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Regulation of DNA damage responses and cell cycle progression by the chromatin remodeling factor CHD4

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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 23 June 2010

Thank you very much for submitting your manuscript to The EMBO Journal. I did receive the assessments from three independent scientists that are enclosed below. We also discussed the criticisms that were raised by the referee's carefully at the editorial level and took into account experimental extensions that you had offered on the phone as well as including feasibility during one round of major revisions. Overall, and given the rather clear recommendation from both refs#1 and #2 we all had to conclude that the currently very descriptive nature and the study that contains two interesting, but rather disjointed sets of observations essentially precludes definitive commitment to the study. We still recognize that the study does have very high potential impact, depending on either clear functional understanding of CHD4 in DNA repair or alternatively, providing data that would in a convincing manner link CHD4-recruitment and its indicated role in cell cycle regulation. However, the presented and mostly negative results indicate that this seems not very straightforward. With this in mind and to not delay potential submission elsewhere, we have at this stage and from our very molecular perspective no other choice than to formerly reject the study. Despite this, and strictly conditioned that you might indeed be able to provide such insight, we would certainly be willing to assess suitability of a new manuscript for publication here. Alternatively, a slightly shortened version of the current study might indeed be suited for rapid publication in for instance our sister journal EMBOreports. Upon your consent, the editors would be able to quickly advise on such an option, presumably based on the current reports. From the perspective and scope of The EMBO Journal however, I am sorry that we are unable to reach a more positive conclusion as we are unable to overrule the very critical though constructive concerns of two expert referees.

I still hope that the comments of our referees as well as my remarks might help either to develop the study or to seek timely publication elsewhere.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

In this manuscript, Polo et al. explore the purpose of CHD4 recruitment to DNA damage sites. First and foremost, they show evidence that the CHD4 chromatin remodeling factor is rapidly and transiently localized to laser-induced DNA damage sites. The recruitment of CHD4 is accompanied by other NuRD complex subunits, in particular, MTA2 and HDAC1. Reciprocal knockdown experiments were then used to show that CHD4 is the cornerstone of NuRD recruitment since CHD4 depletion impairs both MTA2 and HDAC1 recruitment to damage, but depletion of the other NuRD subunits leaves CHD4 recruitment unaffected. The authors then sought to determine which factors are required for CHD4 and NuRD to relocalize to damage sites. While H2AX was not required, interestingly, they found that PARP activity was. Biochemical assays were then utilized to ascertain that CHD4 is indeed able to directly bind PAR chains. Then the authors show that, in an independent mechanism, CHD4 is phosphorylated on S1346 after DNA damage, in an ATM-dependent manner. Neither is this phosphorylation required for PAR binding, nor for CHD4 recruitment to damage. The authors begin to address the biological significance of the CHD4 localization. Through a series of assays with a number of different damage types, they show that CHD4 depletion does not affect various aspects of DNA repair and DDR signaling. There is an effect, however, of CHD4 depletion on both clonogenic survival after DNA damage treatment, and on cell cycle progression, namely the G1/S transition. The effect on G1/S transition is then linked to p53 acetylation status by p300 and transcriptional control of p21.
The results presented here show a very clear mechanism of CHD4 recruitment to DNA damage that is mediated by PAR modifications. CHD4 acts to recruit the entire NuRD complex to these sites, and in addition, is independently phosphorylated by ATM. Unfortunately, neither the biological significance of post-damage CHD4 recruitment, nor its phosphorylation are clear from these results. The authors attempt to link the defect in clonogenic survival after damage to an effect on the p53-p21 pathway, but this is not firmly established and certainly not direct enough to warrant any major conclusions to be made about the role of CHD4 and NuRD at damage sites.

Many of the results in this paper are intriguing and offer a glimpse into the role of CHD4 and chromatin remodeling at the sites of DNA damage. However, the manuscript really reports two half stories. Tthe biological effects of CHD4 from these results are not well established, and there is further work required to link these observations and correlations to produce a real working model for CHD4 function in DNA repair.

Major comments:

1. The authors show CHD4 recruitment to DNA damage solely using laser-induced damage. Does CHD4 relocalize after IR?
2. To test whether repair of damage is proficient in CHD4-knockdown cells, the authors use dissociation of the SSB repair factor XRCC1 from laser damage. Since the majority of the paper is about the repair of DSBs, might it also be relevant to test the dissociation of a DSB repair protein as well? Even though SSBs may be efficiently repaired, what about the DSBs?
3. Similar to the above comment, the authors also use comet assays of cells treated with H2O¨2 to suggest that DNA damage is being repaired in CHD4-depleted cells, and the test clonogenic survival to IR and to H2O¨2. These treatments produce different spectra of DNA damage, it would be nice to see the comet assay carried out with IR-treated cells to ascertain that repair is actually proficient in the CHD4 depletion context.
4. Though they have nice separation-of-function mutations for the phosphoylation (SA mutation) and the PAR-dependent recruitment (C-terminal truncation), these are not utilized to help dissect the distinct roles of CHD4 phosphorylation and localization. The possibility for independent functions are merely brought up and never addressed.
5. When testing the biological significance, the authors did not address whether CHD4

