THE EMBO JOURNAL

Manuscript EMBO-2009-72925

A viral E3 ligase targets RNF8 and RNF168 to control histone ubiquitination and DNA damage responses

Caroline E. Lilley, Mira S. Chaurushiya, Chris Boutell, Sebastien Landry, Junghae Suh, Stephanie Panier, Roger D. Everett, Grant S. Stewart, Daniel Durocher and Matthew D. Weitzman

Corresponding author: Matthew D. Weitzman, Salk Institute

Review timeline:

Submission date:	21 October 2009
Editorial Decision:	26 November 2009
Revision received:	04 December 2009
Accepted:	10 December 2009

Transaction Report:

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

1st Editorial Decision 26 November 2009

I would like to thank you for submitting your manuscript for consideration by The EMBO Journal. Your manuscript has now been evaluated by three referees and I enclose their reports below. As you will see from their comments the referees are all positive regarding the study and require that a number of issues are resolved before publication in the EMBO Journal. These include the effect of ICP0 on RNF8 and RNF168 localization and the specificity of the phosphorylated S1981 ATM antibody. Once these issues are satisfactorily addressed, we would be happy to publish a revised manuscript.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

One of the most intriguing and important virus-host interactions is the mysterious ability of herpes viruses to exist in a latent form in nerves for the life of the host and re-activate upon some form of stress. Recent work in several labs has suggested that the chromatin status of latent viral genomes in neurons differs from that of viral genomes in lytically infected epithelial cells. The switches between these states are complex and not well understood. ICP0, a multifunctional immediate early protein expressed in lytically infected cells, is one determinant of latency, but how it functions is only partially understood. Lastly, herpes viruses have evolved to utilize some aspects of cellular stress responses, but the details are far from clear.

This manuscript deals with the convergence of these disparate areas and provides a fascinating body of evidence indicating at least one way that ICP0 selectively modulates host cell DNA damage signaling to promote viral lytic infection. The first point made is that ICP0 both prevents IR-induced damage foci (IRIF) and induces the disassembly of pre-formed IRIF when the cell is infected with wt virus (HSV) or transfected with ICP0 expression plasmid. Without ICP0 or with mutant ICP0 defective in Ub-ligase activity of ICP0, IRIF remain unperturbed. The target of ICP0 in IRIF appears to be after Mdc1 binding to gH2AX, but prior to 53BP1 recruitment to foci (Fig. 1, 2). Recent evidence from several labs that histone ubiquitylation contributes to IRIF formation via RNF8 Ub ligase led the authors to the observation that ICP0 expression was associated with under-ubiquitylation of histone H2A/AX, and from there to the ICP0 Ub-ligase-dependent and proteasome-dependent reduction of RNF8 and RNF168 levels (Fig. 3, 4). The physical association of RNF8 with ICP0 and the ability of purified ICP0 to ubiquitylate RNF8 in vitro strongly suggest that ICP0 modifies RNF8, limiting histone ubiquitylation in chromatin at sites of IR damage and 53BP1 association to form IRIFs (Fig. 5, 6). Lastly, the authors demonstrate that wt HSV plaque formation in MEFs lacking RNF8 is only slightly reduced by reconstituting the MEFs with RNF8, whereas viral yield of ICP0-mutant viruses is strongly reduced in reconstituted cells relative to RNF8-knockout cells (Fig. 6C). This is the first demonstration of a novel molecular mechanism by which ICP0 benefits viral propagation.

Specific points
1. The description of the experiment in Fig. 2A is confusing both in the results (p. 9) and figure legend. Does mock refer to the IR control, the ICP0 transfection control or both? The results do not state which panels in Fig. 2A are being described or how the result leads to the conclusion drawn. Which panels show endogenous Mdc1 and which show GFP-Mdc1 (or Mdc1-GFP?)- transfected? The plasmid is not described in methods either.
2. Fig. 2E. Title states that ICP0 results in increased post-IR mobility of 53BP1, but actually it prevents the IR-induced decrease in 53BP1 mobility, as judged by looking at the data.
3. p. 10 middle. There is abundant suppl data in this manuscript, but a crucial piece of data is cited here as not shown: that H4-K20me2 level is not altered by ICP0.
4. The discussion is interesting but rambles a bit. Perhaps adding subtitles would pull it together and highlight the authors' points more succinctly. Of particular interest is the link to transcriptional silencing and its re-activation by ICP0 in exit from viral latency and possible correlations with transcriptional silencing during normal cell differentiation.

Referee #2 (Remarks to the Author):

This is an interesting continuation of the systematic effort by the Weitzman lab to explore how viruses subvert the DNA damage machinery of the host cell.

