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Ubiquitin acetylation inhibits polyubiquitin chain elongation

Fumiaki Ohtake, Yasushi Saeki, Kensaku Sakamoto, Kazumasa Ohtake, Hiroyuki Nishikawa, Hikaru Tsuchiya, Tomohiko Ohta, Keiji Tanaka and Jun Kanno

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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.)

Editor: Nonia Pariente

1st Editorial Decision 03 July 2014

Thank you for your submission to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email. As you will see, although all the referees find the topic of interest and in principle suitable for us, they also consider the study preliminary for publication here at this stage.

Given that all referees provide constructive suggestions on how to strengthen the work, I would like to give you the opportunity to revise your manuscript. The crucial issue is that of the physiological relevance of ubiquitin acetylation, and the need to demonstrate that it is enzymatically-mediated. You would need to identify a cellular ubiquitin-dependent process in which ubiquitin acetylation plays a role and/or an endogenous substrate of acetylated ubiquitination. In addition, there are other concerns, raised specially by referee 2, which should be addressed, although addressing this referee's point 4 and 6 would not be a prerequisite for publication.

If the referee concerns can be adequately addressed, we would be happy to accept your manuscript for publication. However, please note that it is EMBO reports policy to undergo one round of revision only and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review.

I look forward to seeing a revised form of your manuscript when it is ready. In the meantime, please contact me if I can be of any assistance.

REFEREE REPORTS:

Referee #1:

In this manuscript, Ohtake et al added potentially a new layer of regulation to the ubiquitin system. Using high-resolution mass spectrometry, the authors identified multiple acetylation sites, including K6, K48, and K63, on ubiquitin. Further biochemical analysis indicted that acetylation on K6/K48 has little effect on charging to E1 and E2 or mono-ubiquitination but it reduces the efficiency of polyubiquitin chain elongation by specific E2 in vitro. Using a model substrate of ubiquitin-fusion degradation (Ub-G76V-GFP), the authors showed that overexpression of acetylated ubiquitin-mimetic mutant could affect the polyubiquitin chain elongation of in cells
Generally, the discovery presented in the report is interesting. A major concern is on the biological significance of ubiquitin acetylation. According to the manuscript, even under the co-treatment of multiple HDAC inhibitors, the endogenous acetylation only occurs on ~0.02% of total ubiquitin (less than 2000 acetylated ubiquitin in per cell). This abundance seems too low to have a global impact on protein ubiquitination. The argument that stoichiometry is also very low for H3K9 acetylation (1%) is not a strong one, as the majority of heterochromatin would be devoid of this modification. Nonetheless, given the novelty and extensive biochemical and mass spectrometric analysis, if evidence supports that the production of Ac-ubiquitin is enzyme-dependent (see comment 1) and its abundance affects a physiological ubiquitin-dependent process (see comment 2), this paper would be suitable for EMBO Report.

1. Non-enzymatic acetylation can occur under certain conditions. Accordingly, there is a concern that a combined inhibition of HDAC and sirtuin family members only increases the abundance of Ac-ubiquitin by less than two fold. To provide much needed confidence that Ac-ubiquitin detected is enzyme-dependent, the authors should determine if Ac-ubiquitin could be induced by the expression of a known acetyltransferase.

2. To gain better support for the functional significance of Ac-Ubiquitin, a more physiological substrate other than Ub-G76V-GFP should be used for the analysis. For example, the authors can investigate the effect of ubiquitin K6R and K6Q on p53 degradation (K48-linked chains) or TAK1 kinase activation (K63-linked ubiquitin chains).

Other minor issues:
1. Legends for Figure for 3B, 5A should be revised to provide more detailed information.
2. Does ubiquitin K6Q mutation has any effect on the ubiquitin chain elongation in vitro?

Referee #2:

The authors of the manuscript "Ubiquitin acetylation modulates polyubiquitin chain elongation" performed a mass spectrometry analysis to identify post-translational modifications on ubiquitin itself. They detected both phosphorylation of T14 as well as acetylation of K6, K48 and K63 on substrate-conjugated ubiquitin, confirming earlier proteomics efforts reporting acetylation of Ub on these residues (Choudhary et al., 2009). Further targeted proteomics experiments by the authors revealed, that the levels of acetylated and substrate-conjugated ubiquitin in cells are upregulated upon HDAC inhibitor treatment. Interestingly, the cellular abundance of AcK6 Ub was low (0.02% of total Ub) being similar to the amount of linear Ub chains, which are transiently assembled in signalling complexes. To characterize the effect of ubiquitin acetylation in vitro, the authors generated recombinant Ub harbouring single site-specific acetyl-lysine residues. They demonstrate that acetylation at K6 or K48 does not impair E1/E2 charging or mono-ubiquitination of the model

substrate Histone2B, whereas free ubiquitin chain synthesis by various E2 enzymes was diminished. Ohtake et al. demonstrate the in vivo significance of their findings by analysing stability and ubiquitination status of Ub-fusion protein UbG76V-GFP and UBB+1, both artificial substrates for poly-ubiquitination in cells, in the presence of WT Ub or AcK6-mimetic K6Q Ub. Taken together, the authors provide evidence that acetylation of ubiquitin might serve as a novel regulatory switch, by neutralizing basic charges on the Ub surface close to its hydrophobic interface. The experimental data in general support the conclusions drawn by the authors. The following questions should be addressed to provide additional evidence for the author's hypothesized model:

Major points:
(1) In figure 2 the authors provide data on AcK6/K48 dynamics upon HDAC inhibitor treatment. Did the authors observe any dynamics upon stimuli (e.g. DNA damage, TNF-alpha)?

(2) Figure 4A: To highlight an inhibitory effect of AcK6 on chain elongation by wild-type ubiquitin, recombinant AcK6/K48 could be titrated into such an in vitro reaction. Furthermore, as a control and also in support to the in vivo data (figure 5), the authors could test if K6R and K6Q Ub show similar effects in vitro as wild type Ub and AcK6 Ub, respectively.

(3) Figure 4C: The conclusions made from the data of figure 4 are crucial to the manuscript. The authors provide evidence that AcK6/K48 represses Ub chain elongation in vitro and claim, that AcK6/48 has an impact on non-covalent Ub-Ub-conjugating enzyme interactions based on published data. To support this hypothesis the authors should provide additional data on the non-covalent interactions between AcK6/K48 and one of the E2 enzymes tested (in comparison to wild type Ub).

(4) It has been recently shown, that post-translational modifications on the interaction surface between ubiquitin/UBLs and UBDs have a strong impact on their interaction - as shown for phosphorylation of p62's UBA domain (Matsumoto et al., 2011). In addition, acetylation of the ubiquitin-like modifier SUMO has been shown to specifically affect SUMO-SIM interactions (Ullmann et al., 2012). Therefore, it would be of great interest to the field, if the authors could provide additional data on acetyl-Ubiquitin-UBD interactions by using ubiquitin-chains harbouring AcK6/K48 or acetylation-mimicking mutations.

(5) In figure 5 A-F Ohtake et al. utilize a model UFD substrate (Ub-G76V-GFP) as a target for polyubiquitination and show, that proteasomal turnover is reduced by co-expression of an acetylation mimicking Ub mutant. Is this mutant (K6Q) recognized by the 26S proteasome? If not, this could be a possible explanation for the accumulation of Ub-G76V-GFP in this assay. Furthermore, is it possible to stabilize Ub-G76V-GFP by HDAC inhibitor treatment or overexpression of dominant-negative HDACs instead of overexpression of Ub mutants?

(6) Figure 5C: Why does K6Q Ub stabilize bulk proteins, as argued in the main text of the manuscript? Does DUB cleavage still occur?
(7) Also related to figure 5: The quantification in (E) is not convincing. Why do the authors not again use targeted proteomics for quantification at this critical point of the manuscript? Quantification of mono- and poly-ubiquitinated Ub-G76V-GFP by mass spectrometry would help to improve the data in (D).

Minor points:
(1) The authors do not discuss the possibility of acetylation of already elongated and conjugated chains. What do they hypothesize based on their observations: does acetylation mainly occur on Ub monomers prior chain formation or also after chain formation on poly-Ub chains? Since the authors immunoprecipitated conjugated ubiquitin they provided no data if free Ub molecules are already acetylated, but use acetylated Ub monomers as substrates for their in vitro assays.

(2) Figure 4A: Due to an excess of free Ub a significant fraction of unreacted Ub remains in the assays. Is there any Ub chain elongation with less substrate Ub in those assays?

(3) Figure 5A: a loading control should be provided.

(4) Methods in suppl.: There is no information on the reaction time for the in vitro ubiquitination

assays (neither in figure 4A/B nor its legend) - but the authors write in the supplementary methods, that the reaction was incubated for "the times indicated".

Referee #3:

The posttranslational modification of cellular proteins with ubiquitin is an essential signaling event in all eukaryotes. Recent work on the ubiquitin ligase Parkin had revealed that ubiquitin itself can be subject to posttranslational modification - in this case, phosphorylation of S65 of ubiquitin occurred in proximity to mitochondria and was utilized to activate Parkin ubiquitin ligase activity in an allosteric manner. In this manuscript, the authors show that endogenous ubiquitin can also be modified by acetylation of Lys6 and Lys48. Consistent with previous structural work, they show that this modification interferes with the activity of chain elongating E2 enzymes, but not with charging by the E1. In vivo, they attempt to mimic acetylation of ubiquitin and suggest that it impairs proteasomal degradation of a model substrate, i.e. a protein that would require a long ubiquitin chain to form.

The identification of acetylated ubiquitin and its quantification were done carefully and reveal an interesting facet of ubiquitin biology. The biochemical characterization was done very carefully, too, as it was based on incorporation of acetylated Lys into ubiquitin, rather than using a mimic. These findings are interesting. However, I have concerns about the physiological relevance of ubiquitin acetylation, and I do think this needs to be addressed in some way, before this manuscript can be accepted for publication.

