THE EMBO JOURNAL

Manuscript EMBO-2011-79220

A New Non-Catalytic Role for Ubiquitin Ligase RNF8 in Unfolding Higher-Order Chromatin Structure

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Review timeline:

Submission date:	21 August 2011
Editorial Decision:	13 September 2011
Revision received:	01 February 2012
Editorial Decision:	13 February 2012
Additional Correspondence:	17 February 2012
Accepted:	23 March 2012

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 13 September 2011

Thank you for the submission of your manuscript to The EMBO Journal. We have just now received the full set of reports from the referees, which I copy below. As all three referees think that your manuscript is interesting and their comments are quite positive, I would like to ask you to revise it according to the referees' comments.

In their reports, besides a number of technical questions that should be addressed, all referees remark that the molecular mechanism of action behind the new RNF8-CHD4 pathway needs further clarification. Particularly, all referees agree that the physical interaction between RNF8 and CHD4 and the mechanism by which RNF8 recruits CHD4 needs a more detailed analysis.

Please be aware that your revised manuscript must address the referees' concerns and their suggestions (as detailed above and in their reports) should be taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is 'The EMBO Journal' policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing

manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your 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 your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

RNF8 is a nuclear E3 ubiquitin ligase that participates in DNA damage response. In this manuscript, Luijsterburg et al identified a novel non-catalytic role of RNF8 in mediating extensive chromatin decondensation. Using an in vitro system, they show that the FHA domain of RNF8 is critical for the chromatin relaxation. Moreover, RNF8-mediated chromatin decondenastion is dependent on the recruitment of CHD4, which also promotes ubiquitin and BRCA1 assembly at the DNA double strand breaks. Although the experimental setting is relatively interesting and the results may lead to understand whether RNF8 mediates chromatin unfolding, it is necessary for authors to provide additional evidence to support their conclusions and examine the molecular mechanism in the RNF8-CHD4 pathway. Current form of the manuscript is too preliminary to be published in EMBO J.

Major point:
1. This reviewer is confused by the statistical analysis of the chromatin array area when RNF8 is expressed in AO3 cells (Fig. 2D). The results indicate that the array area ranges form 0 - 37.5 %. However, in the similar experiments presented in Fig. 4J and K, the error bar is minimal. The authors should unify the statistical analyses in Fig. 2-4. Otherwise, it might cause misleading or misunderstanding. Moreover, if the chromatin array area varies significantly following RNF8 expression (Fig. 2D-F), author could not rule out of the possibility that some of the huge LacR-RNF8 positive area are generated by transient RNF8 overexpression artifact. The FHA domain could form oligomer in vitro. Thus, what observed in this in vitro system could be merely nonspecific aggregates of overexpressed RNF8 in AO3 cells. To rule out this possibility, a tomoxifen-induced nuclear translocation system should be integrated with a line of AO3 cell stably expressing low level of LacR-RNF8.

2. The mechanism by which the RNF8 FHA recruits CHD4 is not clear at all. Does the FHA domain bind CHD4 or its-associated proteins? Which region of CHD4 could be recruited by RNF8? The detailed biochemistry analysis is needed.

3. Authors use overexpressed wild type and mutant CHD4 to claim that CHD4 regulates ubiquitin and BRCA1 assembly at DNA damage site. However, the correct way is to use RNAi to knock-down CHD4 and then examine ubiquitin and BRCA1's localization. RNAi-resistant wild type and mutant CHD4 should be reintroduced into the cell and used for the assay on ubiquitin and BRCA1.

4. This reviewer is confused by the LacR-RNF168 construct, which is indicated that both MIUs are mutated (Fig. 1B). How could mutated RNF168 relocate to DNA damage sites (Fig. S1)? Previous publications clearly demonstrated that second MIU of RNF168 was required to target the whole protein to DNA damage sites (Doil et al, 2009; Stewart et al, 2009).

5. Is CHD4 required for tethering RNF168 into the chromatin in the absence of DNA damage? Please show the evidence using LacO-LacR system.

6. Without helicase activity, how does CHD4 regulate DNA decondensation?

Referee #2

The authors identified that although H2A ubiqutination E3 ligases RNF8, RNF168, RNF2, when tethered to chromatin using an in vivo targeting system, induce local H2A ubiquitination, only tethering of RNF8 to chromatin mediates extensive chromatin decondensation. Furthermore, the authors demonstrated that CHD4, a catalytic component of the NuRD complex, is required for RNF8-mediated chromatin unfolding. CHD4 is recently identified to be recruited to DNA damage sites and is required for cell cycle regulation, DNA repair and cell survival in response to DNA damage. However, how CHD4 exert its function at DNA damage sites and how CHD4 is regulated is not very clear. The authors found that the chromatin remodeling ATPase activity of CHD4 is required for chromatin decondensation, efficient ubiquitin conjugation and BRCA1 assembly at DNA damage sites. In addition, the authors provided evidence that CHD4 recruitment and its function in chromatin decondensation depends on a non-phospho-binding ability of the FHA domain of RNF8 but independent of RNF8's ubiquitin ligase activity. Thus the authors propose a novel E3-ligase independent function of RNF8 in recruiting CHD4 and subsequent chromatin unfolding. The authors suggested that RNF8-dependent recruitment of CHD4 creates chromatin decondensation in a local chromatin environment to promote chromatin ubiquitination and BRCA1 assembly.

This work provided new insights into the RNF8-dependent chromatin modification in response to DNA damage. Using an in vitro tethering system, the authors dissected the catalytic function and non-catalytic function of RNF8. While RNF8 RING domain mutant fails to recruit RNA168 and initiate robust H2A chromatin ubiquitination, it recruits CHD4 and promotes chromatin decondensation. However, it is not clear how these two processes are coordinating with each other. It would be interesting to see whether recruitment of CHD4 and subsequent chromatin unfolding itself promotes H2A ubiquitination. The authors could easily test that for Ub conjugation and BRCA1 assembly in the cells with tethering of CHD4 or tethering of RNF8 RING mutant. In addition, tethering of a RNF8 mutant that does not recruit CHD4 but retains the catalytic activity (RNF8 FHA) would be useful to investigate how efficient the catalytic function of RNF8 is in H2A Ub conjugation and BRCA1 assembly.

Some other comments and suggestions:

Figure 1. The labeling for panel K is a little bit confusing. It would help if there is more explanation in the figure legend for panel K that both WT and MIU mutant of RNF168 were used in the experiment. In addition, while tethering of a ligase inactive mutant of RNF8 (RNF8*) failed to recruit RNF8 or RNF168, there is a significant amount of signal for Ub and uH2A. Does it mean additional ligase be recruited or residual activity of the RNF8 mutant?