Here the authors provide evidence that that the ICP0 protein of the Herpes Simplex Virus type 1 (HSV-1) disrupts regulation of the DNA double strand breaks (DSBs) by targeting RNF8 and RNF168, two ubiquitin ligases that facilitate retention of repair proteins at the DSB-flanking chromatin. It is shown that ICP0 expression triggers a progressive loss of H2A and H2AX ubiquitylation followed by mobilization of 53BP1 (an established mediator of DNA damage repair

and signalling) from the ionizing radiation induced foci. Mechanistically, evidence is provided that ICP0 (itself an E3 ligase) binds and ubiquitylates RNF8 and thereby promotes its degradation by the proteasome. Interestingly, levels of RNF168 also rapidly decline in the ICP0-expressing cells although the exact mechanism of this has not been determined. Finally, it is shown that ICP0-null or RING mutant viruses exhibited reduced plaque-forming efficiency in RNF8WT cells when compared to the RNF8 KO counterparts. This result underscores the physiological relevance of these findings and suggests that ICP0 contributes to activation of latent viruses by subverting histone ubiquitylation and thus mobilizing DNA repair proteins.

Overall, this is an interesting study with implications going well beyond the virology field. In particular, there is growing evidence from this and previous studies that viral proteins often target important pathways involved in genome maintenance. The finding that ICP0 destabilizes the two key ubiquitin ligases involved in coordinating retention of repair factors on damaged chromosomes is important and underscores the emerging significance of regulatory ubiquitylation in the DNA damage response.

I am happy to recommend publication in principle, but several important issues need to be addressed and/or clarified before publication.

Specific points
1) Fig. 1: Using an antibody to phosphorylated S1981 of ATM and concluding that expression of ICP0 'prevented accumulation of activated ATM at IRIF' (p. 7) is misleading and very likely incorrect for several reasons: First, it has been repeatedly observed that most anti-pS1981 antibodies recognize also other proteins phosphorylated by ATM. As a matter of fact, excluding these crossreactions in situ is technically impossible because knockdown of ATM would preclude not only ATM autophosphorylation but also all other downstream phosphorylations. Second, retention of ATM at DSBs has been extensively studied and reported to be mediated by NBS1 and MDC1, respectively. However, both of these factors are recruited to DSBs independently of RNF8/RNF168 and thus the logical prediction would be that degradation of these to E3 ligases by ICP0 should NOT interfere with ATM retention but rather selectively displace proteins operating downstream in this pathway such as 53BP1 (as is clearly shown in Fig. 2). In fact, 53BP1 itself is an ATM target and some of the pS1981-decorated IRIFs in Fig. 1 may well represent it, or other proteins that require ubiquitin for their chromatin retention and are at the same time phosphorylated by ATM. In summary - I am not asking the authors to remove this figure - it is indeed an informative set of data and a good entry point to the whole story; all I am asking is to interpret the data properly. Just one suggestion is as follows: 'ICP0 prevented local, ATM-dependent phosphorylations at the DSBs, suggesting that localization or activation of repair proteins was disrupted'. According to this reviewer's opinion, this is the only correct conclusion that can be drawn from these data and in fact Fig. 2 nicely discriminates between the two possibilities and shows that the 'defect' in ICP0-expressing cells is on the level of retention of 53BP1.
2) Fig. 2: These are very interesting and important results - the only think that remain unexplained and thus can create confusion here (and in all other figures that contain images with overexpressed ICP0) is the sequestration of the ICP0 protein to prominent nuclear speckles. What are these structures? These cannot be viral replication centres because the protein clearly aggregates when transfected alone. It is suggested (p. 14) that these might be 'sites of ICP0 expression' but no evidence is provided to support such claim. Is there a precedent that other viral proteins behave that way? This should be clarified and in general this issue needs to be treated with caution throughout the paper not just because it may create confusion but because it actually has implications for interpreting important experiments - on such case is described in the following comment.
3) Fig. 5A: There are in fact several issues that I do not fully understand here. Firstly, as shown in the previous biochemical assays (Fig. 4), RNF8 becomes progressively degraded after ICP0 transfection. Yet, in Fig. 5A, both proteins co-exist and colocalize in the nuclear speckles. How to explain this? Is it a matter of timing? Would RNF8 become eventually degraded by ICP0 also in these speckles? An alternative (and in fact not uninteresting) possibility is that ICP0 might regulate RNF8 at two levels - one, by degrading the bulk of the protein (Fig. 4) and two, by sequestering a fraction of it to nuclear speckles (Fig. 5A). The latter scenario begs for an important extension of this Figure - specifically, what happens with the ICP0/RNF8 foci after IR? Would ICP0 prevent re-localization of the available RNF8 from the speckles to the DSB sites? And finally, is RNF168 also present in these speckles? The mechanism of how ICP0 destabilizes RNF168 is puzzling (as the authors mention in the text, these two proteins do not bind each other and also RNF8 does not seem

to 'bridge' ICP0 with RNF168...). Perhaps a bit more insight into the RNF168 localization before and after ICP0 expression (and with and without IR) may help elucidate this issue.