Major points:

1. While the authors establish that ubiquitin acetylation can occur in cells, they also show that it occurs at very minor levels. 99.97% of ubiquitin molecules are not acetylated at Lys6, and 99.99% of ubiquitin levels are not acetylated at Lys48. This is very problematic, as they suggest an inhibitory role for ubiquitin lysine acetylation. Ubiquitin chains are often longer than required for signaling (i.e.more than 4 ubiquitin molecules for proteasomal recognition) - so how can acetylation of such a small fraction of ubiquitin molecules have a biologically significant effect (i.e. repress chain formation to conjugates <4 molecules)? They compare it to histone acetylation, but this modification functions positively, as a recruitment factor - so, even a small overall percentage of modified histone molecules can recruit a significant proportion of target proteins to a specific site. What is clearly missing from this paper is a phenotype for acetylated ubiquitin without overexpression of mimics (i.e. not stabilization of an artificial substrate that is 100% modified, but effects on an endogenous ubiquitylation substrate in the face of 0.01% ubiquitin acetylation).

2. Along the same lines, it would help this manuscript immensely, if they could identify a substrate modified with acetylated ubiquitin - maybe for such a single substrate, a higher percentage of modified proteins contain acetylated ubiquitin? While the biochemistry is nice, the in vivo relevance is really limiting in the current version of this paper.

Minor point:

1. Introduction - Rpn10 does not specifically recognized K48-linked chains; remove from text.

1st Revision - authors' response 26 September 2014

Response to the reviewers' comments

We appreciate the reviewers' constructive suggestions, which we feel have significantly improved our study. In the revised manuscript, we have carefully addressed the reviewers' comments with substantial new experimental evidence, as listed in this point-by-point

response letter. We thank the reviewers for their insightful comments that have strengthened our study.
We have reorganized the text and figures according to the reviewers' suggestions and the journal's guidelines.
1) New figures include Figs. 4C, 5A, 5C, 5E, 5F, and 5G.
2) New supplementary figures include Figs. S3D, S4C, S4D, S4E, S6B, S7A, S7D, S8A, and S8B.
3) Content that was previously in Figs. 5B, 5C, 5E, 5F, and part of Figs. 4C and 5A was moved to the supplement due to space limitations.
4) Modified figures: in Fig. 2B, statistical analysis was included; in Fig. 4B, the abundance of chains from the two data sets are shown as normalized values.
5) Due to the 30,000 character length limitation, the manuscript had to be significantly shortened. For clarity, experimental details were described in the supplementary methods section.
6) According to the journal's guidelines, individual data points were shown instead of error bars where appropriate.

Response to Referee #1:

In this manuscript, Ohtake et al added potentially a new layer of regulation to the ubiquitin system. Using high-resolution mass spectrometry, the authors identified multiple acetylation sites, including K6, K48, and K63, on ubiquitin. Further biochemical analysis indicted that acetylation on K6/K48 has little effect on charging to E1 and E2 or mono-ubiquitination but it reduces the efficiency of polyubiquitin chain elongation by specific E2 in vitro. Using a model substrate of ubiquitin-fusion degradation (Ub-G76V-GFP), the authors showed that overexpression of acetylated ubiquitin-mimetic mutant could affect the polyubiquitin chain elongation of in cells
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As suggested by the reviewer, we screened several known histone acetyltransferases (HATs) and HDACs for ubiquitin acetylation. We overexpressed several HATs in 293F cells, quantified the acetylation levels of ubiquitin, and found candidates for ubiquitin-cetylating/deacetylating enzymes (please see **Response Letter Figure #1-1**). The data suggested that ubiquitin acetylation is enzymatically mediated. However, the identification of bona fide acetylation enzymes will clearly require further rigorous biochemical and functional analyses. Because we found that histone H2B was a substrate for acetyl-ubiquitylation as described below, we would prefer to establish the detailed mechanisms for acetylating enzymes and their functional significance in gene regulation using comprehensive methods such as RNA-sequencing and ChIP-sequencing as a next project.

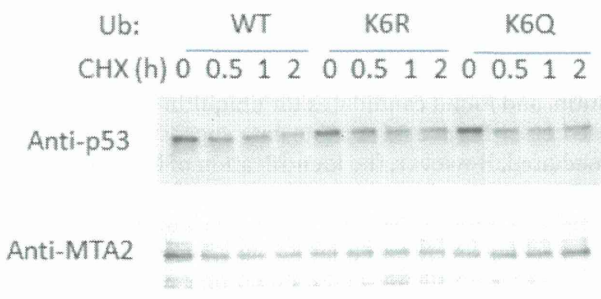
As for the reviewer's concern regarding the significance of HDAC inhibitors on ubiquitin acetylation, we have now included a statistical analysis in Fig. 2A. The effect of combined treatment with TSA+NIA was significant for both AcK6 (P = 2.66E-6) and AcK48 (P = 3.55E-4) as determined by a Student's t-test. This result indicated that cellular acetyl-ubiquitin

is regulated by deacetylating enzyme(s).
Certainly, it is known that acetylation occurs nonenzymatically in bacteria and eukaryotes. But, in eukaryotes, nonenzymatic acetylation in vivo is reported mainly for metabolic enzymes within the mitochondrial matrix (Wagner JBC 2013, Wagner Mol Cell 2014, Newman JBC 2012). By contrast, our SILAC-based search for AcUb (K6Q)-associated proteins revealed the significant enrichment of chromosome-related proteins (P = 1.4E-7), including histone H2B (**new Figure 5E and S8**). We also found that AcK6 was enriched in purified histones and that K6Q ubiquitin enhanced the monoubiquitylation of histone H2B (**new Figure 5F–G**), implying the existence of a regulatory mechanism for ubiquitin acetylayion. Based upon these results, we favor a model whereby the modification of H2B by acetyl-ubiquitin is a regulated process, rather than a nonenzymatic one. Since our current results could not exclude a nonenzymatic mechanism, this possibility is discussed in the revised manuscript.

Data not shown.

2. To gain better support for the functional significance of Ac-Ubiquitin, a more physiological substrate other than Ub-G76V-GFP should be used for the analysis. For example, the authors can investigate the effect of ubiquitin K6R and K6Q on p53 degradation (K48-linked chains) or TAK1 kinase activation (K63-linked ubiquitin chains).

We appreciate this important suggestion. To use a more physiological substrate for analysis, we first screened for K6Q-enriched proteins using SILAC. Gene ontology analysis of the identified proteins revealed that chromosome- and chromatin-regulating proteins were enriched (**new Figure 5E and S8**). Although this assay included both UbK6Q-interacting and UbK6Q-conjugated proteins, the data suggested that the cellular function of acetyl-ubiquitin may be related to chromosome and chromatin regulation.
Within the list, we identified histone H2B among other histone-interacting/modifying proteins (**new Figure S8A**). Mass spectrometric quantification revealed a more than ten-fold enrichment of acetyl ubiquitin in the endogenous histone fraction as compared to the total ubiquitin conjugates (**new Figure 5F**). Analyzing histone H2B as an endogenous substrate of AcK6, we found that expression of nonacetylatable UbK6R decreased cellular endogenous H2B-K120ub levels, while UbK6Q increased them **(new Figure 5G)**. These results suggest that the monoubiquitylation state of endogenous histone H2B could be stabilized by AcUb (K6Q); that result was consistent with the case of the model substrate UbG76V-GFP. These new data suggested a functional role for acetyl-ubiquitin in the regulation of an endogenous substrate, H2B.
We also tested the effect of K6R and K6Q on p53 degradation in MCF7 cells (where p53 regulation is intact). However, p53 degradation was not significantly affected, suggesting that the effect of AcK6 is not global, but specific for its target proteins (**Response Letter Figure #1-2**).



Response Letter Figure #1-2. MCF-7 cells were transfected with the indicated Ub mutants, and a cycloheximide chase was performed.

Other minor issues:
1. Legends for Figure for 3B, 5A should be revised to provide more detailed information.
We are sorry for the insufficient information and have revised the legends to include greater detail.

2. Does ubiquitin K6Q mutation has any effect on the ubiquitin chain elongation in vitro?
We tested ubiquitin K6R and K6Q in ubiquitin chain elongation assays using several E2s (UBCH5 and CDC34). Our data showed that K6Q was inhibitory to chain elongation as compared to K6R or wild-type Ub **(new Figure S4E)**.

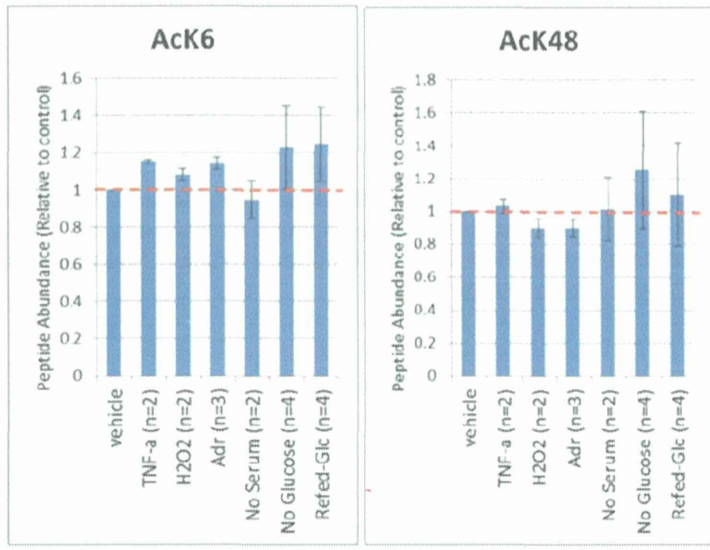
Response to Referee #2:

The authors of the manuscript "Ubiquitin acetylation modulates polyubiquitin chain elongation" performed a mass spectrometry analysis to identify post-translational modifications on ubiquitin itself. They detected both phosphorylation of T14 as well as acetylation of K6, K48 and K63 on substrate-conjugated ubiquitin, confirming earlier proteomics efforts reporting acetylation of Ub on these residues (Choudhary et al., 2009). Further targeted proteomics experiments by the authors revealed, that the levels of acetylated and substrate-conjugated ubiquitin in cells are upregulated upon HDAC inhibitor treatment. Interestingly, the cellular abundance of AcK6 Ub was low (0.02% of total Ub) being similar to the amount of linear Ub chains, which are transiently assembled in signalling complexes. To characterize the effect of ubiquitin acetylation in vitro, the authors generated recombinant Ub harbouring single site-specific acetyl-lysine residues. They demonstrate that acetylation at K6 or K48 does not impair E1/E2 charging or mono-ubiquitination of the model substrate Histone2B, whereas free ubiquitin chain synthesis by various E2 enzymes was diminished. Ohtake et al. demonstrate the in vivo significance of their findings by analysing stability and ubiquitination status of Ub-fusion protein UbG76V-GFP and UBB+1, both artificial substrates for poly-ubiquitination in cells, in the presence of WT Ub or AcK6-mimetic K6Q Ub. Taken together, the authors provide evidence that acetylation of ubiquitin might serve as a novel regulatory switch, by neutralizing basic charges on the Ub surface close to its hydrophobic interface. The experimental data in general support the conclusions drawn by the authors. The following questions should be addressed to provide additional evidence for the author's hypothesized model:

We thank the reviewer for suggesting the literature citation, Choudhary 2009, because consistency with such proteomic studies strengthens the validity of our findings. We have now added the suggested paper as a reference.