depletion in the absence of damage reduces clonogenic survival. The kill curves are normalized and therefore mask this possible effect, this could suggest that the CHD4 effect on clonogenic survival is not just DNA damage-dependent, but that it is a more general defect that is exacerbated by the presence of damage.
6. The clonogenic survival defect in CHD4-depleted cells could be due to a checkpoint recovery defect, that is, escape from the G2/M checkpoint arrest after repair is complete. The authors should address this possibility by looking at cell cycle re-entry after DNA repair.
7. The effect of CHD4 depletion on damage surivival and p53 and p21/cell cycle is correlative at this juncture, and needs to be tested further. Does the rescue of the cell cycle effect by co-depletion of p300 also rescue the clonogenic survival of cells after damage?
8. Since an effect on DNA damage accumulation and CHD4 depletion has been established in the literature, it is imperative that the authors address this experimentally. They claim that the biological effects that they are seeing in CHD4-knockdown cells are not due to accumulation of endogenous DNA damage, but rather, due to some sort of defect in cell cycle control after exogenous DNA damage. However, it needs to be addressed in detail since this affects their major conclusions about the effects of CHD4 depletion.

Minor comments:

1. DNA damage, chromatin remodeling, strand breaks, and cell cycle are both improperly hyphenation often throughout the text.
2. Page 6- CHD4 is written as CDH4
3. It would be nice to see where the CHD4 deletion derivatives map on the primary structure. This could easily be incorporated into the cartoon rendering in Figure 3.
4. In the figure 2, when referring to the C-terminal truncation, it would be more intuitive if the authors labeled the mutant data with a C, not just "C."
5. The authors mention briefly that ATM inhibition actually increases CHD4 localization to breaks. Since a large section of the results also shows that CHD4 is phosphorylated by ATM, it would be useful to address this in the discussion, since it suggests something about the role of this phosphorylation event that they have so clearly established.
6. Not even one mention of antibodies are made in the main Materials and Methods section. The authors use a number of specialty antibodies to make their conclusions. The table definitely belongs in supplemental methods, but a brief mention of the antibodies used for the main figures should appear in the main body of the text.

Referee #2 (Remarks to the Author):

In this manuscript, Polo, S. et al. investigated the function of CHD4 in regulating DNA damage response and genome stability. Their data show that CHD4 is rapidly recruited to DNA damage sites as part of the NuRD complex. The recruitment of CHD4 to DNA damage lesions is dependent on PARP1/2 by binding to PAR chains. They also identified that CHD4 is phosphorylated at Ser-1346 in an ATM-dependent manner upon DNA damage. PARP-dependent CHD4 recruitment to damaged chromatin and ATM-dependent CHD4 phosphorylation, however, are distinct events. Although CHD4 depletion does not impair DNA damage signaling, CHD4 deficient-cells are more sensitive to DNA damage stimuli. By analyzing cell cycle and p53 acetylation, the authors proposed that CHD4 regulates the process of p53 deacetylation and controls the G1/S transition.

The potential involvement of CHD4 and its associated-NuRD complex in DNA damage response is novel and interesting. However, in the current form, this manuscript failed to provide experimental evidence to connect the recruitment of CHD4 to the DNA damage sites to its function in DNA damage response. The effect of CHD4 on p53 deacetylation and p21 regulation is interesting but it does not directly link to its role in regulating DNA damage response.
In short, the functional studies and mechanistic studies were obviously disconnected and this serious weakness significantly diminishes the reviewer's enthusiasm.

Specific comments:

1. CHD4 accumulated at sites of DNA lesions within a few minutes, then quickly declined and was no longer visible after 30 minutes (which is quite striking). CHD4 has such a rapid kinetics at DNA damage sites but it is not required for the initiation of DNA damage signaling. What is the role of this CHD4 accumulation?

2. There is no evidence in the manuscript to indicate the function of CHD4 phosphorylation by ATM in cellular responses to IR. In Fig 4A, phospho-mutants form foci normally at 5 min post IR. Do phospho-mutants show the same kinetics as the wild-type? Is it possible that ATM-dependent phosphorylation of CHD4 regulates its rapid disassociation from DNA damage lesions, which is required for the proper assembly and maintenance of other DNA damage signaling and/or repair proteins at DNA damage sites?

3. Does CHD4 phosphorylation regulate its interaction with the components of NuRD complex, such as HDAC1 and MTA2? Do phosphor-mutants rescue the defective recruitment of HDAC1 or MTA2 foci in CHD4 depleted-cells?

4. CHD4 recruitment is dependent on PARP1/2. Does CHD4 interact with PARP1/2? Is PAR binding of CHD4 sufficient for its recruitment? Also, does CHD4 contain poly ADP-ribosylation modification after IR?

5. The authors showed that nocodazole, a chemical blocking G2/M phase transition, failed to induce G2/M arrest in CHD4-depleted cells, indicating that cells may have been arrested in G1 even without genotoxic stress. If this is the case, in Figure S6A, how could IR induce G2/M checkpoint in CHD4-depleted cells.

6. As p300 depletion rescues the defective G1/S transition after nocodazole treatment, does p300 depletion rescue the hypersensitivity of CHD4 depleted cells to IR?

7. In Fig. 6D, E, CHD4 depleted-cells show normal cellular responses to enhance p53 acetylation and p21 induction after IR. These data raise the question whether the function of CHD4 in regulating p53 acetylation and p21 indeed specifically contributes to the cellular response to DNA damage.

Referee #3 (Remarks to the Author):

Polo et al.
Regulation of DNA damage responses and cell-cycle progression by the chromatin-remodeling factor CHD4

More than 10 years ago CHD4 a component of the NuRD complex has been shown to associate with ATR and thereby a link between CHD4 complex and DNA damage was established. In the present manuscript Polo et al. performed a very detailed analysis of the role of CHD4 in the DNA damage response pathway.

In the first part of the analysis they convincingly demonstrate ATM-dependent CHD4 phosphorylation and PARP-dependent CHD4 recruitment to damaged sites within the chromatin of human cells. In a series of elegant experiments they show that components of the NuRD complex (CHD4, HDAC1 and MTA2) co-localize at damaged chromatin in a CHD4-dependent manner. PARP but not ATM is important for the recruitment and the N-terminus of CHD4 shows (weak) PAR binding activity. Phosphorylation of CHD4 was induced upon DNA damage and dependent on ATM activity but was not required for PAR binding or recruitment to damaged chromatin. In the end of the first part Polo et al. tried to define the role of CHD4at the damage sites. In a number of elaborate experiments they could exclude a role of CHD4 in the recruitment of PARP, MDC1, p53BP1 and BRCA1, in H2AX phosphorylation and focus formation and the G2/M checkpoint. However, CHD4 was shown to promote cell survival upon genotoxic stress.

In the second part of the manuscript the authors turn their attention to a more general role of CHD4 in the regulation of cell cycle progression. Loss of CHD4 resulted in induction of the CDK inhibitor p21 in a way that is to a large extent dependent on p53. Interestingly p53 protein but not RNA levels were increased in the absence of CHD4. Finally the authors suggest that CHD4 might have a function in the control of reversible p53 acetylation.

In my opinion the experiments are of excellent quality and include all the required controls. The data are very convincing and the manuscript is well written. The outcome of the study is novel and is of broad biological significance.

My only criticism concerns Figure 6. It is not clear whether the increase in acetylation of p53 shown in Figure 6E reflects only the increased amount of the p53 protein. Here, a quantification of the signals (p53K382ac versus p53) would help. One could also load lower amounts of the knockdown extracts #1 and #3 containing comparable amounts of p53 (similar to siLuci) to demonstrate an increase in acetylation levels. Based in the discussion on a potential role of HDAC1 (or HDAC2) as p53 deacetylating enzyme it would be important to know whether knockdown of HDAC1 affects p53 acetylation and/or expression levels.

Discussion:

It would be interesting to compare the roles of different chromatin remodelling complexes (SWI/SNF) in DNA damage response.

1st Resubmission 12 July 2010

Referee #1:

In this manuscript, Polo et al. explore the purpose of CHD4 recruitment to DNA damage sites. First and foremost, they show evidence that the CHD4 chromatin remodeling factor is rapidly and transiently localized to laser-induced DNA damage sites. The recruitment of CHD4 is accompanied by other NuRD complex subunits, in particular, MTA2 and HDAC1. Reciprocal knockdown experiments were then used to show that CHD4 is the cornerstone of NuRD recruitment since CHD4 depletion impairs both MTA2 and HDAC1 recruitment to damage, but depletion of the other NuRD subunits leaves CHD4 recruitment unaffected. The authors then sought to determine which factors are required for CHD4 and NuRD to relocalize to damage sites. While gH2AX was not required, interestingly, they found that PARP activity was. Biochemical assays were then utilized to ascertain that CHD4 is indeed able to directly bind PAR chains. Then the authors show that, in an independent mechanism, CHD4 is phosphorylated on S1346 after DNA damage, in an ATM-dependent manner. Neither is this phosphorylation required for PAR binding, nor for CHD4 recruitment to damage. The authors begin to address the biological significance of the CHD4 localization. Through a series of assays with a number of different damage types, they show that CHD4 depletion does not affect various aspects of DNA repair and DDR signaling. There is an effect, however, of CHD4 depletion on both clonogenic survival after DNA damage treatment, and on cell cycle progression, namely the G1/S transition. The effect on G1/S transition is then linked to p53 acetylation status by p300 and transcriptional control of p21.

The results presented here show a very clear mechanism of CHD4 recruitment to DNA damage that is mediated by PAR modifications. CHD4 acts to recruit the entire NuRD complex to these sites, and in addition, is independently phosphorylated by ATM. Unfortunately, neither the biological significance of post-damage CHD4 recruitment, nor its phosphorylation are clear from these results. The authors attempt to link the defect in clonogenic survival after damage to an effect on the p53-p21 pathway, but this is not firmly established and certainly not direct enough to warrant any major conclusions to be made about the role of CHD4 and NuRD at damage sites.

Many of the results in this paper are intriguing and offer a glimpse into the role of CHD4 and chromatin remodeling at the sites of DNA damage. However, the manuscript really reports two half stories. The biological effects of CHD4 from these results are not well established, and there is further work required to link these observations and correlations to produce a real working model for CHD4 function in DNA repair. Major comments: 1. The authors show CHD4 recruitment to DNA damage solely using laser-induced damage. Does CHD4 relocalize after IR?

As a complementary approach to laser-induced damage, we have examined CHD4 redistribution in cells treated with various genotoxic agents. Probably because of the transient nature of CHD4 recruitment to DNA damage sites, we have not been able to detect its localization to IR-induced foci (IRIF). Importantly, however – and consistent with our laser microirradiation data – we have now been able to observe that CHD4 displays enhanced resistance to detergent extraction very early on after cells are treated with the DNA-damaging agent H2O2. These new data are provided in Supplementary Fig S1E of the revised manuscript and are described on page 5.

2. To test whether repair of damage is proficient in CHD4-knockdown cells, the authors use dissociation of the SSB repair factor XRCC1 from laser damage. Since the majority of the paper is about the repair of DSBs, might it also be relevant to test the dissociation of a DSB repair protein as well? Even though SSBs may be efficiently repaired, what about the DSBs? 3. Similar to the above comment, the authors also use comet assays of cells treated with H2O2 to suggest that DNA damage is being repaired in CHD4-depleted cells, and the test clonogenic survival to IR and to H2O2. These treatments produce different spectra of DNA damage, it would be nice to see the comet assay carried out with IR-treated cells to ascertain that repair is actually proficient in the CHD4 depletion context.

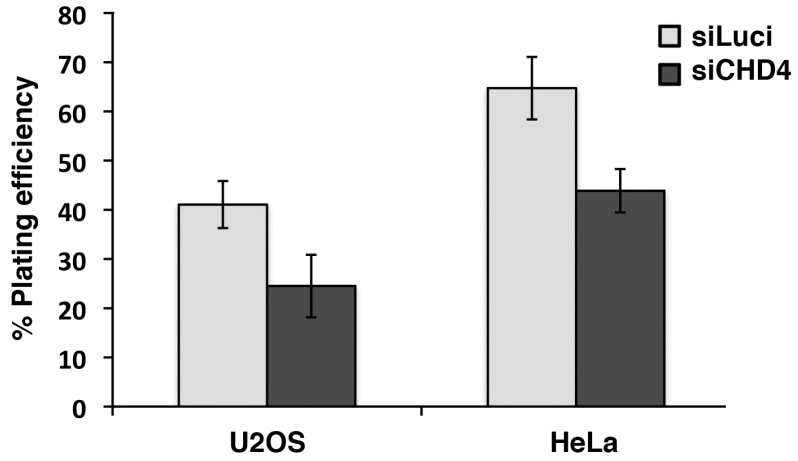
In these two points, the reviewer is asking for more information about the role of CHD4 in DSB repair. In this respect, we now show in Figure 5B of our revised manuscript that CHD4 depletion results in prolonged γH2AX signal following IR exposure, which could indeed reflect defective repair of DSBs. Given the multiplicity of DSB repair factors and the existence of several subpathways for DSB repair, we felt that it would be difficult to assess overall DSB repair by following the recruitment/dissociation kinetics of one factor in particular. Therefore, to directly address DSB repair efficiency upon CHD4 depletion, we have followed the reviewer's suggestion and performed neutral comet assays in cells treated with the radiomimetic agent phleomycin. This experiment, which forms Figure 5D of our revised manuscript, shows that DSB repair is defective in CHD4- depleted cells. These new data are described on page 10 of our revised manuscript.

4. Though they have nice separation-of-function mutations for the phosphoylation (SA mutation) and the PAR-dependent recruitment (C-terminal truncation), these are not utilized to help dissect the distinct roles of CHD4 phosphorylation and localization. The possibility for independent functions are merely brought up and never addressed.

We have already used these mutants to address whether CHD4 phosphorylation and recruitment to DNA damage are inter-dependent, and through a series of complementary experiments presented in Figure 3 we concluded that they are distinct events.

5. When testing the biological significance, the authors did not address whether CHD4 depletion in the absence of damage reduces clonogenic survival. The kill curves are normalized and therefore mask this possible effect, this could suggest that the CHD4 effect on clonogenic survival is not just DNA damage-dependent, but that it is a more general defect that is exacerbated by the presence of damage.

We consistently observe a small effect on cell viability upon CHD4 depletion in undamaged cells (ca. 33%) both in U2OS and HeLa cells, as shown in the figure below (Error bars: s.d. from 4 independent experiments). This reduction in plating efficiency is comparable to those caused by depletion of other well studied DDR factors. The results presented in the survival curves in our manuscript are normalized to plating efficiencies, so as to focus on the effect of CHD4 depletion upon DNA damage. Such normalization methodologies are commonly used when studying cell sensitivity to DNA damage. We have revised our manuscript in the legend of Figure 5 to mention the effects of CHD4 depletion on cell viability.

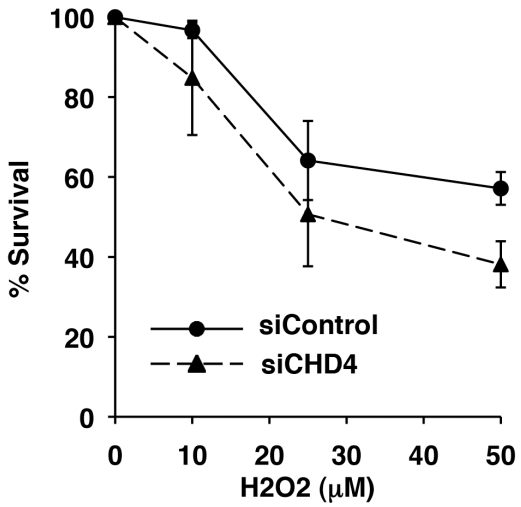


6. The clonogenic survival defect in CHD4-depleted cells could be due to a checkpoint recovery defect, that is, escape from the G2/M checkpoint arrest after repair is complete. The authors should address this possibility by looking at cell cycle re-entry after DNA repair.

We thank this reviewer for pointing out this possibility. To address this, we have analyzed cell cycle re-entry 24 hours after cell exposure to 10 Gy of IR. In Supplementary Fig. S6B of our revised manuscript, we show that recovery from G2/M checkpoint arrest is observed in CHD4- depleted cells and is comparable to control cells. These results taken together with our other data support the effect of CHD4 depletion on DNA damage sensitivity being due to a DNA repair defect rather than a defect in DNA damage checkpoint.

7. The effect of CHD4 depletion on damage surivival and p53 and p21/cell cycle is correlative at this juncture, and needs to be tested further. Does the rescue of the cell cycle effect by codepletion of p300 also rescue the clonogenic survival of cells after damage?

We did not mean to imply that the effect of CHD4 depletion on cell survival following DNA damage is entirely mediated by defective cell cycle control, and we discuss this further in our revised manuscript to make things clearer. Importantly, we have tested the effect of CHD4 on survival after damage in cells with a proficient p53 pathway (U2OS cells in Figure 5E) and in those with a defective p53 pathway (HeLa cells, see figure below), and have found that that CHD4 depletion affects cell survival after DNA damage in both cases. Thus, the role of CHD4 on the p53-p21 pathway cannot entirely account for its function in cell viability after damage. Furthermore, in light of our new data linking CHD4 to DSB repair, we think that it is a repair defect that in large part causes the increased sensitivity of CHD4-depleted cells to DNA damage. Error bars: s.d. from 2 independent experiments



Error bars: s.d. from 2 independent experiments

8. Since an effect on DNA damage accumulation and CHD4 depletion has been established in the literature, it is imperative that the authors address this experimentally. They claim that the biological effects that they are seeing in CHD4-knockdown cells are not due to accumulation of endogenous DNA damage, but rather, due to some sort of defect in cell cycle control after exogenous DNA damage. However, it needs to be addressed in detail since this affects their major conclusions about the effects of CHD4 depletion.

Consistent with a previous report (Pegoraro *et al*, *Nat Cell Biol* 2009), we observe accumulation of endogenous DNA damage upon CHD4 depletion, as shown by slightly increased comet tails in neutral comet assays (Figure 5D). Importantly, while such accumulation of endogenous damage is observed only after several days of siRNA treatment (ie. after several cell cycles with reduced CHD4 expression), the accumulation of p21 leading to G1/S cell cycle arrest appears within a few hours, prior to any detectable increase in γH2AX signal; and p21 induction in CHD4-depleted cells is not prevented by PIKK inhibitors. Thus, we conclude that cell cycle arrest upon CHD4 depletion is not triggered by but precedes the accumulation of endogenous DNA damage. The biological effect of CHD4 depletion on cell survival thereby most likely results from a combination of DNA damage accumulation due to impaired DSB repair and defective cell cycle control.

Minor comments: 1. DNA damage, chromatin remodeling, strand breaks, and cell cycle are both improperly hyphenation often throughout the text. 2. Page 6- CHD4 is written as CDH4

These have been corrected.

3. It would be nice to see where the CHD4 deletion derivatives map on the primary structure. This could easily be incorporated into the cartoon rendering in Figure 3.

The positions of the N and C fragments are now indicated in revised Figure 3A.

4. In the figure 2, when referring to the C-terminal truncation, it would be more intuitive if the authors labeled the mutant data with a C, not just "C."

We have kept WT, N and C for consistency throughout the figures.

5. The authors mention briefly that ATM inhibition actually increases CHD4 localization to breaks. Since a large section of the results also shows that CHD4 is phosphorylated by ATM, it would be useful to address this in the discussion, since it suggests something about the role of this phosphorylation event that they have so clearly established.

It is indeed tempting to speculate that CHD4 phosphorylation by ATM regulates the dissociation kinetics of CHD4 from damage sites. However, we did not notice significant differences in the timing or levels of accumulation of CHD4 wild-type and phospho-mutants to damage sites. Instead, our interpretation of the effect of ATM inhibitor is that it promotes CHD4 accumulation by enhancing the poly-(ADP-ribose) signal (as shown in Supplementary Fig. S1F). We now discuss this in our revised manuscript.

6. Not even one mention of antibodies are made in the main Materials and Methods section. The authors use a number of specialty antibodies to make their conclusions. The table definitely belongs in supplemental methods, but a brief mention of the antibodies used for the main figures should appear in the main body of the text.

We apologize for this omission, which has been rectified in the revised manuscript.

Referee #2:

In this manuscript, Polo, S. et al. investigated the function of CHD4 in regulating DNA damage response and genome stability. Their data show that CHD4 is rapidly recruited to DNA damage sites as part of the NuRD complex. The recruitment of CHD4 to DNA damage lesions is dependent on PARP1/2 by binding to PAR chains. They also identified that CHD4 is phosphorylated at Ser-1346 in an ATM-dependent manner upon DNA damage. PARP-dependent CHD4 recruitment to damaged chromatin and ATM-dependent CHD4 phosphorylation, however, are distinct events. Although CHD4 depletion does not impair DNA damage signaling, CHD4 deficient-cells are more sensitive to DNA damage stimuli. By analyzing cell cycle and p53 acetylation, the authors proposed that CHD4 regulates the process of p53 deacetylation and controls the G1/S transition.

The potential involvement of CHD4 and its associated-NuRD complex in DNA damage response is novel and interesting. However, in the current form, this manuscript failed to provide experimental evidence to connect the recruitment of CHD4 to the DNA damage sites to its function in DNA damage response. The effect of CHD4 on p53 deacetylation and p21 regulation is interesting but it does not directly link to its role in regulating DNA damage response. In short, the functional studies and mechanistic studies were obviously disconnected and this serious weakness significantly diminishes the reviewer's enthusiasm.

Specific comments:

1. CHD4 accumulated at sites of DNA lesions within a few minutes, then quickly declined and was no longer visible after 30 minutes (which is quite striking). CHD4 has such a rapid kinetics at DNA damage sites but it is not required for the initiation of DNA damage signaling. What is the role of this CHD4 accumulation?

It is true that the rapid and transient kinetics of CHD4 recruitment to DNA damage sites suggests an early role for CHD4 in the DDR. Although we did not find a direct function of CHD4 in SSB repair and ATM-dependent signaling, we now provide in Figure 5D of our revised manuscript data showing, by neutral comet assays, that CHD4 promotes the repair of DNA DSBs. These data are also supported by our finding – now included as additional Western-blot panel in revised Figure 5B – that the γH2AX signal after IR treatment persists in CHD4-depleted cells as compared to control cells. Thus, we conclude that a prime function for CHD4 accumulation at DNA damage sites is to ensure efficient repair of DSBs. In our discussion, we speculate about possible mechanisms for this, which could involve chromatin remodeling and/or transcription inhibition by CHD4 to promote DNA repair.

2. There is no evidence in the manuscript to indicate the function of CHD4 phosphorylation by ATM in cellular responses to IR. In Fig 4A, phospho-mutants form foci normally at 5 min post IR. Do phospho-mutants show the same kinetics as the wild-type? Is it possible that ATM-dependent phosphorylation of CHD4 regulates its rapid disassociation from DNA damage lesions, which is required for the proper assembly and maintenance of other DNA damage signaling and/or repair proteins at DNA damage sites?

Given the effect of ATM inhibition, which increases CHD4 accumulation at damage sites, it is indeed tempting to speculate that CHD4 phosphorylation by ATM regulates the dissociation kinetics of CHD4 from damage sites. However, when we examined this possibility, we did not detect significant differences in the timing and levels of accumulation of wild-type CHD4 and CHD4 phospho-mutants at damage sites. Instead, our interpretation of the effect of the ATM inhibitor is that it promotes CHD4 accumulation by enhancing the poly-(ADP-ribose) signal, as shown in Supplementary Fig. S1F and discussed in the revised manuscript.

Regarding the potential impact of CHD4 phosphorylation on other aspects of CHD4 function, we have been able to show that it does not affect the interaction of CHD4 with other NuRD subunits (as explained below**)**. Thus, the function of CHD4 phosphorylation by ATM is still elusive at this stage, and will be the subject of further studies.

3. Does CHD4 phosphorylation regulate its interaction with the components of NuRD complex, such as HDAC1 and MTA2? Do phosphor-mutants rescue the defective recruitment of HDAC1 or MTA2 foci in CHD4 depleted-cells?

As shown in Supplementary Fig. S4C-D of the revised manuscript, we have found that CHD4 interactions with the NuRD complex components HDCA1 and MTA2 are unaffected upon cell exposure to IR, and that the CHD4 phospho-mutants (SA and SE) associate with HDAC1 and MTA2 as does wild-type CHD4. These data therefore suggest that CHD4 phosphorylation does not simply regulate its interactions with other NuRD subunits.

4. CHD4 recruitment is dependent on PARP1/2. Does CHD4 interact with PARP1/2? Is PAR binding of CHD4 sufficient for its recruitment? Also, does CHD4 contain poly ADP-ribosylation modification after IR?

Although we have observed that CHD4 weakly associates with PARP1 in co-immunprecipitation experiments, we do not think that this interaction mediates CHD4 recruitment to DNA damage sites. Indeed, our data show that CHD4 recruitment is impaired both by PARP depletion (Fig. S3D) and PARP inhibition (Fig. 2A), the latter resulting in enhanced accumulation of PARP1 at damage sites (Godon *et al*, *NAR* 2008) because PARylation of PARP1 is required for its dissociation. Therefore,

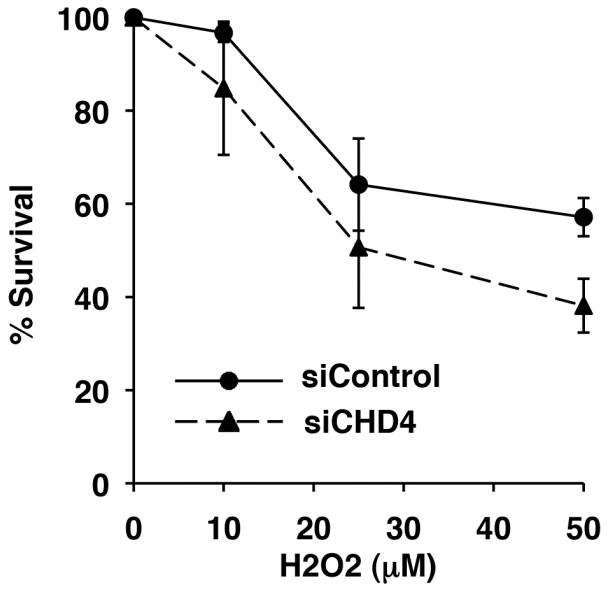
the critical parameter for CHD4 accumulation at DNA damage sites must be the presence of PARylated proteins. We have also looked for PARylation of CHD4 itself after DNA damage but it was undetectable. These data suggest that PARP-dependent recruitment of CHD4 to DNA damage sites is mediated by CHD4 binding to PARylated proteins, including PARP1, present at damage sites rather than via PARylation of CHD4 itself, which we now mention in our revised manuscript. To address whether PAR binding of CHD4 is sufficient for its recruitment, we have examined the accumulation of the CHD4 N fragment to sites of laser-damage. Unfortunately, we noticed that all CHD4 mutants lacking the C-terminal domain are mislocalised and accumulate in the nucleoli, which precludes analysis of their recruitment to laser lines.

5. The authors showed that nocodazole, a chemical blocking G2/M phase transition, failed to induce G2/M arrest in CHD4-depleted cells, indicating that cells may have been arrested in G1 even without genotoxic stress. If this is the case, in Figure S6A, how could IR induce G2/M checkpoint in CHD4-depleted cells.

We are sorry if we did not explain things clearly in our original submission. CHD4-depleted cells arrest at the G1/S transition only if they have a functional p53 pathway. Thus, to analyze the impact of CHD4 depletion on the G2/M checkpoint (Figure S6) we used HeLa cells which have a defective p53 pathway and do not arrest at the G1/S transition, contrary to U2OS cells (Figure 6A). We have now added a note in the legend of Figure S6 to clarify this point.

6. As p300 depletion rescues the defective G1/S transition after nocodazole treatment, does p300 depletion rescue the hypersensitivity of CHD4 depleted cells to IR? 7. In Fig. 6D, E, CHD4 depleted-cells show normal cellular responses to enhance p53 acetylation and p21 induction after IR. These data raise the question whether the function of CHD4 in regulating p53 acetylation and p21 indeed specifically contributes to the cellular response to DNA damage.