Referee #3 (Remarks to the Author):

In this manuscript, Weitzman and colleagues describe the effect of the Herpes simplex viral protein IPC0 on components of the mammalian DNA damage response. They show that IPC0 - a ubiquitin ligase - impacts upon the recently discovered RNF8/RNF168 pathway by triggering the degradation of both RNF8 and RNF168. This leads to a decreased histone H2A and H2AX ubiquitylation and hence, to a defective accumulation of 53BP1 and BRCA1 at sites of DSBs. The authors also claim that this mechanism may lead to the activation of the latent viral genome.
This is a thorough study and the data set is complete and convincing. There is however one major issue that needs attention before I could recommend this manuscript for publication in EMBO J. In Figure 1, the authors conclude that IPC0 has a strong negative effect on the accumulation of phosphorylated ATM at sites of DSBs. I think the data they present do not allow such a conclusion. The mechanism by which the ATM kinase accumulates in/is retained at, chromatin regions flanking DSBs is highly controversial. Some groups suggested that ATM interacts directly with the MDC1 FHA domain and is thus coupled to damaged chromatin (Lou et al., 2006, Mol Cell). Other labs have shown that the NBS1 C-terminus directly interacts with ATM and that this interaction is required for ATM recruitment/accumulation at sites of DSBs (Falck et al., 2005, Nature). None of these studies has indicated that ATM accumulation is downstream of the RNF8/RNF168 pathway. However, the present study indirectly does so, since the authors show that IPC0 impacts upon the RNF8/RNF168 pathway and apparently, also leads to a defective ATM accumulation. If this were true, these results would be a very significant novel finding and would put into question the current models of ATM recruitment (especially given that the authors also show that IPC0 does not have an effect on MDC1 and NBS1 recruitment and foci formation).
In Figure 1, the authors use a phospho-specific antibody against ATM phosphorylated at Ser1981. It has been shown in the past that these phosphospecific antibodies are quite promiscuous, i.e., they recognize other phosphorylated proteins besides the ones they have been raised against. This is usually not very relevant in Western blotting, because this technique also yields information about the size of a protein. However, in immunofluorescence microscopy, this information is lost. I know that some of the S1981P-"specific" antibodies actually efficiently recognize phosphorylated 53BP1 in IF, and not ATM. Thus, if the authors would like to stick to their conclusion in Figure 1, they need to properly control that their antibody does indeed recognize ATM-S981P and not another protein. This is a bit hard to do because even though the signal may get lost in A-T cells (or upon inhibition of the ATM kinase), this does not yet proof that the antibody is specific for ATM, because the other protein(s) it recognizes may be ATM targets. One way to properly test this antibody would be to use an ATM deficient cell line that has been reconstituted with ATM mutated at Ser1981 (e.g. S1981A). Such cell lines have been published, e.g. by the Lavin lab. Unfortunately, ATM S1981A is non functional, which also renders it useless as a control. Thus, in my opinion, the only reliable control would be to use in this experiment an antibody raised against the unphosphorylated ATM kinase.
If the authors however don't wish to go down that road (which in my opinion, would be a wise decision...) they could just simply use an antibody raised against another protein in this set of experiments in Figure 1, e.g. 53BP1. After all this paper is about a viral protein that targets the RNF8/RNF168 pathway and not about the highly controversial issue of how the ATM kinase is retained at sites of DSBs.

Minor issue:

I don't understand why Flag-RNF8 signal is not reduced in GFP-ICP0 transfected cells in Figure 5A.

1st Revision - authors' response 04 December 2009

REFEREE #1
This referee felt that we had provided "a fascinating body of evidence indicating at least one way that ICP0 selectively modulates host cell DNA damage signaling to promote viral lytic replication". He/she stated that our work was the "first demonstration of a novel molecular mechanism by which ICP0 benefits viral propagation". The referee had several specific points which we have addressed below:

1. The referee felt that the experiment in Figure 2A was confusing and not adequately described in the text. He/she also noted that the Mdc1-GFP plasmid used was not described in the methods section. We have replaced the original Figure 2A with a simplified version which does not include the Mdc1-GFP plasmid. We are confident that the point of the panel (initial activation of H2AX is unaffected by expression of ICP0) is clearer in the new figure and we thank the referee for pointing out the potential source of confusion. We have re-written the results section and the figure legend describing this experiment to reflect the changes.
2. The referee correctly notes that our FRAP data actually shows that ICP0 prevents the IR-induced decrease in 53BP1 mobility, rather than causes an increase in post-IR mobility of 53BP1. We have amended the text accordingly.
3. We have now included the data demonstrating that ICP0 expression does not affect levels of H4K20me2 as panel B in Supplemental Figure 5.
4. We have now added subtitles to the discussion as suggested by the referee.

REFEREE #2
This referee stated that our study was interesting and had implications going well beyond the virology field. He/she recommended publication in principle but raised several points that required clarification. These points are addressed below:

1. The referee raised concerns about the specificity of the phospho-specific antibody to ATM phosphorylated at Ser1981 used in Figure 1 and Supplemental Figures S1 and S2. We share the referees concerns and had previously performed control experiments in cells deficient in ATM to confirm that the antibody does not detect any signal in these cells. However, as the referee correctly notes, knockdown of ATM precludes not only ATM autophosphorylation but also all other downstream phosphorylation events so this control experiment is not conclusive. The referee states that Figure 1 is "an informative set of data and a good entry point to the whole story" and recommends that we do not remove it. He/she suggests that we re-word the text describing experiments with this antibody to make it clear that our data show that ICP0 prevents local, ATM-dependent phosphorylation events at IRIF (rather than claiming ICP0 prevents accumulation of activated ATM itself). We have revised all sections describing experiments using this antibody to make this important distinction clear.
2. The referee notes that in many figures, ICP0 is clearly sequestered to prominent nuclear speckles and asks for clarification of what these structures are. The speckled structures containing ICP0 co-localize with ND10 domains. However, these nuclear structures are disrupted by WT ICP0, so the co-localization can be best visualized with a RING mutant version of ICP0 that cannot disrupt ND10 ñ in this case, ICP0 remains co-localized with the major ND10 component, PML. We thank the reviewer for drawing our attention to the fact that we had not explained what these "sites of ICP0 expression" are and we have now amended the text accordingly.
3. The ability of RNF8 to co-exist in the presence of ICP0 in Figure 5A is due to over-expression from the co-transfected RNF8 plasmid relative to ICP0. In Figure 5A, the Flag-RNF8 plasmid was transfected in excess of the GFP-ICP0 plasmid while in Figure 4B, ICP0 is transfected in excess of the RNF8 plasmid. We thank the referee for highlighting the fact that this was not clear and we have amended the text and figure legend describing both figures. The referee raises the possibility that ICP0 both sequesters and degrades RNF8, and he/she suggests looking at ICP0/RNF8 foci after IR to clarify this issue. We have now performed the experiment suggested by the referee and it is included as new Figure 5B. As the referee predicted, the available RNF8 is still not able to localize to IRIF and remains bound to ICP0 at the ICP0 speckles. However, this lack of localization is likely due to the fact that IRIF are not correctly formed in the presence of WT ICP0, since in the presence of the delta RING mutant of ICP0, IRIF appear to be "dominant" over the ICP0 speckles in terms of RNF8 localization. We have amended the text to describe this new experiment and discuss the

potential implications. Although we have observed that RNF168 localization is altered by ICP0, it does not completely co-localize with ICP0 sites. This suggests that the targeting of RNF168 is not as straight forward as the direct binding of RNF8 by ICP0. The mechanism by which RNF168 is degraded is clearly more complex and is the subject of ongoing investigations in the lab.

REFEREE #3
This referee stated that our study was thorough and that the data set was complete and convincing. He/she raised the same point as Referee #2 about the specificity of the phospho-specific antibody to ATM phosphorylated at Ser1981. This referee recommended that we do not pursue experiments to test the specificity of this antibody, since our paper is about a viral protein that targets the RNF8/RNF168 pathway and not about the controversial issue of how ATM is retained at sites of DSBs. We agree with the referee and hope that our careful re-wording of the experiments using this antibody (as suggested by Referee #2) will satisfy his/her concerns. Referee #3 also raised the same concern as Referee #2 regarding the co-expression of RNF8 and ICP0 in Figure 5A. As described in point 3 to Referee #2 above, the ability of RNF8 to co-exist in the presence of ICP0 in Figure 5A is due to over-expression from the transfected RNF8 plasmid relative to ICP0. We have amended the text to make this point more clear.

We feel that we have addressed all the concerns of the referees and that our study has been improved through the review process. We appreciate the refereesí thorough and constructive comments and thank you very much for your careful consideration of this manuscript. We hope that you will now find the manuscript appropriate for publication in EMBO Journal and we look forward to hearing from you in the near future.