Major points:
(1) In figure 2 the authors provide data on AcK6/K48 dynamics upon HDAC inhibitor treatment. Did the authors observe any dynamics upon stimuli (e.g. DNA damage, TNF-alpha)?

As suggested by the reviewer, we tested various signaling pathways, including a DNA-damaging agent (adriamycin) and TNF-alpha. However, these agents did not significantly affect global AcK6 or AcK48 levels (**Response Letter Figure #2-1**). In our revised experiments, we found that acetyl-ubiquitin was strongly enriched in proteins related to chromosome organization, and identified histone H2B as a substrate. For this reason, we speculated that AcK6/K48 modifications may be locally regulated.



Response Letter Figure #2-1. 293F cells were treated as indicated, and were lysed and subjected to immunoprecipitation using an anti-Ub(FK2) antibody. Quantification was performed as in Fig. 2. Data represent the average ± S.E. Adr, Adriamycin; Refed, incubation in normal DMEM media after glucose depletion.

(2) Figure 4A: To highlight an inhibitory effect of AcK6 on chain elongation by wild-type ubiquitin, recombinant AcK6/K48 could be titrated into such an in vitro reaction. Furthermore, as a control and also in support to the in vivo data (figure 5), the authors could test if K6R and K6Q Ub show similar effects in vitro as wild type Ub and AcK6 Ub, respectively.

A) We thank the reviewer for the important suggestion. To our surprise, the titration of wild-type vs. AcK6/AcK48 ubiquitin clearly revealed an inhibitory effect of AcK6 and AcK48 in chain elongation (**new Figure S4D**). The result may partly explain why low abundance AcUbs could inhibit polyubiquitylation in the presence of wild-type ubiquitin.
B) We tested ubiquitin K6R and K6Q in ubiquitin chain elongation assays using several E2s (UBCH5 and CDC34), and observed that K6Q was indeed inhibitory to chain elongation as compared to K6R or wild-type (**new Figure S4E**).

(3) Figure 4C: The conclusions made from the data of figure 4 are crucial to the manuscript. The authors provide evidence that AcK6/K48 represses Ub chain elongation in vitro and claim, that AcK6/48 has an impact on non-covalent Ub-Ub-conjugating enzyme interactions based on published data. To support this hypothesis the authors should provide additional data on the non-covalent interactions between AcK6/K48 and one of the E2 enzymes tested (in comparison to wild type Ub).

Noncovalent interactions between ubiquitin and Uev1a were tested using surface plasmon resonance (SPR), according to a previous study that used the technique to describe the interactions of Ub and UEV1a (Pastushok, FEBS Letter 2007). The measured Kd of wild-type Ub and UEV1a was comparable to the previous observation (Pastushok, 2007). We found that the Kd of Ub-UEV1a was ~4-fold or ~10-fold higher with AcK6 or AcK48, respectively, as compared to wild-type Ub [**new Figure 4C(iii) and S6B**]. These results indicated that the acetylation of K6/48 had an impact on noncovalent Ub-E2 interactions.

(4) It has been recently shown, that post-translational modifications on the interaction surface

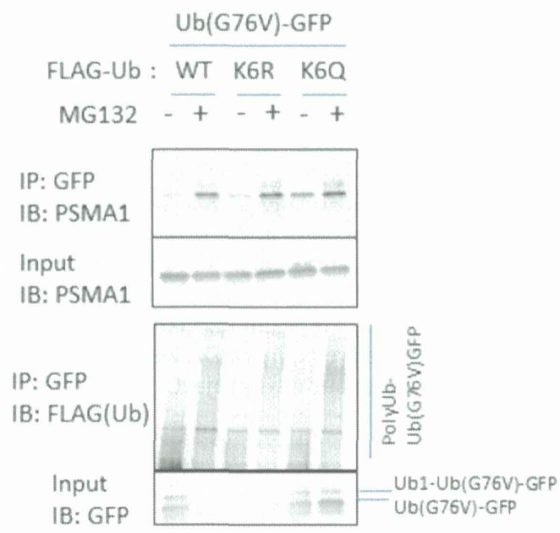
between ubiquitin/UBLs and UBDs have a strong impact on their interaction - as shown for phosphorylation of p62's UBA domain (Matsumoto et al., 2011). In addition, acetylation of the ubiquitin-like modifier SUMO has been shown to specifically affect SUMO-SIM interactions (Ullmann et al., 2012). Therefore, it would be of great interest to the field, if the authors could provide additional data on acetyl-Ubiquitin-UBD interactions by using ubiquitin-chains harbouring AcK6/K48 or acetylation-mimicking mutations.

We thank the reviewer for the interesting suggestion, and agree that AcUb may alter interactions with some specific classes of UBDs, since there are at least sixteen UBDs and three distinct UBD-interacting surfaces within Ub (Hurley, 2006). In a pilot experiment, two well-established UBDs, Dsk2 (TUBE1) and Rad23 (TUBE2), were used in a quantification analysis. As shown in **Response Letter Figure #2-2**, the result implied some specificity or preference of UBDs for acetyl-ubiquitin. Since there are many UBDs, we would like to screen various UBDs for their interactions with acetyl-Ub in our next project.

Data not shown.

(5) In figure 5 A-F Ohtake et al. utilize a model UFD substrate (Ub-G76V-GFP) as a target for polyubiquitination and show, that proteasomal turnover is reduced by co-expression of an acetylation mimicking Ub mutant. Is this mutant (K6Q) recognized by the 26S proteasome? If not, this could be a possible explanation for the accumulation of Ub-G76V-GFP in this assay. Furthermore, is it possible to stabilize Ub-G76V-GFP by HDAC inhibitor treatment or overexpression of dominant-negative HDACs instead of overexpression of Ub mutants?

A). We agree that the stabilization of UbK6Q-modified substrates may generally involve reduced polyubiquitylation or proteasomal targeting or both. For the immunoprecipitation analysis of Ub(G76V)GFP, the interaction of the proteasome component C2 (PSMA1) correlated with the levels of polyubiquitylated Ub(G76V)GFP (**Response Letter Figure #2-3**). This indicated that the recognition of polyubiquitylated Ub(G76V)GFP by the proteasome was not severely affected by K6Q.
B). Consistent with the results of acetyl-mimetic Ub expression, we found that treatment of cells with the HDAC inhibitor, trichostatin A (TSA), stabilized Ub-G76V-GFP (**new Figure S7D**).



Response Letter Figure #2-3. Immunoprecipitation was performed in 293F cells. When similar amounts of polyubiquitylated Ub(G76V)GFP were loaded (where the monoubiquitylated form is lower in K6R-expressed cells), levels of co-precipitated PSMA1 correlate to levels of polyubiquitylated Ub(G76V)GFP.

(6) Figure 5C: Why does K6Q Ub stabilize bulk proteins, as argued in the main text of the manuscript? Does DUB cleavage still occur?

Because the stability of total ubiquitin conjugates was unaltered, we speculate that UbK6Q-modified endogenous proteins may be stabilized. However, we could not identify the composition of those proteins at this stage. Therefore, we have toned down this claim to avoid confusing the readers.
As for DUB cleavage, we tested USP2cc for cleavage of the *C*-terminal tag; its efficiency was unaltered (Fig. 3B). However, as described in a recent paper (Mevissen, Cell 2013), there are various DUBs with remarkable diversity in terms of specificity to polyubiquitin chains. Therefore, it seems likely that some specific DUBs may have a preference for acetyl-ubiquitin. We have included this point in the discussion.

(7) Also related to figure 5: The quantification in (E) is not convincing. Why do the authors not again use targeted proteomics for quantification at this critical point of the manuscript? Quantification of mono- and poly-ubiquitinated Ub-G76V-GFP by mass spectrometry would help to improve the data in (D).

Because the substrate UbG76V-GFP itself contains a ubiquitin sequence, the abundance of monoubiquitylation and total substrate could not be distinguished in this system. Nevertheless, mass spectrometric quantification clearly showed that K48-linked polyubiquitin chains conjugated to UbG76V-GFP (as a percentage of total ubiquitin) were increased upon the expression of UbK6R, but were decreased by the expression of UbK6Q, supporting our data (**new Figure 5C**). Contrary to the significant alteration of K48-chains, the abundance of K29-chains and K63-chains was not significantly changed. This result was consistent with the notion that the UFD pathway encompasses both initial K29-linked mono/oligo ubiquitylation and subsequent K48-linked polyubiquitylation. Due to a mutation at the K6 residue, K6-chains and K11-chains were not accurately quantifiable; however, based upon the results from wild-type ubiquitin, the abundance of K6/11-chains was low as compared to that of K48- or K29-chains (data not shown). Collectively, these quantifications support our observation that UbK6R enhances, and UbK6Q represses, polyubiquitylation on UbG76V-GFP.

Minor points:
(1) The authors do not discuss the possibility of acetylation of already elongated and conjugated chains. What do they hypothesize based on their observations: does acetylation mainly occur on Ub monomers prior chain formation or also after chain formation on poly-Ub chains? Since the authors immunoprecipitated conjugated ubiquitin they provided no data if free Ub molecules are already acetylated, but use acetylated Ub monomers as substrates for their in vitro assays.

We performed quantification of monomer Ub, and found that a fraction of monomer Ub was also acetylated (**new Figure S3D**). These data suggest that the acetylated Ub monomer could be conjugated to substrates. As we could not exclude the possibility that a certain fraction of Ub is also acetylated after conjugation to substrates, we included this point in the discussion section.

(2) Figure 4A: Due to an excess of free Ub a significant fraction of unreacted Ub remains in the assays. Is there any Ub chain elongation with less substrate Ub in those assays?

Following the reviewer's suggestion, we repeated the in vitro ubiquitylation assays for UBCH5 and CDC34 with less substrate. In our hands, a significant fraction of free Ub remained, even with less substrate. Nevertheless, the results showed that the inhibitory effect of AcK6/K48 on chain elongation was observed at similar levels with different substrate concentrations (**new Figure S4C**).

(3) Figure 5A: a loading control should be provided.

We have now included loading controls (**new Figure 5A and S7A**).

(4) Methods in suppl.: There is no information on the reaction time for the in vitro ubiquitination assays (neither in figure 4A/B nor its legend) - but the authors write in the supplementary methods, that the reaction was incubated for "the times indicated".

We are sorry for the insufficient information and have now included detailed information on the incubation times in the figures or the figure legends.

Response to Referee #3:

The posttranslational modification of cellular proteins with ubiquitin is an essential signaling event in all eukaryotes. Recent work on the ubiquitin ligase Parkin had revealed that ubiquitin itself can be subject to posttranslational modification - in this case, phosphorylation of S65 of ubiquitin occurred in proximity to mitochondria and was utilized to activate Parkin ubiquitin ligase activity in an allosteric manner. In this manuscript, the authors show that endogenous ubiquitin can also be modified by acetylation of Lys6 and Lys48. Consistent with previous structural work, they show that this modification interferes with the activity of chain elongating E2 enzymes, but not with charging by the E1. In vivo, they attempt to mimic acetylation of ubiquitin and suggest that it impairs proteasomal degradation of a model substrate, i.e. a protein that would require a long ubiquitin chain to form.

The identification of acetylated ubiquitin and its quantification were done carefully and reveal an interesting facet of ubiquitin biology. The biochemical characterization was done very carefully, too, as it was based on incorporation of acetylated Lys into ubiquitin, rather than using a mimic. These findings are interesting. However, I have concerns about the physiological relevance of ubiquitin acetylation, and I do think this needs to be addressed in some way, before this manuscript can be accepted for publication.

Major points:

1. While the authors establish that ubiquitin acetylation can occur in cells, they also show that it

occurs at very minor levels. 99.97% of ubiquitin molecules are not acetylated at Lys6, and 99.99% of ubiquitin levels are not acetylated at Lys48. This is very problematic, as they suggest an inhibitory role for ubiquitin lysine acetylation. Ubiquitin chains are often longer than required for signaling (i.e.more than 4 ubiquitin molecules for proteasomal recognition) - so how can acetylation of such a small fraction of ubiquitin molecules have a biologically significant effect (i.e. repress chain formation to conjugates <4 molecules)? They compare it to histone acetylation, but this modification functions positively, as a recruitment factor - so, even a small overall percentage of modified histone molecules can recruit a significant proportion of target proteins to a specific site. What is clearly missing from this paper is a phenotype for acetylated ubiquitin without overexpression of mimics (i.e. not stabilization of an artificial substrate that is 100% modified, but effects on an endogenous ubiquitylation substrate in the face of 0.01% ubiquitin acetylation).

We appreciate this insightful suggestion. We hypothesized that the biological effect of acetylated ubiquitin, which we observed in such low abundance, might be exhibited locally, not globally. We searched for acetyl-ubiquitin–related proteins using SILAC, where Ub-K6Q or Ub-K6R was expressed in cells cultured with heavy-isotope– or light-isotope–containing media, respectively, and the relative abundance of K6Q/K6R-associated proteins was quantified. From two independent experiments, we identified ~50 proteins that preferentially interacted with Ub-K6Q (**new Figure 5E**). Gene ontology analysis revealed the significant enrichment of chromosome/chromatin-related proteins (P = 1.4E-7) in the K6Q-interacting protein set (**new Figure S8)**. Further mass spectrometric analysis of purified endogenous histones identified histone H2B as a substrate for acetyl-ubiquitylation, as described in the response to Point 2 (**new Figure 5F**).

Using histone H2B as an endogenous substrate of AcK6, we found that expression of nonacetylatable UbK6R decreased cellular endogenous H2B-K120ub levels, while expression of UbK6Q increased them (**new Figure 5G**). Because proteasomal inhibition rapidly decreases histone monoubiquitylation to compensate for the free ubiquitin pool (Dantuma, JCB 2006), we could not determine if the observed stabilization of monoubiquityl-H2B involved the repression of steady-state polyubiquitylation or other mechanisms, such as deubiquitylation. Nonetheless, these results suggested that acetyl-ubiquitin could stabilize the monoubiquitylation state of H2B at a certain step(s) in its dynamic regulation. Taken together, we propose that one of the physiological effects of ubiquitin acetylation might be the stabilization of monoubiquityl-histone H2B.

From these observations, we speculate that acetyl-ubiquitin is concentrated at specific chromatin regions and regulates the local monoubiquitin state of H2B. Supporting this model, H2B-K120ub mark has diverse molecular functions at specific chromatin regions; it promotes H3 K4 methylation, H3 K79 methylation, and transcriptional elongation by cooperating with FACT, as well as counteracting nucleosome compaction, and regulating DNA repair responses (reviewed in Brawn, EMBO Rep 2012). Concerning the molecular mechanism, various histone modifications are also classified as 'inhibitory' at the molecular levels: H3R2 methylation inhibits H3K4 methylation, and H3S10 phosphorylation prevents the association of HP1, a regulator of heterochromatin (reviewed in Lee, Cell 2010). These clues point to the fact that a small fraction of inhibitory modifications could still have a biologically significant effect within the local environment. We appreciate the reviewer's critical suggestions and are aiming to reveal the precise roles of acetyl-ubiquitin in chromatin regulation by using comprehensive methods such as RNA-sequencing and ChIP-sequencing in our next project.

2. Along the same lines, it would help this manuscript immensely, if they could identify a substrate modified with acetylated ubiquitin - maybe for such a single substrate, a higher percentage of modified proteins contain acetylated ubiquitin? While the biochemistry is nice, the in vivo relevance is really limiting in the current version of this paper.

We thank the reviewer for this great suggestion. Accordingly, we screened candidate substrates using SILAC, and identified histone H2B, among other histone-interacting/-modifying proteins (**new Figure 5E**). Mass spectrometric quantification showed that acetyl-ubiquitin was enriched more than ten-fold in the endogenous ubiquityl-histone fraction,

suggesting that histone H2B, and possibly also H2A, is an endogenous substrate for ubiquitin acetylation (**new Figure 5F**).

Minor point:

1. Introduction - Rpn10 does not specifically recognized K48-linked chains; remove from text.

We have removed this description from the text.

2nd Editorial Decision 03 November 2014

Thank you for your patience while we have reviewed your revised manuscript. As you will see from the reports below, although referee 3 still has some concerns regarding the physiological relevance of the findings, referees 1 and 2 are now positive about publication in EMBO reports. On balance and after further discussion, we have decided to move forward with the publication of your study, which is the first biochemical characterization of the effects of ubiquitin acetylation and identification of an endogenous substrate.

I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor issues/corrections have been addressed, as follows.

- Supplementary figure 4D should be moved to the main manuscript, ideally to figure 4. In addition, response letter figure 2-3 is an important control and should be included in the supplementary informaiton of your article.

- It is a precondition for publication in EMBO reports that authors agree to make all data freely available, where possible in an appropriate public database. In the case of mass spectrometry datasets, they should be deposited in a machine-readable format (e.g. mzML if possible) in one of the major public database, for example Pride (http://www.ebi.ac.uk/pride/) or PeptideAtlas(http://www.peptideatlas.org) and authors should follow the MIAPE recommendations (http://www.psidev.info/index.php?q=node/91).

This should be specified in the main text in the first instance where the data are mentioned, with the relevant accession code (which can also be included in the Methods section under the appropriate subheading).

- The Materials and Methods section is very succinct. Some informaiton regarding the in vitro ubiquitylation and E1/E2 charging assays, as well as the SPR experiments, should be included in the main text.

After all remaining corrections have been attended to, you will receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports.

REFEREE REPORTS:

Referee #1:

The revision has addressed all major comments.

Referee #2:

no further questions. the authors have done a good work in responding to questions.

Referee #3:

The authors addressed some of the issues raised in the first round of reviews, but my major concerns remain. It still needs to be established whether ubiquitin acetylation is a physiological significant event in cells that occurs on specific proteins.

1. I am not convinced that 0.01% of acetylated ubiquitin has a significant consequence on ubiquitin-dependent signaling. The histone H2B data is not convincing - histones are very abundant proteins, so if the acetylation of ubiquitin should protect histones from turnover, then one should see much higher levels of acetylated ubiquitin. Also, this acetylated ubiquitin should be found at particular chromosomal locations.

2. The experiments in Fig. 5 might suggest that histones could be substrates for acetylated histone, but the evidence is very weak and descriptive. Histones were identified as preferentially binding to Ub-K6Q (not acetylated ubiquitin), but not through a covalent purification of substrates. Thus, whether endogenous substrates exist for this modification remains unclear.

2nd Revision - authors' response 19 November 2014

Thank you very much for your consideration to our work. We greatly appreciate your evaluation of our manuscript for supporting the publication in *EMBO Reports*. Following your instructions, we have revised our manuscript and provided some information as follows:

1) We have moved previous supplementary Figure S4D to Figure 4B in the main manuscript, and previous Response Letter Figure #2-3 to supplementary Figure S8C, in the revised manuscript.

2) Mass spectrometry datasets have been deposited to PeptideAtlas, with identifier PASS00617 (password; YJ4592nm). The accession code was mentioned in the methods section of the main text.

3) We have modified methods section for more detailed information following your advice. Some information regarding *in vitro* ubiquitylation and E1/E2 charging assays and SPR experiments has been included in the main text.

We sincerely hope that our manuscript is now deemed acceptable for publication in *EMBO Reports*.

3rd Editorial Decision 20 November 2014

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

Thank you for your contribution to EMBO reports and congratulations on a successful publication.

Oncogene (2014), 1–9

www.nature.com/onc

ORIGINAL ARTICLE

Rad18 and Rnf8 facilitate homologous recombination by two distinct mechanisms, promoting Rad51 focus formation and suppressing the toxic effect of nonhomologous end joining

S Kobayashi[1], Y Kasaishi[1], S Nakada[2,5], T Takagi[3], S Era[1], A Motegi[1], RK Chiu[4], S Takeda[1] and K Hirota[1,3]

The E2 ubiquitin conjugating enzyme Ubc13 and the E3 ubiquitin ligases Rad18 and Rnf8 promote homologous recombination (HR)-mediated double-strand break (DSB) repair by enhancing polymerization of the Rad51 recombinase at γ-ray-induced DSB sites. To analyze functional interactions between the three enzymes, we created *RAD18*$^{-/-}$, *RNF8*$^{-/-}$, *RAD18*$^{-/-}$/*RNF8*$^{-/-}$ and *UBC13*$^{-/-}$ clones in chicken DT40 cells. To assess the capability of HR, we measured the cellular sensitivity to camptothecin (topoisomerase I poison) and olaparib (poly(ADP ribose)polymerase inhibitor) because these chemotherapeutic agents induce DSBs during DNA replication, which are repaired exclusively by HR. *RAD18*$^{-/-}$, *RNF8*$^{-/-}$ and *RAD18*$^{-/-}$/*RNF8*$^{-/-}$ clones showed very similar levels of hypersensitivity, indicating that Rad18 and Rnf8 operate in the same pathway in the promotion of HR. Although these three mutants show less prominent defects in the formation of Rad51 foci than *UBC13*$^{-/-}$cells, they are more sensitive to camptothecin and olaparib than *UBC13*$^{-/-}$cells. Thus, Rad18 and Rnf8 promote HR-dependent repair in a manner distinct from Ubc13. Remarkably, deletion of Ku70, a protein essential for nonhomologous end joining (NHEJ) significantly restored tolerance of *RAD18*$^{-/-}$ and *RNF8*$^{-/-}$ cells to camptothecin and olaparib without affecting Rad51 focus formation. Thus, in cellular tolerance to the chemotherapeutic agents, the two enzymes collaboratively promote DSB repair by HR by suppressing the toxic effect of NHEJ on HR rather than enhancing Rad51 focus formation. In contrast, following exposure to γ-rays, *RAD18*$^{-/-}$, *RNF8*$^{-/-}$, *RAD18*$^{-/-}$/*RNF8*$^{-/-}$ and *UBC13*$^{-/-}$cells showed close correlation between cellular survival and Rad51 focus formation at DSB sites. In summary, the current study reveals that Rad18 and Rnf8 facilitate HR by two distinct mechanisms: suppression of the toxic effect of NHEJ on HR during DNA replication and the promotion of Rad51 focus formation at radiotherapy-induced DSB sites.

Oncogene advance online publication, 24 November 2014; doi:10.1038/onc.2014.371

INTRODUCTION

Anticancer therapeutic DNA-damaging agents including ionizing radiation, camptothecin (a topoisomerase I poison) and olaparib (an inhibitor against poly(ADP ribose)polymerase) kill cycling cells by inducing double-strand breaks (DSBs). These agents create DSBs in distinct manners, as ionizing radiation generates DSBs in genomic DNA in a higher-order chromatin structure, whereas camptothecin and olaparib generate DSBs in one of the two sister chromatids during DNA replication. Ionizing radiation-induced DSBs are repaired by both homologous recombination (HR) and nonhomologous end joining (NHEJ),[1–4] whereas DSBs induced by camptothecin and olaparib are repaired exclusively by HR.[5–9] The two DSB repair pathways compete with each other as evidenced by the observation that the embryonic mortality caused by a defect in the HR factor BRCA1 is suppressed by the additional mutation of 53BP1, a factor involved in NHEJ.[10]

Vital roles for Rad18 and Rnf8 in genome maintenance have been suggested from a number of studies on cells treated with small interfering RNA (siRNA) for transient depletion of Rad18 or Rnf8. However, the strong effects caused by such transient depletion require careful interpretation as both the *RAD18*$^{-/-}$ and *RNF8*$^{-/-}$ mice develop normally without showing prominent defects in meiotic HR or NHEJ-dependent V(D)J recombination in lymphoid precursors.[11–15] Lack of any prominent phenotype in these mice is partly attributable to difficulty in precise phenotypic analysis of DNA damage response using primary culture cells. Moreover, the acute effect caused by siRNA-mediated transient depletion can be greater than the long-term effect caused by the loss of functional proteins, due to compensation for the loss by upregulation of other proteins in the long term. To analyze the effects caused by the loss of ubiquitylation enzymes involved in DNA damage responses, we have generated isogenic mutants from a single parental cell line, DT40 cells. In addition, to precisely distinguish the catalytic role from the non-ubiquitylation role of Rad18 and Rnf8, we created ubiquitylation-dead mutants (*RAD18*$^{C29F/-}$ and *RNF8*$^{C398F/-}$ cells) as well as null mutants (*RAD18*$^{-/-}$ and *RNF8*$^{-/-}$ cells; Table 1).

In this study, we analyzed the capability of *RAD18*$^{-/-}$, *RNF8*$^{-/-}$, *RAD18*$^{-/-}$/*RNF8*$^{-/-}$ and *UBC13*$^{-/-}$clones to perform HR to repair DSBs, and made the following three conclusions. First, Rad18 and Rnf8 facilitate DSB repair by HR using two distinct mechanisms: promoting Rad51 focus formation at γ-ray-induced DSB sites and

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Table 1. DT40 mutants used in this study

Cell line	*Selection marker for gene disruption*		*Reference*
	First gene	*Second gene*	
RNF8$^{-/-}$	*bsr/puro*	—	This study
UBC13$^{-/-}$	*bsr/his*	—	16
RAD18$^{-/-}$	*his/hygro*	—	1, 3
RAD18$^{-/-}$/*RNF8*$^{-/-}$	*his/hygro*	*bsr/puro*	This study
KU70$^{-/-}$	*his/bsr*	—	4
RNF8$^{-/-}$/*KU70*$^{-/-}$	*his/bsr*	*puro/neo*	This study
RAD18$^{-/-}$/*KU70*$^{-/-}$	*puro/hygro*	*his/bsr*	3
RNF8$^{C398F/-}$	*bsr*/Cre-excised *puro*	—	This study
RAD18$^{C29F/-}$	*neo*/Cre-excised *puro*	—	This study
RAD18$^{-/-}$/*RNF8*$^{C398F/-}$	*his/hygro*	*bsr*/Cre-excised *puro*	This study
RNF8$^{-/-}$/*RAD18*$^{C29F/-}$	Cre-excised both *bsr* and *puro*	*neo*/Cre-excised *puro*	This study

suppressing the toxic effect of NHEJ on HR during the repair of DSBs induced by camptothecin and olaparib. Second, Rad18 and Rnf8 promote Rad51 focus formation independently of each other, whereas the two enzymes function in the same pathway in suppressing the toxic effect of NHEJ on HR. Third, the non-catalytic functioning of Rad18 and Rnf8 significantly contributes to Rad51 focus formation.

RESULTS

RNF8$^{-/-}$ and *UBC13*$^{-/-}$ cells display distinctly different phenotypes

To disrupt the chicken *RNF8* locus, we constructed two targeting vectors, *RNF8-puro* and *RNF8-bsr* (Supplementary Figure 1A), and sequentially transfected these constructs into wild-type DT40 cells. Targeted disruption of the *RNF8* gene was verified by Southern blot analysis of *Xba*I-digested genomic DNA with the use of an external 3′ probe (Supplementary Figure 1B). Reverse transcription-PCR analysis showed the loss of RNF8 expression confirming disruption of the *RNF8* gene (Supplementary Figure 1C). *RNF8*$^{-/-}$cells proliferated with normal kinetics, whereas *UBC13*$^{-/-}$ cells proliferated with slower kinetics due to spontaneously occurring apoptosis, compared with wild-type cells[16] (Figure 1a). The extent of apoptosis during the cell cycle was closely correlated with the number of spontaneously arising mitotic chromosomal breaks among isogenic DNA repair-deficient DT40 mutants.[17] In agreement with the close correlation, *RNF8*$^{-/-}$ cells exhibited no increase in the number of spontaneous chromosomal breaks, whereas *UBC13*$^{-/-}$ cells exhibit a significant increase (Figure 1b). The severe genome instability of *UBC13*$^{-/-}$ cells agrees with the embryonic lethality of *UBC13*$^{-/-}$ mice.[18,19] These observations indicate that Ubc13 is able to contribute to genome maintenance independently of Rnf8.

To determine in which DNA repair pathways Rnf8 has a role, we measured cellular survival after exposure of cells to DNA-damaging agents. *KU70*$^{-/-}$ cells, but not *RNF8*$^{-/-}$ cells, were sensitive to ICRF193,[20,21] a catalytic inhibitor of topoisomerase II (Figure 1c), indicating that Rnf8 is not involved in the promotion of canonical NHEJ. We then measured sensitivity to camptothecin (a topoisomerase I poison) and olaparib (a poly (ADP ribose) polymerase inhibitor), two chemotherapeutic agents that induce replication fork collapse. The subsequent restart of replication requires HR with the intact sister chromatid.[5–9] *RNF8*$^{-/-}$ cells showed greater sensitivity to both camptothecin and olaparib than *UBC13*$^{-/-}$ cells, and the sensitivity was completely reversed by complementation with a *RNF8* transgene (Supplementary Figures 1D and F). The hypersensitivity in *RNF8*$^{-/-}$ cells to camptothecin is consistent with results of a previous study.[22] From the data of Figures 1d and e, we conclude that Rnf8 can function independently of Ubc13 in the cellular tolerance to the chemotherapeutic agents.

The Rad18 and Rnf8 ubiquitin ligases are compensatory for each other in genome maintenance and promotion of Rad51 polymerization at γ-ray-induced DSB sites

To analyze the functional relationship between Rad18 and Rnf8, we generated *RAD18*$^{-/-}$/*RNF8*$^{-/-}$ double mutant cells. Spontaneously arising chromosome breaks in *RAD18*$^{-/-}$/*RNF8*$^{-/-}$ double mutant were increased fourfold as compared with either *RAD18*$^{-/-}$ or *RNF8*$^{-/-}$ single-mutant cells (Figure 1b). This result reveals substantial functional redundancy between Rad18 and Rnf8 in genome maintenance during the cell cycle.

We next measured the sensitivity of *RAD18*$^{-/-}$/*RNF8*$^{-/-}$ cells to ICRF193, camptothecin, olaparib and γ-rays. *RAD18*$^{-/-}$, *RNF8*$^{-/-}$ and *RAD18*$^{-/-}$/*RNF8*$^{-/-}$ cells showed no sensitivity to ICRF193 (Figure 2a); however, they were similarly sensitive to camptothecin (Figure 2b). Moreover, the three types of cells showed similar sensitivity to olaparib (Figure 2c). In sharp contrast, *RAD18*$^{-/-}$/*RNF8*$^{-/-}$ cells showed significantly greater sensitivity to γ-rays than observed with *RAD18*$^{-/-}$ or *RNF8*$^{-/-}$ cells (Figure 2d). We therefore conclude that Rad18 and Rnf8 ubiquitin ligases operate in the same pathway in the HR-mediated repair of DSBs induced by the two chemotherapeutic agents, whereas Rad18 and Rnf8 can facilitate HR-mediated repair of γ-ray-induced DSBs independently of one another. To explore the role of the two ubiquitin ligases in HR, we measured Rad51 focus formation following γ-rays irradiation. The Rad51 focus formation of *RAD18*$^{-/-}$/*RNF8*$^{-/-}$ cells was reduced 2.5-fold as compared with either *RAD18*$^{-/-}$ or *RNF8*$^{-/-}$ cells (Figure 2e and Supplementary Figure 2B). This phenotype agrees with the γ-ray sensitivity of *RAD18*$^{-/-}$, *RNF8*$^{-/-}$ and *RAD18*$^{-/-}$/*RNF8*$^{-/-}$ cells. Thus, the two ubiquitin ligases independently promote the function of Rad51 at γ-ray-induced DSBs.

To investigate whether the functional redundancy seen in the DT40 clones were relevant to human cells, we analyzed γ-ray-induced Rad51 focus formation in human HCT116 cells. The depletion of Rnf8 was assessed by analyzing the disappearance of 53BP1 foci[23–25] (Supplementary Figure 3). Similar to the observation in *RAD18*$^{-/-}$ mouse embryonic fibroblasts,[26] *RAD18*$^{-/-}$ HCT116 cells displayed only a slight defect in Rad51 focus formation. Although the depletion of Rnf8 caused only a mild reduction of Rad51 foci formation in the *RAD18*$^{+/+}$ HCT116 cells, it significantly decreased the number of Rad51 foci in the *RAD18*$^{-/-}$ cells (Figure 2f and Supplementary Figure 3). We therefore conclude that Rad18 and Rnf8 independently contribute to the Rad51 focus formation at γ-ray-induced DSBs in the chicken and human cells.

The inactivation of NHEJ in *RNF8*$^{-/-}$ and *RAD18*$^{-/-}$ cells significantly restores their tolerance to camptothecin and olaparib

The epistatic relationship between *RAD18* and *RNF8* in cellular tolerance to camptothecin and olaparib (Figures 2b and c) led us to investigate whether Rnf8 suppresses the toxic effect of NHEJ on HR as does Rad18.[3] We inactivated NHEJ by disrupting the *KU70* gene in *RNF8*$^{-/-}$ cells, and found that the loss of Ku70 in *RNF8*$^{-/-}$ as well as *RAD18*$^{-/-}$ cells completely restored their cellular tolerance to camptothecin (Figure 3a). Similarly, *RNF8*$^{-/-}$/*KU70*$^{-/-}$ and *RAD18*$^{-/-}$/*KU70*$^{-/-}$ cells were more tolerant to olaparib than *RAD18*$^{-/-}$ and *RNF8*$^{-/-}$ cells (Figure 3b). Thus, Rad18 and Rnf8 might suppress the toxic effect of NHEJ on HR. To verify that the observed cellular tolerance represents the capability of cells to repair one-end breaks, which occur as a consequence of replication over broken template strands,

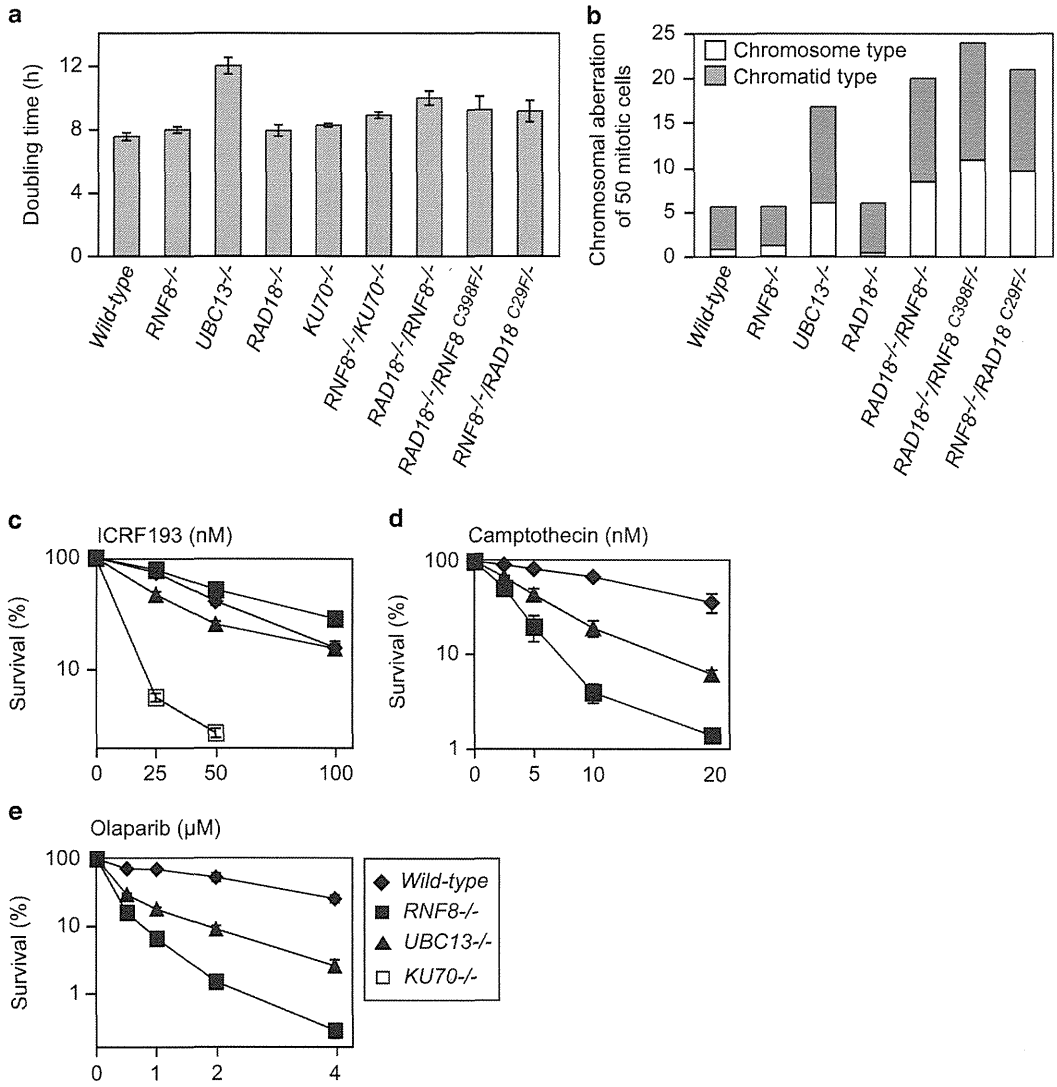


Figure 1. *RNF8*$^{-/-}$ and *UBC13*$^{-/-}$ cells display distinctly different phenotypes. (**a**) The average doubling time for the indicated genotypes. Error bars show the s.e. in at least three independent experiments. (**b**) Spontaneously arising chromosomal aberrations in 50 mitotic cells. Two breaks at the same site of both sister chromatids are defined as chromosome-type breaks (white rectangle), whereas breaks at one of the two sister chromatids are chromatid-type (gray rectangle). (**c**–**e**) Cellular sensitivity to ICRF193 (**c**), camptothecin (**d**) and olaparib (**e**) was analyzed. Survival rate was calculated as the percentage of surviving cells treated with DNA-damaging agents relative to the untreated surviving cells. The concentration or dose is displayed on the horizontal axis on a linear scale, whereas the survival rate is displayed on the *y* axis on a logarithmic scale. Error bars show the s.e. of the mean in at least three independent experiments.

we examined chromosomal aberrations in mitotic cells having been treated with camptothecin. *RNF8*$^{-/-}$ and *RAD18*$^{-/-}$ cells showed significant increases in the number of chromosomal aberrations compared with wild-type cells, whereas such increases were not seen in *RNF8*$^{-/-}$/*KU70*$^{-/-}$ and *RAD18*$^{-/-}$/*KU70*$^{-/-}$ cells (Figure 3c). Thus, cellular survival (Figure 3a) represents the capability of cells to repair one-end breaks. Taken together, we conclude that the collaboration between Rad18 and Rnf8 suppresses the toxic effect of NHEJ on HR-dependent repair of one-end breaks (Figure 3c).

There are three possible mechanisms underlying the toxic effect by NHEJ. The Ku proteins might suppress HR by inhibiting the polymerization of Rad51 at one-end breaks or by interfering with subsequent steps such as homology search and strand exchange. The third mechanism involves aberrant NHEJ of two one-end breaks derived from neighboring stalled replication forks. We measured Rad51 focus formation following exposure of cells to camptothecin, and found that the loss of Ku70 did not restore the Rad51 focus formation of *RNF8*$^{-/-}$ cells (Figure 3d and Supplementary Figure 2A). Thus, Rnf8 suppresses the toxic effect of NHEJ on HR presumably by facilitating homology search and/or strand exchange. To address the third mechanism, we counted the number of radial chromosomes (Figures 3c, e and f), which arises mainly as a consequence of aberrant NHEJ,[10] as NHEJ-deficient *KU70*$^{-/-}$ cells exhibited a few times lower radial chromosomes events when compared with wild-type cells (Figure 3f). The number of radial chromosomes in *RAD18*$^{-/-}$/*RNF8*$^{-/-}$ cells was very similar to that in *RNF8*$^{-/-}$ and *RAD18*$^{-/-}$ cells. Strikingly, the loss of Ku70 in *RNF8*$^{-/-}$ and *RAD18*$^{-/-}$ cells greatly reduced the number of radial chromosomes (Figures 3c and f). We conclude that Rad18 and Rnf8 work together to promote HR-dependent repair of one-end breaks by inhibiting both aberrant NHEJ of two one-end breaks and a suppressive effect of NHEJ on HR.

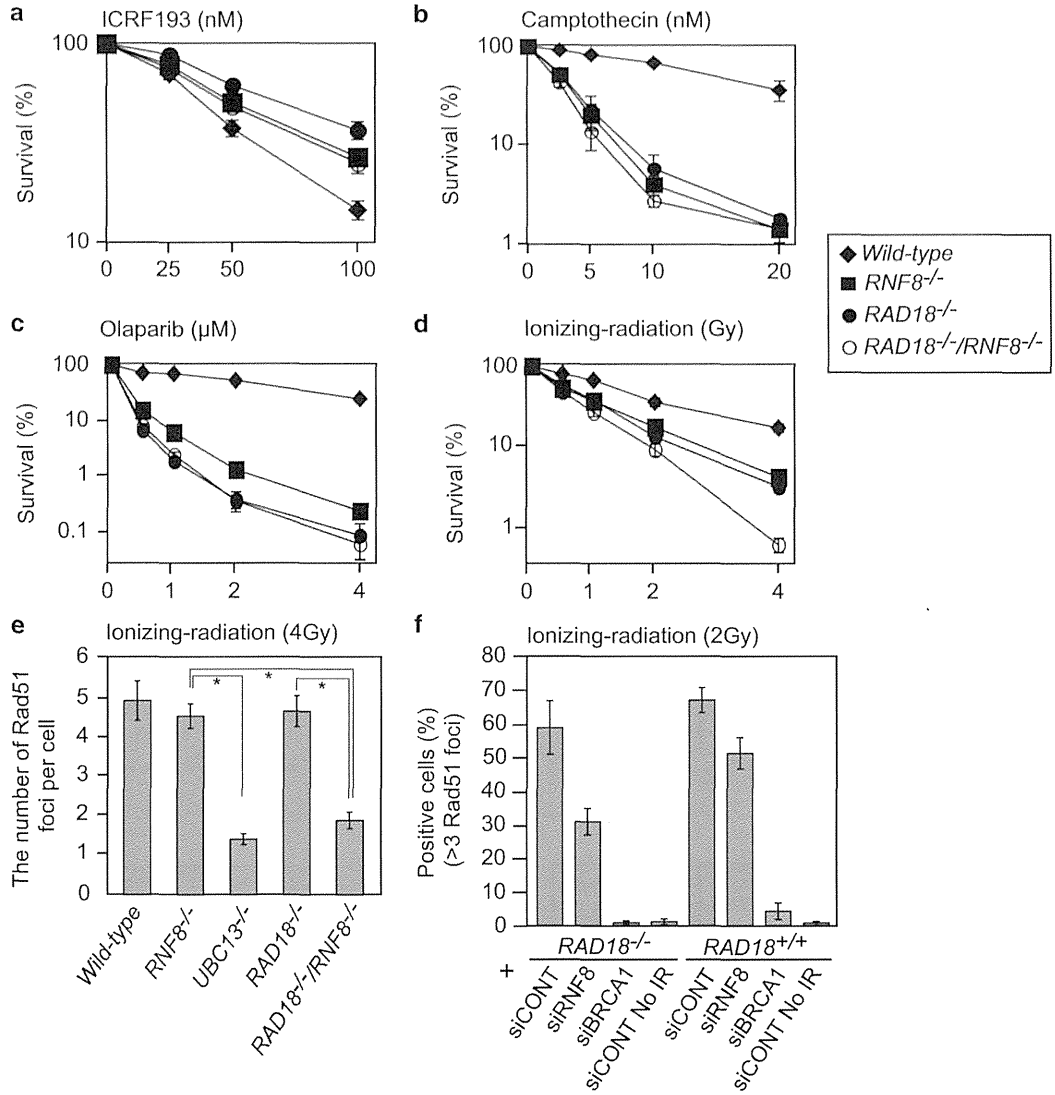
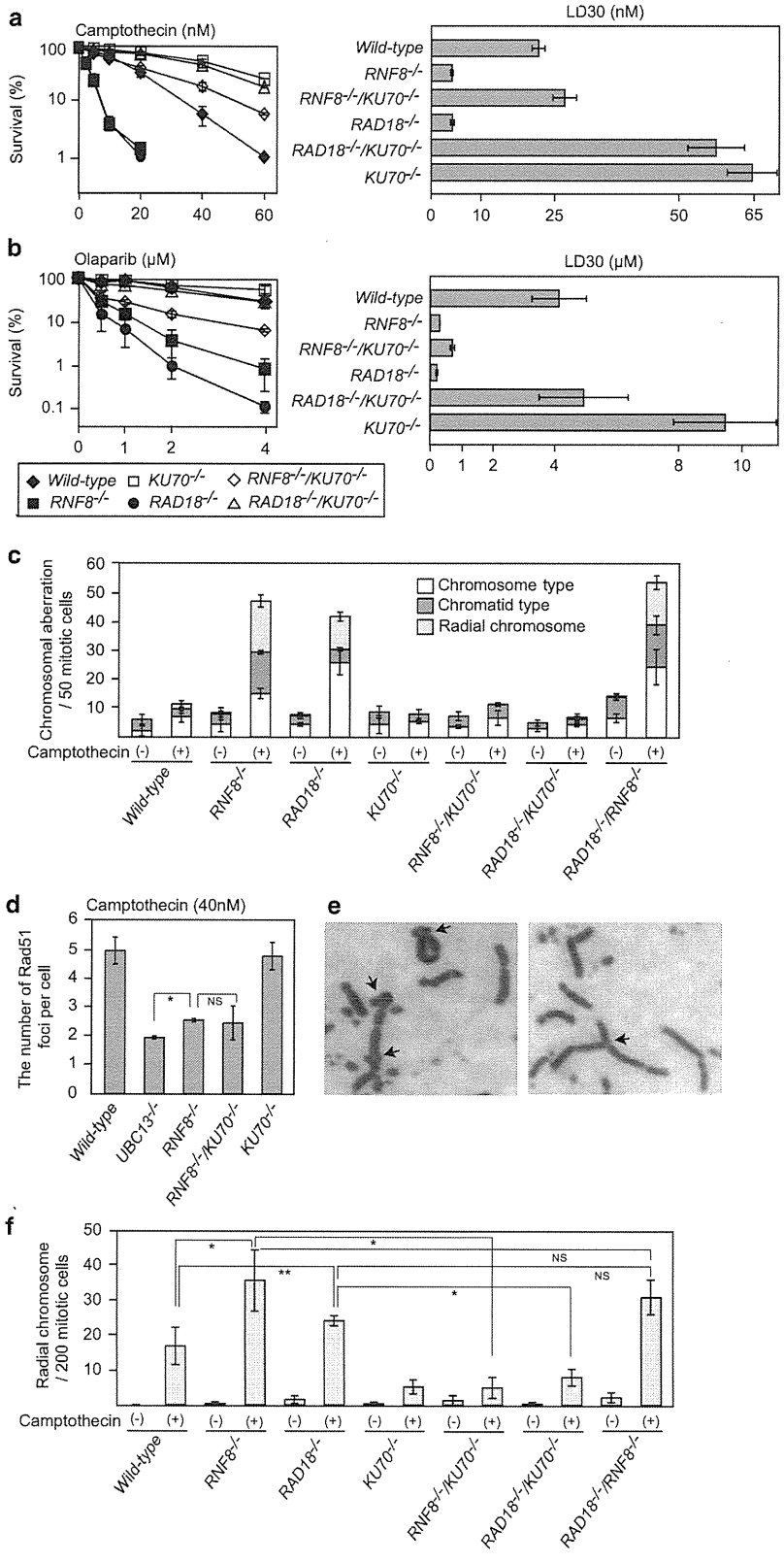


Figure 2. The Rad18 and Rnf8 ubiquitin ligases operate in the same pathway in cellular tolerance to camptothecin and olaparib, whereas they promote Rad51 recruitment to γ-ray-induced DSB sites independently of each other. (**a**–**d**) Cellular survival of the indicated DNA-damaging agents is shown as in Figure 1. ICRF193 (**a**), camptothecin (**b**), olaparib (**c**) and ionizing radiation (^{137}Cs γ-ray) (**d**). (**e**) The average number of γ-ray-induced Rad51 foci per cell. Cells were fixed 1h after 4 Gy γ-rays irradiation and then stained with anti-Rad51 antibody. Each histogram represents the value obtained by subtracting the number of Rad51 foci in non-irradiated controls from observed foci 1 h after irradiation. The actual number of the number of Rad51 foci is shown in Supplementary Figure 2B. Cells (100–200) were counted and error bars represent s.e. calculated from at least three independent experiments. Statistical significance was determined by a Student's *t*-test: $^{*}P < 0.01$. (**f**) HCT116 cells transfected with the indicated siRNAs were exposed to 2 Gy ionizing radiation. Six hours after irradiation (IR), cells were fixed and subjected to Rad51 and 53BP1 immunofluorescence analysis. Cells treated with siRNA for 2 days were used in this experiment. Depletion of RNF8 was assessed by the disappearance of 53BP1 foci (Supplementary Figure 3). Cells with more than three Rad51 foci were counted as positive cells. Cells (300) were counted. Data are represented as the mean ± s.d. ($n = 3$).

