defect caused by lagging chromosome as did their PWWP2A depletion.*

Although, we would love to perform such an assay, it is technically extremely challenging. Up to now, no human H2A.Z knockout cell lines are available, most likely because this variant is essential for cell proliferation and many other biological processes. Supporting this notion is the study by Rangasamy et al, and the observation that chicken DKO cells die rapidly upon inducible H2A.Z depletion (Kusakabe et al., Genes Cells, 2016). Consistent with our PWWP2A knockdown results was the observation that loss of H2A.Z in DT40 cells leads to an increase in the number of mitotic cells due to problems in mitotic progression. Please see also our response to the previous point #3.

5. PWWP2A seems to regulate the expression of genes involved in developmental process regulation and cell morphogenesis. Maybe it would be interesting to investigate the effect of the expression of the PWWP2A mutants (ΔPWWP) or the PWWP2A deletions on the regulation of those genes. This could also investigate if a region of PWWP2A is essential for its role in regulation of gene expression. Also, this experiment would allow to identify the regions of PWWP2A which are essential for recruitment or interaction with target and downstream proteins.

In order to determine which regions/domains in PWWP2A are essential for its function we performed rescue experiment in frogs (see also our response to point 15 of referee #1) and results are shown in new Fig. 8A and new Appendix Table S4. Interestingly, I_S_PWWP and DPWWP protein variants ameliorate the pwMO phenotype, although at slightly lower efficiency than full length PWWP2A, in contrast to the DIC variant, which cannot rescue this defect any more. This result strongly suggests that Xenopus pwwp2a protein exerts its function in craniofacial development through interaction with H2A.Z without a strong influence of the PWWP domain. Concerning a rescue experiment in human cells upon RNAi and measuring gene expression changes, this is technically difficult and will need lots of time to establish. We would need to redesign new siRNAs with target sequences in the UTR regions, since the siRNAs we are currently using recognize mRNA sites within the P2-, IN- and IC-coding regions. Consequently, all our current siRNAs would also affect expression of our exogenous GFP-constructs and we will therefore not be able to perform a rescue experiment. Alternatively, we would like to develop an inducible system to knock-out PWWP2A. We are currently working on it but it will take much more time to establish these cells, which also need to be thoroughly tested. Therefore, we hope the editor and the

referee understand that such an experiment, albeit important, is not technically feasible at the moment.

6. Figure 6F: the names PW#1 and PW#2 are partially overlapping the figure.

We thank the referee for his/her attentiveness and have changed the figure accordingly.

7. Discussion: Although the discussion brings new elements and clarifications about the structure and function of PWWP2A, almost half of the discussion is focused on the quantitative mass spectrometry approach and the identification of H2A.Z interactors which led to the identification of PWWP2A. I understand that the identification of H2A.Z interactors is a really important part of the study but given that the identification of H2A.Z mononucleosome binders was part of previous work (Vardabasso et al., 2015) and that the majority of the experiments address the characterization of PWWP2A, this should reflect in the discussion.

We have shortened the part on the identification of the H2A.Z network and have focused our discussion on the experiments addressed to functionally characterize PWWP2A.

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In sum, we have reworked many parts of our manuscript and included many additional data sets to essentially address all of the concerns raised by all three referees. Since all three referees found the discovery of PWWP2A and its fist functional characterization highly interesting, and suggested many insightful experiments, which we performed and included into our revised manuscript to extend our initial discoveries, we hope that you look favorably on a final decision regarding our manuscript. Thank you for your time and attention.

2nd Editorial Decision 09 May 2017

Thank you for submitting your revised manuscript for our editorial consideration. It has now been once more assessed by two of the original referees, who both consider the study substantially improved and the initial key concerns satisfactorily answered. We shall therefore be happy to eventually publish the study in The EMBO Journal. Before we will be able to proceed with formal acceptance and production of the manuscript, there are however a number of important editorial issues that still need to be addressed.

REFEREE REPORTS

Referee #1: I am completely satisfied with the revisions and therefore recommend publication of this important study.

Referee #2: My questions have been fully addressed in the revised manuscript. It is now appropriate to publish the manuscript in EMBO J.

2nd Revision - authors' response 18 May 2017

With great happiness have we received your letter informing us about the positive decision concerning a potential publication of our manuscript. We have now included all suggested editorial changes and have uploaded the revised text documents and high-quality figures, as well as the requested source data for all key gels/blots/autoradiographs. We hope that these changes have now satisfactorily addressed all of your concerns and the manuscript is now ready for publication in The EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name:	Sandra B. Hake
Journal Submitted to:	EMBO JOURNAL
Manuscript Number:	EMBOJ-2016-95757

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- If n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com	Antibodypedia
http://1degreebio.org	1DegreeBio
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http://oba.od.nih.gov/biosecurity/biosecurity_documents.html	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Due to its explorative nature, this study did not include a power analyis in the experimental design.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	At least three biological replicates are done for each experiment. Three different females and 2-3 different males were used to generate embryos. The numbers of counted embryos are given in the figures.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Preestablished condition: Experiments, in which more than 30% of control embryos failed to develop wildtype morphology, were discarded in total.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	The study did not include randomization.
For animal studies, include a statement about randomization even if no randomization was used.	The study did not include randomization.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	The study did not include blinding.
4.b. For animal studies, include a statement about blinding even if no blinding was done	The study did not include blinding.
5. For every figure, are statistical tests justified as appropriate?	Mass spectrometry analyses contained statistical tests and Xenopus experiments (Figure 8): The performed two-sample t-test is justified as appropriate.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Normal distribution of the data is assumed but not tested for. From experience, protein intensities from pulldown experiments measured by mass spectrometry are usually normally distributed.
Is there an estimate of variation within each group of data?	The variation within the groups is considered in the t-test, but not individually assessed.
Is the variance similar between the groups that are being statistically compared?	The variation within the groups is considered in the t-test, but not individually assessed.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All antibody information is available in the Material and Methods part.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cell lines are well established (ATCC) and were tested regularly with a PCR-based kit for mycoplasm contamination.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Frog experiments were carried out with outbred adult X. laevis and X. tropicalis from Nasco Wisconsin.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Ethics statement: Animal work has been conducted in accordance with Deutsches Tierschutzgesetz; experimental use of Xenopus embryos has been licensed by the Government of Oberbayern (AZ: 55.2.1.54-2532.6-7-12).
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Embryos were generated by in vitro fertilisation and randomly selected for microinjection. For every condition, number of embryos are detailed in the manuscript

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA

14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Data has been deposited at GEO (Accession number GSE78009)
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	GEO Accession number GSE78009
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA