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Recognition of UbcH5c and the Nucleosome by the Bmi1/Ring1b Ubiquitin Ligase Complex

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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 27 May 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. We have now received the reports of three expert referees, which are copied below. As you will see, the referees generally agree on the general interest and overall quality of the work, and at least two of them also consider the new findings in the paper significant and would thus in principle support publication, pending adequate revision of a number of specific points. In this respect, referee 2 raises mostly minor presentational aspects, but there are also several more substantive points in the comments of referee 3 that need to be satisfactorily answered.

I would thus like to invite you to prepare a new version of the manuscript, revised along the lines of the referees' comments copied below. When preparing your letter of response, please be reminded that our policy to allow only a single round of major revision will necessitate diligent and comprehensive answering, and also bear in mind that this letter will form part of the Peer Review Process File available online to our readers in the case of publication (for more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html). Finally, when editing the revision please also amend the manuscript text with a brief 'Author Contribution' section.

Following adequate revision, we should be happy to ultimately consider the manuscript for publication. Should you have any further questions in this regard, please do not hesitate to get back to me directly.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This is a clearly written manuscript reporting structural, biochemical and biophysical characterizations of the RING domains of Bmi1 and Ring1b, a histone H2A ubiquitin E3 ligase complex, in complex with the E2 enzyme UbcH5c. The structure of the Bmi1-Ring1b was previously published, and the complex with UbcH5c revealed pretty much what is expected between an E2 enzyme and a RING domain E3 enzyme. The new finding is that the Bmi1-Ringb heterodimer binds roughly ~10 bp DNA in a sequence independent manner, and the DNA binding ability is important for ubiquitination of nucleosomal H2A. While the manuscript contains some interesting results, such as free and octameric histone H2A are not substrates of the Bmi1-Ring1B complex, my overall judgment is that the work will interest specialists in the field, and manuscript might be more suitable is a specialized journal.

Referee #2 (Remarks to the Author):

This is an interesting manuscript describing the mechanism of H2A ubiquitylation by the E3 ligase activity of PRC, the Bmi1-Ring1b complex in association with the E2 UbcH5c.

The authors have determined a crystal structure of the Bmi1-Ring1b-UbcH5c complex and used the structural information to understand how the ternary complex binds to nucleosomes to ubiquitylate H2A. The authors find that DNA is required for H2A ubiquitylation. The complex binds to DNA of minimal length 10 bp, but binding is sequence independent. Both the DNA interactions and the interactions of UbcH5 with Bmi1-Ring1b are salt dependent. Mutations of certain conserved basic residues both abolish E3 ligase activity and nucleosome binding.

The manuscript is well written and the experiments are well-designed and carefully controlled. The overall results and conclusions are interesting and significant. I have only a few minor comments.

1. Since three proteins are being discussed, it would be useful to distinguish these proteins when referring to specific amino acids, eg use of a superscript B for Bmi1, R for Ring1b, U for UbcH5 etc.
2. Figure 2, label lanes.
3. Page 8, residues mutated to test DNA binding and ubiquitylation should be indicated in Supplementary Figure 4.
4. In the MSA of Supplementary Figure 4B, residue numbers should be given, at least the range shown in the alignment.

Referee #3 (Remarks to the Author):

The authors describe the crystal structure, biophysical binding, ubiquitin ligase activity, and modeled nucleosome interaction(s) of the Bmi1/Ring 1b/UbcH5c ternary complex. The overall structure displays a 1:1:1 stoichiometry with the UbcH5c E2 enzyme docked exclusively with the Ring 1b subunit. This conformation observed in the crystal structure confirms previous data suggesting the location of the interaction interface and low affinity binding. Fluorescence polarization experiments and ubiquitin ligase assays help to discern the nucleosomal components targeted by the intact complex. Lastly, computational docking reveal a possible model for the mono-ubiquitination of H2AK11C within nucleosomes, which has been previously shown responsible for gene silencing in vivo.
The X-ray crystal structure of the UbcH5c-Bmi1/Ring 1b ternary complex is presented clearly and concisely. Although, the structures of the individual components of this complex have been previously solved and display almost identical conformations the new information comes from the interaction interface between UbcH5c and Ring 1b. The interface is small, but this may help explain

the relatively low affinity for the ternary complex. Ubiquitin ligase experiments with the Bmi1/Ring 1b complex show specificity of ubiquitination on H2A requires a nucleosome substrate (although K118 is the residue targeted). Much attention was given to the ability of the Bmi1/Ring 1b complex to non-specifically bind DNA constructs of at least 10 bp and the effects on ligase activity. Additionally, mutational analysis implies that separate binding sites are used to form the ternary complex and to bind substrate. Computational modeling with the HADDOCK 2.0 program gives attractive views into a possible mechanism of nucleosome binding and H2AK119C ubiquitination. In all, the work here is presented well and provides novel insight into the E2-E3 interaction of UbcH5c-Bmi1/Ring 1b and its function on nucleosomal substrates. A few points to consider are listed below

Specific Points to Consider:

1. The DNA constructs used in the binding experiments are meticulously described and characterized, but the histones and nucleosome constructs are hardly mentioned. More detail should be given as to their source, method of purification, and handling. What is the length of DNA on the nucleosomes used? Does this correlate with the construct used in the modeling? Quality control of the nucleosomes must be provided, at the very least in the form of a gel.
2. A concern relating to point 1, is that histone octamer is extremely sensitive to salt concentration and will begin to dissociate below 2M NaCl concentration. The ubiquitin ligase assay is performed at 100 mM NaCl, thus the histone "octamer" is not an octamer but H2A-H2B dimers and (H3-H4)2. Thus it is impossible to conclude whether or not the intact octamer can be an active substrate for the reaction.

A more reasonable experiment would be to do the assay on H2A-H2B dimers and (H3-H4)2 tetramers alone and associated with DNA. Either complex can readily form stably interactions with DNA near physiological conditions. It would be interesting to see whether this proposed H4 acidic patch interaction site might be sufficient for activity.

3. The authors could speculate as to the stringent H2A119C specificity of the site of modification in vivo, yet the in vitro ligase assay shown here seems to readily modify H2A118C. Could this be due to the use of truncated RING/RING versions of the Bmi1/Ring 1b complex?
4. Many of the computationally modeled interfaces for the ternary complex with the nucleosome seem to make biochemical sense (e.g. the basic saddle of Bmi1/Ring 1b binding the nucleosomal DNA). One noted interaction, the UbcH5c/histone tail interaction is described more casually. Which histone tail are the authors referring to...H2A? Also, does this make sense on the UbcH5c molecule, for instance, does it have an acidic groove or tail that could provide a logical binding partner for the highly basic histone tails?

1st Revision - authors' response 28 June 2011

Response to comments from Referee 1:
This referee did not suggest any changes to the manuscript.

Response to comments from Referee 2:

1. Since three proteins are being discussed, it would be useful to distinguish these proteins when referring to specific amino acids, eg use of a superscript B for Bmi1, R for Ring1b, U for UbcH5c etc.
Superscripts have been added to residue numbers throughout as suggested by the referee.

2. Figure 2, label lanes.
Figure 2 has been significantly revised, with the inclusion of an additional experiment to address the concerns of Referee 3. In revising the figure, we labeled all lanes as requested.

3. Page 8, residues mutated to test DNA binding and ubiquitylation should be indicated in Supplementary Figure 4.

A new panel C has been added to the Supplementary figure (now Figure S5) showing all the residues mutated mapped onto the surface of the Bmi1/Ring1b complex.

4. In the MSA of Supplementary Figure 4B, residue numbers should be given, at least the range shown in the alignment.

Residue numbers have been added beside each sequence to indicate the range of residues shown in the alignment.

Response to comments from Referee 3:

1. The DNA constructs used in the binding experiments are meticulously described and characterized, but the histones and nucleosome constructs are hardly mentioned. More detail should be given as to their source, method of purification, and handling. What is the length of DNA on the nucleosomes used? Does this correlate with the construct used in the modeling? Quality control of the nucleosomes must be provided, at the very least in the form of a gel.

We have added a section to the Materials and Methods ("Histone and nucleosome preparation") to address the referee's questions. All histones (H2A, H2B, H3.1, H4, H2A/H2B dimer, and (H3/H4)2 tetramer) were purchased from New England BioLabs. Nucleosomes were isolated from HeLa cell nuclei according to an established protocol, which we have now referenced. Nucleosomes were fractionated by gel filtration, and the fraction used in our assays contains oligonuclosomes, rather than mononucleosomes. We have provided an additional Supplementary figure (Figure S2), in which we show both an SDS-PAGE gel (to confirm the presence of the 4 histone proteins), and an agarose gel (to confirm the presence and length of DNA). The average length of DNA on these nucleosomes is ~ 400 bp.
To answer the referee's question about the correspondence between the nucleosome substrate and the length of DNA in the structure used for modeling (which in fact are not the same), we added a comment to the Discussion about the accessibility of the modeled site in more complex nucleosome structures and models: "Furthermore, our model appears largely compatible with the low-resolution structure of a tetranucleosome core particle and derived chromatin fiber models (Schalch et al, 2005), although some remodeling might be required for PRC1 to fully access sites in the fiber." In the published fiber models, the H2A modification sites point toward an interior superhelical axis. There is some open space that the RING domains might occupy (approximately our modeled binding site), but it is not evident that the complex could enter these sites by diffusion.
Docking explicitly to these more complex structures would be computationally very intensive (and so we did not attempt to do so).

2. A concern relating to point 1, is that histone octamer is extremely sensitive to salt concentration and will begin to dissociate below 2M NaCl concentration. The ubiquitin ligase assay is performed at 100 mM NaCl, thus the histone "octamer" is not an octamer but H2A-H2B dimers and (H3-H4)2. Thus it is impossible to conclude whether or not the intact octamer can be an active substrate for the reaction. A more reasonable experiment would be to do the assay on H2A-H2B dimers and (H3-H4)2 tetramers alone and associated with DNA. Either complex can readily form stably interactions with DNA near physiological conditions. It would be interesting to see whether this proposed H4 acidic patch interaction site might be sufficient for activity.

The referee raises an excellent point, and we thank the referee for pointing this out. Accordingly, we have performed the suggested experiment and included it in a substantially revised Figure 2. The H2A substrate alone or in combination with other histones (H2A/H2B dimer, or a mixture of 2 equivalents of H2A/H2B plus (H3/H4)2) was subjected to the ubiquitin ligase assay in the presence or absence of added DNA (in this case a 146-bp DNA duplex that corresponds to the sequence from a published mononucleosome crystal structure). None of the combinations with DNA, including those components the referee notes will associate with DNA, was a substrate for Bmi1/Ring1b (as assessed by both H2A and Ub Western blot, the latter performed to detect any non- H2A Ub

modifications). We therefore conclude that Bmi1/Ring1b modifies not just histones associated with DNA, but H2A in a well-ordered nucleosome.

3. The authors could speculate as to the stringent H2A119C specificity of the site of modification in vivo, yet the in vitro ligase assay shown here seems to readily modify H2A118C. Could this be due to the use of truncated RING/RING versions of the Bmi1/Ring 1b complex?

There are two questions imbedded in the request to speculate: first is H2A ubiquitination strictly specific for K119 in vivo, and second, does the in vitro reaction produce a different result?
It is generally assumed that H2A ubiquination by Bmi1/Ring1b occurs at K119, and the usual citation given is Wang, 2004 (also in our reference list). In this particular case, the reaction was done in vitro, and a peptide consistent with the K119 modification was detected by MS. It was not shown or discussed whether any other modified peptides (for example, K118-Ub) were present, nor was evidence for unmodified K118 peptides described. The data in this paper are therefore consistent with K119 modification but do not demonstrate strict specificity for the site. A much earlier paper, (Goldknopf and Busch, 1977) identified K119 as a site of H2A modification (clearly by Ub, but predating the name). The modified peptide was identified as one differing in mobility from H2A tryptic peptides by paper chromatography and electrophoresis, and its N-terminus was identified as H2A K119(GG) by Edman degradation. Because K118 is not present in this peptide, the assumption that K118 is not modified is based on the authors not having noticed a second mobility-shifted peptide and identified its composition. Again, this is an absence of evidence for heterogeneity, rather than clear evidence for strictly site-specific modification. In our opinion, therefore, the commonly cited references do not rule out a proportion of K118 Ub modification. Our data (showing modification of K118) are also consistent with the idea of a product mixture, as they do not rule out modification of K119. We do not believe any evidence suggests that the in vivo and in vitro reactions produce markedly different products. K118 and K119 are (obviously) close to one another, are present on a flexible histone tail, and the lysine side chains are themselves conformationally flexible. It would be remarkable indeed if only one of them could approach the E2 closely enough for Ub transfer (would likely require the specific E2- H2A tail docking interaction the referee posits in point 4 below). We chose not to include speculation on these points in the manuscript, as it would add significant length without much chance of a satisfactory resolution of the matter.
In any case, our intention was not to claim that the modification was strictly specific for either K118 or K119. The specificities we find interesting and sought to explain are the specificity for monoubiquitination compared to chain formation and specificity for modification of H2A K118/K119 compared to the many other lysines present in histones. To be clearer about our meaning, we have modified language throughout the text when describing the specificity of the reaction. We have also reworded the caption to the MS figure (Fig. S3), so that it is clearer that we do not rule out K119 modification.

4. Many of the computationally modeled interfaces for the ternary complex with the nucleosome seem to make biochemical sense (e.g. the basic saddle of Bmi1/Ring 1b binding the nucleosomal DNA). One noted interaction, the UbcH5c/histone tail interaction is described more casually. Which histone tail are the authors referring to...H2A? Also, does this make sense on the UbcH5c molecule, for instance, does it have an acidic groove or tail that could provide a logical binding partner for the highly basic histone tails?

The histone tail in question is the C-terminal tail of histone H2A, and the text has been modified to make this point more clearly (including also residue numbers). The referee's question about potential E2-histone tail interactions is intriguing; however, our docking can not fully address this. In published nucleosome crystal structures, much of the C-terminal tail of H2A is disordered and therefore not observed. We used as a docking template one of the few structures that includes the sites of modification (118/119), but this structure does not include the 9 residues beyond position 120. We allowed flexibility of residues 118-120 so that geometry and any specific contacts near the modification site might be optimized during docking, but even these few degrees of freedom greatly increased computation time. Including the entire H2A C-terminus would not have been feasible. Even so, there is an acidic patch on the surface of UbcH5c that potentially could engage in

electrostatic interactions with substrates (but something similar is also present on E2-25k, an E2 that binds to Bmi1/Ring1b, yet will not support Ub transfer to H2Aósee Buchwald et al., 2006). Whether there is any functional importance to E2 acidic patches (beyond interacting with ubiquitin) is an interesting question for future studies.

Acceptance letter 30 June 2011

Thank you for submitting your revised manuscript for our consideration. I have now had a chance to look through it and to assess your responses to the comments raised by the original reviewers, and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

You shall receive a formal letter of acceptance shortly.

Yours sincerely,
Editor
The EMBO Journal