Figure 2. It is not very clear to me what the color coding means. The authors mentioned in the figure legend that GFP-NLS was used to measure the surface area of the nucleus, yet from the figures, it appears that Hoechst (or DAPI, blue) was used for showing the nucleus surface area. Also, what does it mean when a signal is much more brighter (white) than others for panels A-H. This information is not provided in the material and methods either.
In addition, for NIH2/4 cells that contain 10kb of the lacO arrays, quantification of percentage of cells vs. relative array area should also be presented as it was presented for AO3 cells for comparison since the images presented in Fig1. with tethering of RNF8 in the same cells didn't show much of decondensed chromatin.

Figure 3, panel A, too much back ground staining for both LacR-RNF8 and anti-CHD4. Some labels are hard to read.

Figure 6 and Figure 7, Since the authors suggested that there is a portion of CHD4 was recruited in a poly(ADP-riobse) dependent manner independent of RNF8 and this recruitment of CHD4 is not required for Ub conjugation and BRCA1 assembly, it would be helpful to monitor the BRCA1 assembly in cells treated with both RNF8 shRNA and PARPi in Figure 6.

Referee #3

Review of Luijsterburg et al. For EMBO J.

This paper highlights a new non-catalytic role for the ubiquitin ligase RNF8 in unfolding higher-order chromatin structure. The authors show that RNF8 specifically mediates chromatin decondensation, that CHD4 plays a role in this decondensation, and that its recruitment to sites of DNA damage is via the FHA domain of RNF8. They claim that this work reveals a new mechanism of chromatin remodelling-assisted ubiquitylation in the creation of chromatin environments which are permissive for checkpoint and repair machinery.

We already know that CHD4 promotes efficient ubiquitin conjugation and BRCA1 assembly at DSBs (Larsen D H et al. J Cell Biol 2010; 190:731-740) so I do not find this work to be particularly novel.
What's interesting is that it's before RNF168 involvement that CHD4 is important, and that it's not because of ubiqutin ligase activity but an interaction through the FHA domain. Also the fact that they show that RNF8 and CHD4 promote chromatin decondensation is important. In this respect this paper does demonstrate strong evidence for these conclusions and is likely important to a specific field.
I think it may be a step too far to claim that this study reveals a new mechanism of chromatin remodelling-assisted ubiquitylation because the affore mentioned paper already highlighted this.

If just tethering RNF168 can allow for ubiquitylation and BRCA1 recruitment, and it doesn't recruit CHD4 to do so then surely its presence is not required unless the critical function only is the initial recruitment of RNF168. The authors do touch on this point however there is an important experiment missing. To determine if RNF8 and CHD4 work to promote recruitment and/or stable retention of RNF168 at DSBs they should induce DNA damage and assess whether RNF168 can accumulate at this point while CHD4 is knocked down. If it happens that they see less accumulation of RNF168 then this should be quantified in some way.

The authors don't really touch on how an open chromatin environment and transcriptional silencing fit together in this scenario. What about the presence of HDAC1, what could that be doing there? The authors should comment on this including reference to any previous publications on the issue.

- Part one of the results is a nice characterisation of the system.
- Overall this paper needs to be checked to ensure there are no errors, including in figures. For example when a figure is referenced please ensure it is as the text says it is (e.g. Fig 1B, include mCherry in your illustration).
- Please elaborate on catalytically inactive ligases which are mentioned (line 100-101).
- For 1J should it be vice versa? i.e. you say data not shown but I think that actually is shown, and the reciprocal expt is not.
- The experiment to look at RNF168 in NIH2/4 cells (lines 143-150) was not performed or at least is not documented, why not? I think it's a necessary control.
- The fact that it seems to be PARP-independent is interesting but I don't think that figure 6E strongly supports the claim made in the reference to it (lines 301-302).
- Can you really say the situation is either/or, PARP/ non-catalytic RNF8? Is there sufficient evidence to make this claim?
- It seems to come out of nowhere that you speculate that PARP-dependent recruitment of CHD4 might play a role in the deacetlyation of H3K56 during NHEJ? Is there any evidence for this?
- In general figure legends appear to be lengthy and the discussion is lacking.

Referee #4

In this manuscript the authors demonstrate that ubiquitin ligase RNF8 specifically induces chromatin decondensation. They then provide some mechanism to explain this phenomenon by showing that RNF-8 promotes chomatin recruitment of CHD4, a component of the NURD complex,

which is known to mediate chromatin remodeling.
Moreover they showed that this activity takes place at double strand breaks, suggesting that this mechanism operates during physiological responses to DNA damage.
Interestingly, and quite surprisingly the ligase activity of RNF8 is not involved in these events.
Overall the experiments were logically performed and presented. The findings are original and potentially suitable for publication. However, as it stands the work is largely descriptive in nature, as no direct molecular mechanism has been provided. The authors should make an effort to identify direct molecular interactions between the main players. For example, do RNF8 and CHD4 interact directly in vitro and in vivo? This should be addressed by reciprocal co-immunoprecipiation experiments of endogenous and/or overexpressed proteins. Direct interaction with recombinant proteins and protein domains should also be assessed. These experiments are necessary in order to validate the data presented with immunofluorescence and to exclude that other proteins mediate the effects between RNF8 and CHD4. This information would also provide a direct measurement of the strength of these interactions by comparing the level of input proteins and the relative amount of interaction. This quantification, I am afraid, cannot be to obtained by imaging analysis presented and would be essential to ensure that the model presented is solid enough to justify publication.

1st Revision - authors' response 01 February 2012

Response to comments of the reviewers

Reviewer #1

'This reviewer is confused by the statistical analysis of the chromatin array area when RNF8 is expressed in AO3 cells (Fig. 2D). The results indicate that the array area ranges form 0 - 37.5 %. However, in the similar experiments presented in Fig. 4J and K, the error bar is minimal. The authors should unify the statistical analyses in Fig. 2-4. Otherwise, it might cause misleading or misunderstanding.'

See new Fig 2I-J, right panels and Fig 4B-F, right panels (distribution graphs)

We agree that the different ways of representing the effect of LacR fusions on the array may have been unnecessarily confusing. Therefore, as requested by the reviewer, we have unified the analyses and now show for each of the conditions the distribution graphs which, in our opinion, most accurately show both the effect on the array size as well as the observed variation.