Figure 3. Collaboration between Rad18 and Rnf8 suppresses the toxic effect of NHEJ on HR and contributes to the cellular tolerance to camptothecin and olaparib. (**a**, **b**) Cellular sensitivity to camptothecin (**a**), olaparib (**b**) and γ-rays (**d**) is shown as in Figure 1. Lethal dose 30% (LD30) is the concentration of DNA-damaging agents that reduces cellular survival to 30% relative to cells non-treated with DNA-damaging agents. (**c**) Chromosomal aberrations in cells treated with 10 nM camptothecin. The data represent the mean and s.d. from three independent counts each analyzing 50 mitotic cells. (**d**) The average number of Rad51 foci per cell in camptothecin-treated cells. Cells were exposed to 40 nM camptothecin for 1h and then stained with anti-Rad51 antibody. Each histogram represents the value obtained by subtracting the number of Rad51 foci in non-irradiated controls from observed foci 1h after irradiation. The actual number of Rad51 foci is shown in Supplementary Figures 2A and B. Cells (100–200) were counted in each experiment, and error bars represent s.e. calculated from at least three independent experiments. *P*-value was calculated by a Student's *t*-test: $^{*}P < 0.01$ and NS (not significant). (**e**) Representative images of radial chromosomes are shown. Arrows indicate radial chromosomes. (**f**) Formation of radial chromosomes in cells treated with 100 nM camptothecin. The data represent the mean and s.d. from three independent experiments each analyzing 200 mitotic cells. *P*-value was calculated by a Student's *t*-test: $^{*}P < 0.01$, $^{**}P < 0.05$ and NS (not significant).



In summary, Rnf8 and Rad18 facilitate HR in two distinctly different ways, the promotion of Rad51 polymerization at DSB sites and suppression of the toxic effect by NHEJ on HR. Moreover, the functional relationship between Rad18 and Rnf8 differs depending on DNA-damaging agents, as the two enzymes are compensatory for each other in the promotion of Rad51 polymerization at γ-ray-induced DSBs, whereas the two enzymes operate in the same pathway in maintaining replication fork progression when cells are exposed to camptothecin and olaparib.

The ubiquitylation activity of Rad18 and Rnf8 is essential for cellular tolerance to camptothecin and olaparib

Accumulating evidence has suggested a non-catalytic (non-ubiquitylation) function of Rad18 and Rnf8 in the initial step of HR-dependent DSB repair.[26–28] To address possible non-ubiquitylation roles of Rad18 and Rnf8, we selectively inactivated the ubiquitylation activity by mutating C398F and C29F in the *RNF8* and *RAD18* genes, respectively,[26,27,29] and generated $RNF8^{C398F/-}$ (Supplementary Figure 4A), $RAD18^{C29F/-}$ (Supplementary Figure 5A), $RAD18^{-/-}/RNF8^{C398F/-}$ and $RNF8^{-/-}/RAD18^{C29F/-}$ clones. The *RNF8* and *RAD18* knock-in mutations were confirmed by reverse transcription-PCR (Supplementary Figures 4B and 5B) and sequence analysis. The C398F mutation completely inactivates the ubiquitylation activity of Rnf8, as $RNF8^{C398F/-}$ as well as $RNF8^{-/-}$ cells showed no ubiquitin foci at γ-ray-induced DSB sites (Supplementary Figure 4C). The C29F mutation also inhibits the ubiquitylation activity of Rad18, as UV-induced monoubiquitylation of proliferating cell nuclear antigen (PCNA) was not increased in $RAD18^{C29F/-}$ or $RAD18^{-/-}$ cells (Supplementary Figure 5C).

We first evaluated the effects of the C29F and C398F mutations on the maintenance of genome integrity (Figure 1b). The number of spontaneously arising mitotic chromosomal breaks in $RNF8^{-/-}/RAD18^{C29F/-}$ and $RAD18^{-/-}/RNF8^{C398F/-}$ clones was very similar to that in $RAD18^{-/-}/RNF8^{-/-}$ cells, indicating that the catalytic functioning of the two enzymes is critical for genome stability. Next we evaluated the effects of the C29F and C398F mutations on the cellular tolerance to camptothecin and olaparib (Figures 4a and b). The C29F and C398F mutations had the same effect on the cellular tolerance to both camptothecin and olaparib, as did the null mutations of the *RAD18* and *RNF8* genes (Figures 4a and b). Therefore, the ubiquitylation activity of both Rad18 and Rnf8 is essential for repressing the toxic effect of NHEJ on HR-dependent DSB repair.

The contribution of non-ubiquitylation roles played by Rad18 and Rnf8 in initiating HR at γ-ray-induced DSB sites

We next evaluated the non-catalytic functions of Rad18 and Rnf8 in the repair of γ-ray-induced DSBs as well as γ-ray-induced Rad51 focus formation. The *RAD18* C29F mutation had the same effect on γ-ray sensitivity as the $RAD18^{-/-}$ null mutation (Figure 4c). Similarly, the *RNF8* C398F mutation had the same effect on γ-ray sensitivity as the $RNF8^{-/-}$ null mutation (Figure 4c). Thus, the non-ubiquitylation roles played by Rad18 and Rnf8 contribute only marginally, if any, to HR-dependent repair of γ-ray-induced DSBs. To assess the role of ubiquitylation by Rnf8 in Rad51 focus formation, we counted the number of Rad51 foci in ionizing irradiation treated $RAD18^{-/-}$, $RAD18^{-/-}/RNF8^{C398F/-}$ and $RAD18^{-/-}/RNF8^{-/-}$ clones after 1 h. The Rad51 focus formation of $RAD18^{-/-}/RNF8^{C398F/-}$ cells was lower than that of $RAD18^{-/-}$ cells while higher than $RAD18^{-/-}/RNF8^{-/-}$ cells (Figure 4d). This result indicates that Rnf8 promotes Rad51 polymerization through both non-catalytic and protein ubiquitylation, as suggested previously,[27] particularly when Rad18 is absent. Similarly, the Rad51 focus formation of $RNF8^{-/-}/RAD18^{C29F/-}$ cells was an intermediate between those of $RNF8^{-/-}$ and $RAD18^{-/-}/RNF8^{-/-}$ clones (Figure 4d and Supplementary Figure 2B), indicating that the non-catalytic function of Rad18 has an important role in Rad51 focus formation.

DISCUSSION

In this study, we demonstrated that Rad18 and Rnf8 facilitate HR by two distinct mechanisms, promotion of Rad51 focus formation and suppression of the toxic effect of NHEJ on HR. The latter mechanism does not require the promotion of Rad51 focus formation as the loss of Ku70 reversed the cellular tolerance of $RNF8^{-/-}$ cells to camptothecin without changing Rad51 focus formation (Figure 3d). Thus, Rad18 and Rnf8 contribute to cellular tolerance to antimalignant therapies with more complex mechanism than previously appreciated.

We also revealed complex functional relationships between the Rad18 and Rnf8 ubiquitin ligases. The two enzymes operate in the same pathway in cellular tolerance to camptothecin and olaparib (Figures 2b and c), whereas they work independently in both cellular tolerance to radiotherapy (Figure 2d) and promoting the function of Rad51 at DSBs induced by γ-rays (Figures 2e and f). Moreover, the catalytic role of Rad18 and Rnf8 is required for the cellular tolerance to camptothecin, olaparib and radiotherapy (Figures 4a–c), whereas the non-catalytic function of both enzymes contributed to Rad51 focus formation (Figure 4d). In summary, the functional relationship between Rad18 and Rnf8 in DNA damage responses is distinctly different depending on the type of DNA damage.

Although a large number of studies previously suggested pivotal roles of Rad18 and Rnf8 in DNA damage responses, mice deficient in either Rad18 or Rnf8 display only modest phenotypes and are capable of performing meiotic HR.[13,30] The moderate phenotypes imply that other related enzymes potentially compensate for the absence of Rad18 or Rnf8. We demonstrated in this study that the two enzymes compensate for one another in the maintenance of chromosomal DNA, as only $RAD18^{-/-}/RNF8^{-/-}$ cells but not $RAD18^{-/-}$ or $RNF8^{-/-}$ cells showed severe genome instability (Figure 1b) as observed in $UBC13^{-/-}$ cells.[16] Conceivably, the dramatic phenotype of $RAD18^{-/-}/RNF8^{-/-}$ and $UBC13^{-/-}$ cells results from combined defects of HR-dependent DSB repair coupled with defects in post-replicational repair. In summary, Rad18 and Rnf8 have a substantially redundant role in genome maintenance.

HR has a dominant role over NHEJ in DSB repair of *Saccharomyces cerevisiae*, whereas HR and NHEJ contribute equally to the whole DSB repair outcome in metazoan cells. Given that in metazoans, a DSB is targeted by both HR and NHEJ, the two pathways could potentially interfere with each other and prevent effective DSB repair (reviewed in Chapman *et al.*[31]). To avoid such interference, NHEJ must be actively inhibited, in particular for the repair of the DSBs that occur in one of the two sister chromatids during DNA replication. This is because camptothecin and olaparib induce numerous DSBs associated with replication and these DSBs are repaired accurately by HR but not by NHEJ. Recent study has reported that the loss of 53BP1 in Brca1-deficient cells rescues HR defect, indicating that Brca1 antagonizes 53BP1 and promotes end resection in S phase.[10] Our current study further revealed that a collaboration between Rad18 and Rnf8 prevent the function of NHEJ and thereby facilitates HR-mediated repair of DSBs induced by camptothecin and olaparib. Moreover, NHEJ causes an increase in the number of radial chromosomes in both $RNF8^{-/-}$ and $RAD18^{-/-}$ cells (Figures 3c and f). We also revealed that the inhibition of NHEJ requires functional ubiquitylation capability of the two enzymes (Figures 4a and b), perhaps monoubiquitylation by Rad18 followed by polyubiquitylation by Rnf8. The precise identity of the Rad18 and Rnf8 ubiquitylation substrate remains an important unresolved question. Although ubiquitylation of 53BP1, Ku80 and proteins involved in the Fanconi anemia pathway by Rad18[32,33] was reported, they might not contribute to cellular