These two points relate to the possibility that CHD4 role in p53-dependent cell cycle regulation contributes to cell survival post damage. We did not mean to imply that the effect of CHD4 depletion on cell survival is entirely mediated by defective cell cycle control and we discuss this further in our revised manuscript to avoid this confusion. In fact, we have tested the importance of CHD4 for survival after damage in cells with a proficient p53 pathway (U2OS cells, Figure 5E) or defective p53 pathway (HeLa, see figure below) and found that CHD4 depletion affected cell survival after DNA damage in both cases. Thus, the role of CHD4 on the p53-p21 pathway cannot entirely account for its function in promoting cell viability after DNA damage. Furthermore, in light of our new data linking CHD4 to DSB repair (Figure 5B, D), we think that it is a repair defect that in large part causes the increased sensitivity of CHD4-depleted cells to DNA damage. Error bars: s.d. from 2 independent experiments



Error bars: s.d. from 2 independent experiments

Referee #3 :

Polo et al. Regulation of DNA damage responses and cell-cycle progression by the chromatin-remodeling factor CHD4

More than 10 years ago CHD4 a component of the NuRD complex has been shown to associate with ATR and thereby a link between CHD4 complex and DNA damage was established. In the present manuscript Polo et al. performed a very detailed analysis of the role of CHD4 in the DNA damage response pathway.

In the first part of the analysis they convincingly demonstrate ATM-dependent CHD4 phosphorylation and PARP-dependent CHD4 recruitment to damaged sites within the chromatin of human cells. In a series of elegant experiments they show that components of the NuRD complex (CHD4, HDAC1 and MTA2) co-localize at damaged chromatin in a CHD4-dependent manner. PARP but not ATM is important for the recruitment and the N-terminus of CHD4 shows (weak) PAR binding activity. Phosphorylation of CHD4 was induced upon DNA damage and dependent on ATM activity but was not required for PAR binding or recruitment to damaged chromatin. In the end of the first part Polo et al. tried to define the role of CHD4at the damage sites. In a number of elaborate experiments they could exclude a role of CHD4 in the recruitment of PARP, MDC1, p53BP1 and BRCA1, in H2AX phosphorylation and focus formation and the G2/M checkpoint. However, CHD4 was shown to promote cell survival upon genotoxic stress.

In the second part of the manuscript the authors turn their attention to a more general role of CHD4 in the regulation of cell cycle progression. Loss of CHD4 resulted in induction of the CDK inhibitor p21 in a way that is to a large extent dependent on p53. Interestingly p53 protein but not RNA levels were increased in the absence of CHD4. Finally the authors suggest that CHD4 might have a function in the control of reversible p53 acetylation.

In my opinion the experiments are of excellent quality and include all the required controls. The data are very convincing and the manuscript is well written. The outcome of the study is novel and is of broad biological significance.

My only criticism concerns Figure 6. It is not clear whether the increase in acetylation of p53 shown in Figure 6E reflects only the increased amount of the p53 protein. Here, a quantification of the signals (p53K382ac versus p53) would help. One could also load lower amounts of the knockdown extracts #1 and #3 containing comparable amounts of p53 (similar to siLuci) to demonstrate an increase in acetylation levels.

p53 acetylation is intimately linked to its stabilization, since acetylation prevents its ubiquitindependent degradation. It is thus difficult to analyze them separately. What we want to stress is that, upon CHD4 depletion, we readily detect acetylation on stabilised p53, while p53 phosphorylation is almost undetectable. The rescue of cell cycle progression by combining sip300 with siCHD4 also supports the importance of acetylation in cell cycle arrest. We have modified the text of our manuscript on pages 11-12 to try to make these issues clearer.

Based in the discussion on a potential role of HDAC1 (or HDAC2) as p53 deacetylating enzyme it would be important to know whether knockdown of HDAC1 affects p53 acetylation and/or expression levels.

While we agree that investigating the potential impact of HDAC1 and HDAC2 on p53 acetylation would be worthwhile, we feel that such studies are beyond the scope of the present work.

Discussion: It would be interesting to compare the roles of different chromatin remodelling complexes (SWI/SNF) in DNA damage response.

In our revised discussion, we speculate that the different chromatin remodelling factors recruited to DNA damage sites may have synergistic and/or antagonistic functions in the regulation of chromatin compaction at damage sites.

2nd Editorial Decision 16 July 2010

The paper has been reviewed with the following comments:

In this paper Jackson and colleagues provide convincing evidence for a direct role for the chromatin remodeling factor CHD4 in DNA double strand break repair. They demonstrate that CHD4 is a target of the ATM kinase, that it is recruited together with other components of the NuRD complex to breaks and, surprisingly, the mechanism involves binding to PARylated proteins at break sites. They also demonstrate the CHD4 regulates the G1/S transition through regulating p53 deacylation.

This study provides a major advance in our understanding of how chromatin remodeling regulates the DNA damage response. The authors have done an excellent job to address the reviewers' comments, through their demonstration that CHD4 promotes the repair of DNA double strand breaks. This clearly shows the biological significance of CHD4 recruitment to DNA breaks, which was the major criticism of the previous reviews. I strongly recommend publication in EMBO Journal.