'Moreover, if the chromatin array area varies significantly following RNF8 expression (Fig. 2D-F), author could not rule out of the possibility that some of the huge LacR-RNF8 positive area are generated by transient RNF8 overexpression artifact. The FHA domain could form oligomer in vitro. Thus, what observed in this in vitro system could be merely nonspecific aggregates of overexpressed RNF8 in AO3 cells. To rule out this possibility, a tomoxifen induced nuclear translocation system should be integrated with a line of AO3 cell stably expressing low level of LacR-RNF8.'

See new Fig S2 and S3 and Fig I in the rebuttal (below). Text line 176-177, 179-188.

The reviewer is concerned that the observed chromatin decondensation may be an artifact caused by the presence of non-specific RNF8 aggregates. The reviewer argues that in particular under conditions of RNF8 over-expression its FHA domain may form oligomers in AO3 cells. This point is well taken since we and others have shown that RNF8 can form oligomers. To address the concern of the reviewer we have studied oligomerization of RNF8 in AO3 cells and found that oligomerization of RNF8 is strictly dependent on its RING domain (**Fig. S3**). This is not surprising since RING-dependent dimerization has also been observed for other RING ubiquitin ligases. Since the mutant RNF8*RING does not form oligomers but shows a qualitatively very similar decondensation as wild-type RNF8 (**Fig. 2E**), we can exclude possibility that this effect can be attributed to non-specific RNF8 aggregates. Our observations also imply that, in the case of RNF8*RING, the number of bound molecules at the array will be limited by the number of LacO sequences integrated in the

genome. Thus, a relatively high expression level of the LacR-ubiquitin ligase fusion proteins will not result in increased binding at the array as the number of binding sites is limited. For example, in NIH2/4 cells, there are approximately 512 binding sites for the LacR per cell regardless of the expression level of the LacR. Also in these cells, we find that the binding of LacR-RNF8 proteins triggers unfolding of the array, while the binding of LacR or LacRRNF168 molecules does not.

We nevertheless felt that a cell line that stably expresses a tethered RNF8 could be an important tool for future investigations. As the reviewer pointed out such a system has to be based on inducible tethering to avoid constitutive activation of the DDR. The suggestion of the reviewer to use a tamoxifen-inducible tethering system was very much appreciated and encouraged us to generate such cell lines. Tamoxifen-inducible LacR-RNF8 and LacR were generated by introducing the ligand binding domain of the estrogen receptor (ER). Although analysis of ER-GFP-LacR-RNF8 in transiently transfected cells confirmed that localization of the fusion at the array was induced by tamoxifen, we unfortunately found that even in the absence of tamoxifen, a considerable fraction of the cells showed already recruitment of the fusions at the array (**Fig Ia** at the end of the rebuttal). In another attempt, we generated a fusion of RNF8 with the reverse tetracycline repressor protein (rTetR) and expressed this fusion in a U2OS cell line carrying ~200 copies of a TetO-containing cassette integrated in the genome. In transiently transfected cells, we confirmed that the tetracycline analog doxycycline induced recruitment of the fusion to the TetO array, but also with this approach the fusion was clearly detectable at the array in a large fraction of the cells in absence of doxycycline, albeit at low levels (**Fig Ib** at the end of the rebuttal).

Attempts to make stable cell lines have so far been unsuccessful. We think that there are two important complicating factors. First, stably introducing plasmids in cells carrying these LacO and TetO arrays is quite challenging since the arrays themselves cause genetic instability. Second, as mentioned above, the tamoxifen- and doxycycline-inducible systems were found to be leaky in the context of our tethering approach and it is likely that the constitutive activation of the DDR by the residual tethering of RNF8 hinders the generation of stable cell lines.

Notably, in support of our model, we found a significant decondensation of the array in GFPrTetR-RNF8 expressing cells in the presence of doxycycline (**Fig. S2**). We have included this data set since it shows that also an alternative means of tethering RNF8 to chromatin triggers decondensation further consolidating the robustness of our findings.
In summary, our demonstration that the RNF8*RING mutant does not form oligomers in AO3 cells while being fully competent in causing chromatin decondensation excludes the possibility that non-specific RNF8 aggregates are responsible for the observed effects.

'The mechanism by which the RNF8 FHA recruits CHD4 is not clear at all. Does the FHA domain bind CHD4 or its-associated proteins? Which region of CHD4 could be recruited by RNF8? The detailed biochemistry analysis is needed.'

See new Fig. 3H-J. Text line 247-262.

We have now performed several biochemical assays to characterize in more detail the interaction between RNF8 and CHD4. Our results confirm that RNF8 interacts with endogenous CHD4 in immunoprecipitation experiments (**Fig. 3H**). The RNF8*RING*FHA mutant bound more avidly to CHD4 confirming that this binding is independent of the RING domain and does not involve canonical phospho-FHA interactions (**Fig. 3H**). We also show now that CHD4 interacts with recombinant His-tagged RNF8 in vitro (**Fig. 3I**). Finally, we used a series of GST-CHD4 fusion proteins spanning the full-length protein to identify the region responsible for the interaction with RNF8 and found that recombinant RNF8 binds to a recombinant CHD4 fragment encompassing amino acids 1222-1507 (**Fig. 3J**).

'Authors use overexpressed wild type and mutant CHD4 to claim that CHD4 regulates ubiquitin and BRCA1 assembly at DNA damage site. However, the correct way is to use RNAi to knock-down CHD4 and then examine ubiquitin and BRCA1's localization. RNAi-resistant wild type and mutant CHD4 should be reintroduced into the cell and used for the assay on

ubiquitin and BRCA1.'

See new Fig. 7C-F. Text line 381-396.

We used RNA interference to knockdown endogenous CHD4 and simultaneously expressed siRNA-resistant wild-type CHD4 or CHD4K757R mutant (**Fig. 7C**). Next we analyzed the assembly of RNF168 (**Fig. 7D**), the formation of ubiquitin conjugates (**Fig. 7E**) and the recruitment of BRCA1 (**Fig. 7F**) to ionizing radiation-induced foci. These data show that wild-type CHD4 but not CHD4K757R mutant facilitates efficient recruitment of RNF168, ubiquitin and BRCA1 to DSBs underscoring a central role for the chromatin remodeling activity of CHD4 in this process.

'This reviewer is confused by the LacR-RNF168 construct, which is indicated that both MIUs are mutated (Fig. 1B). How could mutated RNF168 relocate to DNA damage sites (Fig. S1)? Previous publications clearly demonstrated that second MIU of RNF168 was required to target the whole protein to DNA damage sites (Doil et al, 2009; Stewart et al, 2009).'

Text line 89-97.

This is indeed surprising. However, we consistently found that mutations of the two MIUs did not abrogate sequestration of RNF168 at DSBs. We think that a likely explanation for this observation is the recent identification of a third ubiquitin binding domain in RNF168 that suffices to localize RNF168 at DSBs (Pinato et al, 2011). This is mentioned in the revision.

'Is CHD4 required for tethering RNF168 into the chromatin in the absence of DNA damage? Please show the evidence using LacO-LacR system.'

See new Fig. S5A-G. Text line 400-423.

This is a very good point of the reviewer as it directly probes into the functional significance of CHD4-dependent remodeling in the DDR. We and others have found that the effect of CHD4 is mainly quantitative in nature and hence it is feasible that the tight association of the LacR-RNF8 with the LacO may bypass the need for CHD4 to optimize recruitment of downstream factors. It is noteworthy that RNF8 has a short retention time at DSBs and this situation will be essentially different when tethered by the LacR. We used two different approaches to address this question. In the first set of experiments, we tethered either wildtype RNF8 or an RNF8 mutant that lacked the FHA domain required for CHD4 binding and quantified enrichment of ubiquitylated H2A (**Fig. S5A**) as well as recruitment of BRCA1 (**Fig. S5B**) and RNF168 (**Fig. S5C**) to the array. In the second approach, we reduced the levels of endogenous CHD4 by RNA interference (**Fig S5D**) and studied its effect on enrichment of ubiquitylated H2A (**Fig. S5E**) as well as recruitment of BRCA1 (**Fig. S5F**) and RNF168 (**Fig. S5G**) to tethered LacR-RNF8. We found that the tight association of RNF8 with the LacR indeed abrogated the need for CHD4 in the recruitment of these downstream factors. Our results suggest that CHD4 promotes efficient initiation of the DDR by transiently-bound RNF8 at damaged chromatin and that artificially prolonging the chromatin retention time of RNF8 bypasses the requirement for CHD4.

'Without helicase activity, how does CHD4 regulate DNA decondensation?'

Like most SNF2-related chromatin remodelers, CHD4 harbours a conserved ATPase domain and a conserved helicase domain. We found that the ATPase activity of CHD4 is essential for its ability to mediate unfolding of the array upon tethering RNF8 and for promoting the assembly of RNF168 and BRCA1 and the conjugation of ubiquitin at DSBs. Whether CHD4 actually has helicase activity and to what extent this putative helicase function contributes to chromatin remodeling is unclear. In fact, none of the CHD proteins have been shown to actually possess helicase activity. Our findings suggest that the ATPase domain of CHD4 is sufficient to mediate efficient chromatin unfolding in the targeting system, which does not require the conserved helicase motifs V and VI. Like other remodeling enzymes, the DNAdependent ATPases activity of CHD4 may be activated by nucleosomal DNA, and the enzyme may subsequently move along the DNA template and destabilize histone protein–

DNA interactions.

Reviewer #2

'Figure 1. The labeling for panel K is a little bit confusing. It would help if there is more explanation in the figure legend for panel K that both WT and MIU mutant of RNF168 were used in the experiment.'

We thank the reviewer for this suggestion and we have clarified the figure legend to explain panel K more clearly.

'In addition, while tethering of a ligase inactive mutant of RNF8 (RNF8) failed to recruit RNF8 or RNF168, there is a significant amount of signal for Ub and uH2A. Does it mean additional ligase be recruited or residual activity of the RNF8 mutant?'*

Text line 122-125.

The reviewer is correct that a small fraction of the cells do show enrichment of ubiquitin and ubiquitylated H2A at the array even when catalytically inactive ubiquitin ligases are tethered. However, the same level of enrichment was observed in control cells that only expressed LacR and therefore we conclude that this truly reflects a background level that cannot be attributed to the catalytically inactive ubiquitin ligases. It is important to point out that ubiquitylated H2A is a very abundant histone modification and it has been estimated that as much as 10% of the total population of histone H2A is ubiquitylated. Given the abundance of this modification, it is not surprising that we observe at a low frequency local enrichment of ubiquitylated H2A and ubiquitin at the array independent of the targeting of an H2A ubiquitin ligase. We rephrased the text to clarify this issue.

'Figure 2. It is not very clear to me what the color coding means. The authors mentioned in the figure legend that GFP-NLS was used to measure the surface area of the nucleus, yet from the figures, it appears that Hoechst (or DAPI, blue) was used for showing the nucleus surface area. Also, what does it mean when a signal is much more brighter (white) than others for panels A-H. This information is not provided in the material and methods either.'

Text line 157-159.

We apologize for not being clear on the usage of the look up table (LUT) coding. We now explicitly mention in the result section and in the materials section what the color coding means. The original 8-bit grayscale images were converted to colored images based on their pixel intensities. In an 8-bit image, the intensities range from 0 to 256 (2 to the power 8) and these values were replaced with different colors in which black represents 0 and white represents 256. The blue color refers to the corresponding intensity (pixel intensities of around 30-40) of the LacR fusion in the LUT table and should not be mistaken for Hoechst staining. A white signal corresponds to higher pixel intensities (close to 256) according to the LUT. LUT tables are shown with each of the micrographs. Cells only expressed the mCherry-LacR fusions combined with GFP-NLS for identification of the nucleus in order to quantify the size of the nuclei, but the GFP-NLS signal is not shown in the micrographs. This is now mentioned in the figure legends.

'In addition, for NIH2/4 cells that contain 10kb of the lacO arrays, quantification of percentage of cells vs. relative array area should also be presented as it was presented for AO3 cells for comparison since the images presented in Fig1. with tethering of RNF8 in the same cells didn't show much of decondensed chromatin.'

See new Fig. 2I-K, right panels.

We have changed the figure accordingly. See also response to reviewer #1._ The reviewer points out that whereas the quantitative data shown in Fig. 2I-K show that the decondensation in the NIH2/4 cells is comparable with the effect observed in AO3 cells, this is not evident

from the micrographs shown in the panels in Fig. 1 where the array appears as an equally large spot regardless of the nature of the tethered protein. However, the microscope settings that were used to capture the images shown in Fig. 1 have been optimized for detecting colocalization and are not reliable for quantitative assessment of the surface occupied by the array. For co-localization analysis, we typically used a larger or open pinhole (thicker optical confocal section). For a quantitative assessment of the surface area occupied by the LacO we used different settings with a small pinhole (thin optical confocal section) to avoid saturation of the signal at the array. A drawback of these settings is that we can only detect the signal at the array but not in the nucleoplasm, which is why the images in Fig. 2I-K are shown merged with a nuclear counterstaining to indicate the size of the nuclei. Thus only the high-resolution micrographs that have been collected for quantitative assessment are suitable to appreciate the impact of RNF8 on chromatin condensation, these have been added as new panels in **Fig. 2IK, right panels**

'Figure 3, panel A, too much back ground staining for both LacR-RNF8 and anti-CHD4. Some labels are hard to read.'

See Fig. 3.

We are afraid that this may have been due to conversion of the images to the PDF format. For clarity we enlarged the font in this panel.

'Figure 6 and Figure 7, Since the authors suggested that there is a portion of CHD4 was recruited in a poly(ADP-riobse) dependent manner independent of RNF8 and this recruitment of CHD4 is not required for Ub conjugation and BRCA1 assembly, it would be helpful to monitor the BRCA1 assembly in cells treated with both RNF8 shRNA and PARPi in Figure 6.'

See new Fig 8B. Text line 425-440.

Indeed our model predicts that inhibition of PARP will not further reduce BRCA1 recruitment to DSBs in cells that have been depleted of RNF8. We have performed this experiment and examined the accumulation of BRCA1 into IRIF in untreated and PARP-treated RNF8 knockdown cells. In agreement with previous findings (Mailand et al, 2007), we observed a decrease in BRCA1 recruitment in cells that had been treated with RNF8-specific siRNA.

However, treatment of these cells with PARP inhibitor did not further decrease BRCA1 accumulation at DSBs.

Reviewer #3

'We already know that CHD4 promotes efficient ubiquitin conjugation and BRCA1 assembly at DSBs (Larsen D H et al. J Cell Biol 2010; 190:731-740) so I do not find this work to be particularly novel. What's interesting is that it's before RNF168 involvement that CHD4 is important, and that it's not because of ubiqutin ligase activity but an interaction through the FHA domain. Also the fact that they show that RNF8 and CHD4 promote chromatin decondensation is important. In this respect this paper does demonstrate strong evidence for these conclusions and is likely important to a specific field. I think it may be a step too far to claim that this study reveals a new mechanism of chromatin remodelling-assisted ubiquitylation because the affore mentioned paper already highlighted this.'

It is true that the study by Larsen and co-workers (Larsen et al, 2010) and also Smeenk and co-workers (Smeenk et al, 2010) shows that CHD4 promotes efficient ubiquitin conjugation and BRCA1 recruitment. In our original submission, we already referred to this study that encouraged us to further investigate the role of CHD4 in the DDR with the help of our tethering based activation of the RNF8 pathway. Importantly, while the earlier study did implicate CHD4 as an important player in this pathway, it did not provide evidence on how CHD4 mechanistically stimulated the DDR. Although it may be expected based on the fact that CHD4 is a chromatin remodeler that chromatin (de)condensation is involved in this process, it is not at all trivial since CHD4 is typically associated with chromatin condensation,

which seems hard to reconcile with its stimulating activity in a process that more likely would require chromatin decondensation. Moreover, the fact that a protein that can remodel chromatin plays a role in the assembly of BRCA1 and RNF168 does not necessarily mean this also involves its chromatin remodeling activity. For example, recent studies have shown that knock-down of CHD4 impacts the G1 to S transition (Larsen et al, 2010; Polo et al, 2010; Smeenk et al, 2010). However, this phenotype can be rescued by expression of an ATPasedead version of CHD4 and, hence, does not appear to involve the chromatin remodeling activity of CHD4. In the revised manuscript, we now depleted cells for endogenous CHD4 and show that ectopic expression of ATPase-dead CHD4 does not rescue defects in ubiquitin conjugation and recruitment of BRCA1 and RNF168 at DSBs, while ectopic expression of wild-type CHD4 does rescue these defects. To the best of our knowledge, this is the first study that provides a mechanistic explanation for a central role of the chromatin decondensation activity of CHD4 in the DDR.

'If just tethering RNF168 can allow for ubiquitylation and BRCA1 recruitment, and it doesn't recruit CHD4 to do so then surely its presence is not required unless the critical function only is the initial recruitment of RNF168. The authors do touch on this point however there is an important experiment missing. To determine if RNF8 and CHD4 work to promote recruitment and/or stable retention of RNF168 at DSBs they should induce DNA damage and assess whether RNF168 can accumulate at this point while CHD4 is knocked down. If it happens that they see less accumulation of RNF168 then this should be quantified in some way.'

See new Fig. 7D. Text lines 381-396.

This is a very good point. We have addressed this question by analyzing recruitment of RNF168 to IR foci in cells in which we simultaneously knocked down endogenous CHD4 and expressed an siRNA-resistant wild-type CHD4 or mutant CHD4K757R. We found that knockdown of CHD4 resulted in a significant reduction in the accumulation of RNF168 into ionizing radiation-induced foci. Moreover, substituting endogenous CHD4 with siRNAresistant wild-type CHD4 rescued this defect, while ATPase-dead CHD4 failed to do so (**Fig. 7D**). As proposed by the reviewer, this result is consistent with a role of the ATPase activity of CHD4 in accommodating stable retention of RNF168 at sites of DSBs. This is discussed in the text.

'The authors don't really touch on how an open chromatin environment and transcriptional silencing fit together in this scenario. What about the presence of HDAC1, what could that be doing there? The authors should comment on this including reference to any previous publications on the issue.'

Text line 498-501.

Two recent papers from the Jackson lab touch upon this issue. The first one by Polo et al. show that CHD4 is required to recruit HDAC1 and HDAC2 to DSBs (Polo et al, 2010). The other by Miller et al. shows that HDAC protein are required for the deacetylation of acetylated H3K56 and H4K16 during the DDR (Miller et al, 2010). Moreover, the latter study implicates the activities of HDAC in repair by NHEJ, presumably by restricting the localization of the NHEJ-initiating KU complex proximal to break sites. Whether CHD4 is also required for the deacetylation of H3K56/H4K16 and whether this requires its chromatin remodeling activity remains to be clarified. This issue is discussed in the text.

'Overall this paper needs to be checked to ensure there are no errors, including in figures. For example when a figure is referenced please ensure it is as the text says it is (e.g. Fig 1B, include mCherry in your illustration).'

See Fig. 1B. mCherry has been added to the schematic drawing in Fig. 1B. We have checked the paper for errors.

'Please elaborate on catalytically inactive ligases which are mentioned (line 100-101).'
Text lines 106-107. We mention now the exact position of the inactivating mutations.
'For 1J should it be vice versa? i.e. you say data not shown but I think that actually is shown,

and the reciprocal expt is not.'

This refers now to **Fig S1F.** The statement in the paper was correct. Tethered RNF2 does not recruit RNF8 (**Fig. S1F**) and tethered RNF8 does not recruit RNF2 (data not shown). We have rephrased this statement to avoid misunderstanding.

'The experiment to look at RNF168 in NIH2/4 cells (lines 143-150) was not performed or at least is not documented, why not? I think it's a necessary control.'

See new Fig 2K and L.

The requested experiment has been performed and confirms that tethered RNF168 does not cause chromatin decondensation in NIH2/4 cells.

'The fact that it seems to be PARP-independent is interesting but I don't think that figure 6E strongly supports the claim made in the reference to it (lines 301-302).'

Our experiments show that knock-down of RNF8 or treatment of cells with PARP inhibitors each result in reduced accumulation of CHD4 at DSBs. However, combining RNF8 knockdown and PARP inhibition results in an additive reduction of CHD4 at DSBs, suggesting that two mechanistically different pathways contribute to CHD4 recruitment to DSBs. If the PARP-dependent and RNF8-dependent recruitment would be part of the same pathway, there should not be an additive negative effect on CHD4 recruitment to DSBs. To provide more insight into the PARP- and RNF8-dependent recruitment of CHD4, we analyzed the effect of PARP inhibitors on BRCA1 recruitment. We find that CHD4 as well as RNF8 knock-down affect the recruitment of BRCA1. However, inhibition of PARP did not affect BRCA1 recruitment in wild-type cells and did not further reduce BRCA1 recruitment in RNF8 knockdown cells. Based on these findings, we propose that PARP and RNF8 constitute mechanistically distinct ways to recruit CHD4 to DSBs.

'Can you really say the situation is either/or, PARP/ non-catalytic RNF8? Is there sufficient evidence to make this claim?'

As mentioned above, our findings show that RNF8 and PARP contribute to the recruitment of CHD4 to DSBs. Based on our quantitative measurements, it seems that combining RNF8 knock-down with PARP inhibitors has an additive negative effect on CHD4 recruitment, suggesting that these two mechanisms act in parallel to recruit CHD4 to DSBs. Moreover, we have now added a dataset showing that knock-down of RNF8 or CHD4 results in reduced recruitment of BRCA1 to DSBs, which is a well-known factor that binds downstream of RNF8. However, PARP inhibition does not affect BRCA1 recruitment and does not further reduce BRCA1 recruitment in RNF8-depleted cells, suggesting that the PARP-dependent recruitment of CHD4 does not contribute to the regulation of BRCA1 recruitment. These findings are in line with the notion that the RNF8- and PARP-dependent recruitment of CHD4 act in parallel and have functionally different outcomes.

'It seems to come out of nowhere that you speculate that PARP-dependent recruitment of CHD4 might play a role in the deacetlyation of H3K56 during NHEJ? Is there any evidence for this?'

There is indeed evidence supporting this suggestion. Two recent papers from the Jackson lab showed that CHD4 is required for the recruitment of HDAC1/HDAC2 to DSBs (Polo et al.) and another study (Miller et al) showed that HDAC1/2 regulate deacetylation of H3K56 and H4K16 during NHEJ. It seems logical to assume, based on these studies, that CHD4, by regulating the recruitment of HDAC1/2, may have an impact on H3K56/H4K16 deacetylation and NHEJ. We discuss this issue in the text and comment that it is unclear to what extent CHD4 is involves in this process and whether this requires the chromatin remodeling activity of CHD4.

'In general figure legends appear to be lengthy and the discussion is lacking.'
We have extended the discussion and checked the figure legends for redundant descriptions.

Reviewer #4

'Overall the experiments were logically performed and presented. The findings are original and potentially suitable for publication. However, as it stands the work is largely descriptive in nature, as no direct molecular mechanism has been provided. The authors should make an effort to identify direct molecular interactions between the main players. For example, do RNF8 and CHD4 interact directly in vitro and in vivo? This should be addressed by reciprocal co-immunoprecipiation experiments of endogenous and/or overexpressed proteins. Direct interaction with recombinant proteins and protein domains should also be assessed. These experiments are necessary in order to validate the data presented with immunofluorescence and to exclude that other proteins mediate the effects between RNF8 and CHD4. This information would also provide a direct measurement of the strength of these interactions by comparing the level of input proteins and the relative amount of interaction. This quantification, I am afraid, cannot be to obtained by imaging analysis presented and would be essential to ensure that the model presented is solid enough to justify publication.'

See new Fig. 3H-J. Text lines 247-262.

A set of biochemical experiments has been performed that shows that endogenous CHD4 interacts with RNF8 and RNF8*RING*FHA (**Fig. 3H**). Consistent with the interaction being independent of the RING domain or canonical phospho-FHA binding, we found that the mutant RNF8*RING*FHA interacted more avidly with CHD4. Additionally, CHD4 isolated from cell lysates binds to recombinant RNF8 in vitro suggesting that the two proteins directly interact (**Fig. 3I**). Using a panel of recombinant CHD4 fragments, we found that the domain corresponding with amino acids 1222-1507 was able to directly bind recombinant RNF8 (**Fig. 3J**). Together these data suggest that RNF8 and CHD4 directly interact with each other.

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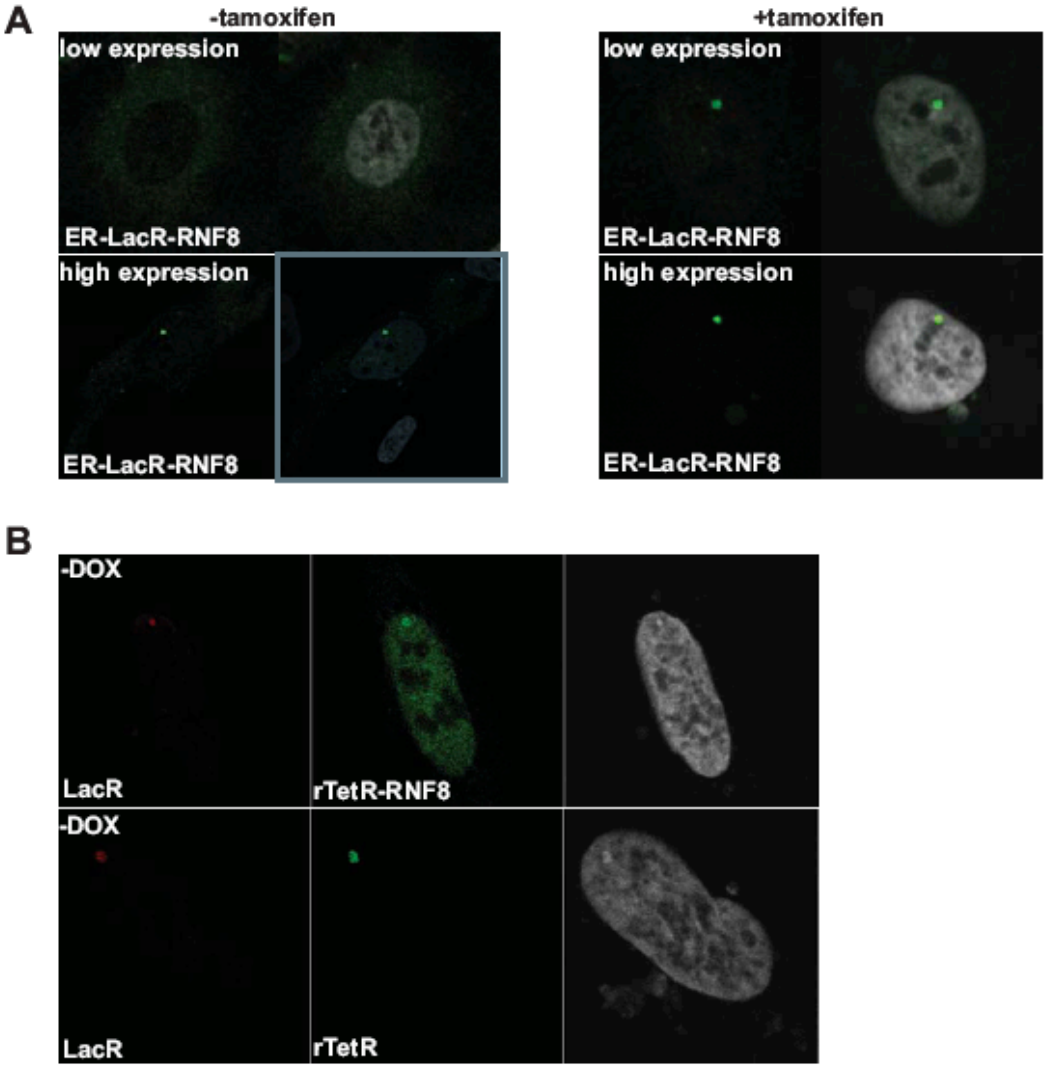
Fig I.



U2OS 263 cells expressing a) ER-LacR-RNF8 in absence or presence of tamoxifen b) rTetR-RNF8 without doxycycline

2nd Editorial Decision 13 February 2012

Thank you for the submission of your revised research manuscript to The EMBO Journal. We have now received the comments on your manuscript from referees #1 and #3, who raised most of the major concerns regarding the previous version of the manuscript.

Without repeating here their arguments that you will find below, referee #3 still raises some concerns regarding the general interest of your study, but in general considers that this revised version properly addresses his/her major concerns. However, referee #1 considers that the functional relationship between RNF8 and CHD4 still remains unclear.

As this was a fundamental problem already raised by the referees during the first round of review and given the concerns about the general interest of the manuscript, taking into account our policy of allowing a single major revision I am afraid that we cannot call for yet another revised version of your manuscript at this stage and therefore we cannot offer to publish it. Please keep in mind that we can only offer to publish papers that receive a strong support from the referees, which is unfortunately not the case here.

I am sorry that I have to disappoint you this time. I hope, however, that the referee comments will be helpful in your continued work in this area and I thank you once more for the opportunity to consider your manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

The authors tried to address most questions in the revised manuscript and the rebuttal letter. The current form of the manuscript is significantly improved. However, this reviewer is still not satisfied with the following points.
1. It is the FHA domain that may form aggregates (Xu et al., MCB, 2002; Li et al., JBC, 2008; Cai et al., Mol. Cell, 2009; Liu et al., NAR, 2012). Authors have to exclude this possibility.
2. It is not clear whether RNF8 specifically interacts with CHD4. Authors performed binding assays using recombinant proteins (Figure 3J). However, the amount of GST-CHD4 fragments in the assays was much more than that of His-RNF8, which did not reflect the stoichiometric binding. Moreover, authors failed to demonstrate which region of RNF8 interacts with CHD4. A separate-function mutation in the FHA domain that abolishes the interaction with CHD4 but not affects pThr-binding is needed to validate their model.
3. Since the catalytic site of ATPase is part of the helicase domain, it is more likely that the helicase C-terminus of CHD4 is not important for its intrinsic helicase activity. Authors need to be very cautious to draw the conclusion.

Referee #3

" Overall I am satisfied that the authors have dealt with the issues I raised previously. The main issue I continue to have with the paper is that it is not as novel as the authors claim it to be. I still think that previous publications (Larsen et al, 2010 in particular) highlighted that CHD4 was required for efficient ubiquitin conjugation and assembly of repair proteins at DSBs.
However this paper does in fact demonstrate how it is required.
I would like to see some changes made to the manuscript.
1) Page 6, line 2, please introduce the GFP-tagged ubiquitin you mention.
2) I would be interested in a comment on why the effect seen in the TetR-based array (Figure S2) is not as large as the LacR-based array.
3) One extra experiment I would be interested in seeing is the effect HDAC1 depletion might have on chromatin decondensation.
4) I am not convinced that Figures 4 G, H and I show failure of recruitment of CHD4, please address this.
5) A comment on why the helicase mutant (Figure 5D) shows less chromatin decondensation even though the authors say the helicase function is not required is also necessary I think.
6) I think that this paper is also quite long and some results sections e.g. the characterisation of the FHA domain-CHD4 interaction and the section highlighting that the interaction is independent of the ubiquitin ligase activity of RNF8 could be dealt with together.
The fact that prolonging the retention time for RNF8 bypasses the
requirement for CHD4 raises some questions and a sort of "so what?" reaction however I appreciate that to investigate this in more detail is beyond the remit of this paper. But it is necessary I think for the authors to determine what effect does RNF8 tethering have on chromatin decondensation.As mentioned previously the novelty aspect is somewhat lacking and I do not think it is of broad biological significance. In conclusion however, with some changes I think that this paper is of importance within this specific field and does show strong evidence for the conclusions that are drawn. "

Additional Correspondence 17 February 2012

With this writing we want to appeal the rejection of our manuscript entitled 'A New Non-Catalytic Role for Ubiquitin Ligase RNF8 in Unfolding Higher-Order Chromatin Structure' (EMBOJ-2011-79220). Although it is disappointing to get a final rejection of a paper after several groups have intensively worked for four months to address all the concerns of the four reviewers, we do understand that EMBO Journal follows a rigorous review process that favors papers with strong support of the reviewers. Having said that, we do believe that it is important to critically evaluate the arguments on which the support, or lack of support, is based. We are happy to see that EMBO Journal follow these very same principles as they state in their instructions to referees 'Furthermore, editorial decisions are not a matter of counting votes or numerical rank assessments, but rather are based on an evaluation of the strengths of the arguments raised by each referee and by the authors.'

In this particular case, it is unclear to us how the strengths of the arguments from both sides have been evaluated since the concerns raised by the reviewers are plainly incorrect or irrelevant. When it comes to the additional experiments we would also like to emphasize that none are crucial for the conceptual advantage of our study but more suggestions for potential follow-up experiments that are outside the scope of the present study. For several of the requested experiments we do already have data available, but decided to omit these data in our already extensively revised manuscript since the reviewers had not asked for these experiments.

We feel our manuscript has been given a very unbalanced and unfair review process. We carried out all the experiments asked for during the initial review of our paper and generated a large body of additional evidence, which corroborates our original conclusions. It appears that the reviewers have either missed or ignored major parts of our paper. Therefore, we believe that it is important to have a closer look at the concerns of the reviewers before a final decision is taken. In particular, we believe that it could be informative to discuss their concerns with the scientists whose work the reviewers refer to in their statements.

Below we summarize some of the inadequacies in the statements of the reviewers:

Referee #1 is misinterpreting the literature. None of the papers cited by the reviewer show that the FHA domain of RNF8 forms oligomers. They show that the FHA domains of two unrelated proteins can do so. To the best of our knowledge, there is no evidence supporting the oligomerization of the FHA domain in RNF8 in the literature and, more importantly, our own data exclude this possibility. Please check the citations listed by the reviewer.

Referee #1 ignores our demonstration that RNF8-RNF8 dimerization is dependent on the RING domain and does not involve the FHA domain. We clearly show in Figures S1 and S3 that a single amino acid substitution in the RING domain of RNF8 completely disrupts RNF8-RNF8 dimerization, while the FHA domain is clearly not involved in this event.

Referee #1 missed/ignored that we, in fact, did identify the FHA domain in RNF8 as being responsible for binding to CHD4 (all of Figure 4 is dedicated to mapping the RNF8 domain that is required for the CHD4 interaction). Also here we do have additional data that show that smaller fragments of the FHA domain do not recruit CHD4. However, they have not been included since the reviewer did not ask to identify which part of the FHA domain is involved in the binding. If the reviewer would have asked, we would of course have included these data that strongly suggest that the full-length FHA domain is required for the non-canonical interaction with RNF8.

Referee #1 is mistaken that the catalytic ATPase site of CHD4 is part of the helicase domain. Please see for example Clapier CR, Cairns BR (2009) Annu Rev Biochem 78: 273-304 for an extensive review on chromatin remodelling enzymes and their domain composition.

Referee #3. We believe that the novelty of our study is highly underestimated by reviewer #3, especially given that our work is well received at scientific meetings. This is the first paper that shows that a ubiquitin ligase recruits a chromatin remodeler independently of its catalytic activity, a process that we refer to as 'chromatin remodeling-assisted ubiquitylation'. To the best of our knowledge, there are no other reports that identified a similar molecular mechanism. The earlier

JCB paper of Dr. Jiri Lukas as well as the back-to-back paper of Dr. Haico van Attikum, who is a co-author of the present study, implicates CHD4 in the DNA damage response but these studies do not provide any mechanistic insights into how CHD4 promotes the DNA damage response. Because our paper connects several broad, high impact topics (ubiquitylation, chromatin remodeling and DNA damage response) and reveal a novel mechanism we remain convinced that this will attract the attention of a broad audience of scientists like the readership of the EMBO Journal.

Referee #3 thinks that it would be interesting to see the effect of HDAC1 knockdown. Why was that not asked during the initial review of our manuscript? We do have data showing that HDAC as well as HAT inhibitors do not affect the decondensation but this has not been included since the reviewers did not ask for it.

Referee #3 feels that we should investigate the effect of RNF8 on chromatin decondensation. I am puzzled: is that not what the entire paper is about?

Additional Correspondence 23 March 2012

This is just to inform you of the latest steps taken in the editorial process of your manuscript. We re-sent your rebuttal letter to referees #1 and #4, whose comments are enclosed below. In light of their opinions we decided to contact an external expert advisor that finally agreed with referee #4 in the conclusiveness and interest of your manuscript.

Congratulations again on a successful publication.

Kind regards,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

This reviewer does not try to ignore the findings of this manuscript. However, the FHA domain of RNF8 has been well characterized to bind phospho-MDC1. The binding mode between RNF8 FHA domain and phoshpo-MDC1 is also conserved in those between other FHA domains and their phospho-peptides. Although reports have shown that some FHA domains may have multiple substrates, these substrates all dock into the same phospho-binding pocket. If the binding between RNF8 and CHD4 reported here is specific and is pThr-independent, this will be the first report to show that FHA domains can bind to its partner using the pocket other than the one for pThr binding. Therefore, compelling evidence is needed to validate the specific binding between RNF8 and CHD4. The authors cannot just use a casual binding assay with huge amount of recombinant proteins in the test tube to demonstrate a specific protein-protein interaction. Instead, the binding pocket needs to be identified and stoichiometric interaction has to be determined. Otherwise, all these results could be merely generated from non-specific interactions with the high concentration of protein in the assays.

The illustration of the domain architecture of CHD4 is misleading. The helicase/ATPase domain of CHD4 contains two sub-domains: the DEAH helicase superfamily domain (motifs I-III) and the helicase superfamily C-terminal domain (motifs IV-VI). Such domain architecture is conserved in many helicase family proteins, as well as many chromatin remodelers including SWI/SNF, ISWI, CHD and INO80 family proteins. For SWI/SNF chromatin remodelers, it is well known that the first sub-domain plays a role in the ATP hydrolysis, while the second sub-domain is required for energy transduction, in which energy from ATP hydrolysis is used for DNA translocation. Importantly, these two sub-domains fold together, and both of them are required for the proper function of DNA/RNA helicases/chromatin remodelers. However, the authors show that the first subdomain (ATPase domain named in the manuscript), but not the second subdomain (helicase domain named in the manuscript) is important for chromatin decondensation. Thus, the molecular mechanism of

this DEAH-like helicase domain is totally unclear and unexplainable based on the previous results from many other groups. The authors argued that only ATPase activity but not helicase activity of CHD4.was important. However, ATPase activity itself does not explain anything. It is totally unclear what could be the new function of the first subdomain in this DEAH helicase box. Again, without compelling evidence, it is too preliminary to challenge the established knowledge.

Referee #4

The authors have adequately answered most of the critiques raised. I would still recommend the authors to tone down the novelty claims. Following this minor revision the paper should be ready for publication.

