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## **E2~Ub conjugates regulate kinase activity of the Shigella effector OspG during pathogenesis**

Jonathan N Pruneda, F. Donelson Smith, Angela Daurie, Danielle L. Swaney, Judit Villen, John D. Scott, Andrew W. Stadnyk, Isolde Le Trong, Ronald E. Stenkamp, Rachel E. Klevit, John R. Rohde, Peter S. Brzovic

*Corresponding author: Peter S. Brzovic, University of Washington*

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### **Review timeline:**

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*Editor: Hartmut Vodermaier*

### **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.)

Editor:

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1st Editorial Decision

27 August 2013

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Thank you again for submitting your manuscript on OspG activation by E2-ubiquitin conjugates to The EMBO Journal. We have now received the comments from three expert referees, which you will find copied below. I am pleased to inform you that all of them find this work interesting and important, especially regarding the structural and mechanistic analyses. We shall therefore be happy to consider this work further for publication, following adequate revision of the various specific points raised by the reviewers. In this respect, most of referee 1 and 3's questions should be rather straightforward to address, however referee 2 has some more serious concerns regarding the mouse work (see their major points 4-6). I realize that the primary interest of this work lies with the novel mechanistic and structural insights, but at the same time feel that the data supporting the physiological importance of these findings would also at least have to be technically solid. Therefore, I would appreciate if you could get back to me with some proposals as to how you might address these issues, once you will have had the chance to carefully consider these points and to discuss them with your collaborators. Also, please do not hesitate to contact me should you have any other questions regarding the reports or this revision.

We generally allow three months as standard revision time - should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension. It is our policy that competing manuscripts published during this period will have no negative impact on our final assessment of your revised study, however please contact the editor as soon as possible upon publication of any related work to discuss how to proceed. Finally, please note that we generally allow for only a single round of major revision, making it important to

diligently respond to all points raised at this stage.

Thank you again for the opportunity to consider this work for The EMBO Journal! I look forward to receiving your final version.

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REFEREE REPORTS:

Referee #1:

Pruneda et al. present a superb manuscript describing a novel reciprocal regulation of an E2~ubiquitin (Ub) intermediate and a Shigella effector kinase (OspG). The authors first find that OspG binds several E2s, and determine the structure of an OspG/UbcH5c~Ub complex. The structure shows that OspG bears much resemblance to a eukaryotic kinase, and the UbcH5c~Ub stabilizes a conformation activated for MgATP binding. Indeed, UbcH5c~Ub stimulates intrinsic OspG kinase activity measured by autophosphorylation and phosphorylation of a promiscuous substrate (Histone H1). By contrast, OspG binds both UbcH5c and Ub in the intermediate so as to splay them apart in an inactive conformation. Accordingly, covalent attachment of Ub enhances E2 affinity for OspG, and OspG reduces reactivity of an E2~Ub intermediate toward free lysine. The authors used a Shigella infection model dependent on exogenous ospG rescue of an ospG deletion strain to validate that structure-based mutations impair Shigella infection. Interestingly, it appears that other bacterial effector kinases, NleH1 and NleH2 function differently from OspG, implying more exciting mechanisms to be identified from studies of bacterial effectors. Taken together with other recent studies of cellular E2~Ub intermediates influencing DUB activities, the novel function for an E2~Ub complex revealed here raises the fascinating question of how often E2~Ub complexes function in ways other than to mediate Ub transfer. I have no major concerns and recommend rapid publication in EMBO Journal.

Minor suggestions for improvement, which I suggest should be up to the authors' discretion as to whether or not they are addressed:

1. Supplemental Figure 2 c and d might be more clear if the structures were identical in size/orientation.
2. This paper seems aimed at experts in kinases, and the analyses of OspG mechanism are fascinating. However, OspG may well have a major role influencing ubiquitin conjugation, and a figure comparing OspG/UbcH5c~Ub and other E2~Ub conjugate structures may enhance the influence of the paper.

Referee #2:

The manuscript "E2~Ub conjugates regulate the kinase activity of the bacterial effector OspG during Shigella pathogenesis" by Pruneda et al. aims to understand the biochemical and structural basis of Shigella OspG kinase activation by a host E2-Ub complex. Having identified 10 distinct E2-Ub complexes that bind directly to OspG, they solve the X-ray crystal structure of UbcH5c-Ub with OspG. Analysis of this structure indicates that E2-Ub conjugate stabilizes the protein kinase in an active conformation and enhances its catalytic activity. The structural insights are confirmed through biochemical studies. Both the affinity and kinase activation properties of the E2-Ub are substantially greater than E2 and Ub alone, which helps to confirm observations in the crystal structure (which shows independent sites of interactions of both E2 and Ub). Using a mouse model of infection, the authors demonstrate that OspG down regulates acute disease in a manner that requires OspG interaction with the E2-Ub interaction. Overall, the structural and biochemical information is intriguing and important, and will be of interest to a broad audience. However, the mouse studies are not convincing. Several questions concerning the study should be addressed prior to publication.

Major concerns:

- (1) The authors attempt to determine which E2-Ub complexes bind to OspG. They identify 10 interacting complexes, all by two of which are found using two different approaches. However, these studies do not address binding specificity since it is possible that these specific E2-Ub complexes are highly abundant in multiple cell types. The authors should perform control experiments testing if other E2-Ub complexes, not identified by mass spec, interact with OspG.
- (2) In the pulldown assays shown in Figure 1B and S1B, all of the interaction appear to be sub-stoichiometric. While this is not a problem per se, there are no negative controls demonstrating the GST-alone or beads do not pellet the E2~ub complex. The experiments without ATP are nice, but they do not answer the question if the complex is non-specifically associating with GST.
- (3) The survival curves in Figure 6A are difficult to evaluate. These should be presented in a higher quality graph with colors that are more distinct or do not perfectly overlap.
- (4) There are not experiments demonstrating that the complemented strains express or translocate equal amounts OspG (and mutant).
- (5) In general, the mouse data is not convincing. The phenotypes observed with the OspG mutants are poorly characterized. There are no differences between bacterial count or histology between WT and ospG- infected mice. Perhaps competitive index measurements would reveal differences in growth or survival that are not observed in the current experimental set up. Also, the analysis of the mouse colons are concerning. The authors show increased inflammation in colon (Figure 6B) in OspG mutants, but this is not readily apparent in the histology sections. For Fig 6 D: representative images for complemented strains are missing. To show differences in inflammatory responses to the Shigella strains, blinded scoring for histopathological changes should be performed on the sections.
- (6) While the mouse studies suggest a role of E2-ub binding for in vivo function of OpsG, it is unclear if this is mediated by kinase activation, or alternatively, by regulation of E2-Ub interaction with E3s (as predicted by the crystal structure). The authors should conduct experiments with kinase-dead version of OspG, which should give a similar phenotype to loss of function E2-Ub binding.

Minor points:

- (1) In the introduction, the authors list the contributions of Kim Et al. The authors should also mention the contributions of Zhao et al. (Plos One) that recently demonstrate that OspG can be activated by Ub. Additional discussion about the discrepancy between these findings and those presented here would be helpful.
- (2) Figure 1A does not provide much information could be moved to supplemental data. More importantly, the gel stain or a table representing the mass spec analysis would be much more pertinent to the study.
- (3) the "clinical illness" metric used in the study is poorly defined in the materials and methods.

Referee #3:

The manuscript by Pruneda et al describes structural and functional data to explain the basis for the reciprocal regulation of E2~Ub conjugates and the OspG protein kinase from Shigella. The authors make numerous important and interesting findings, including:

1. Identification of specific E2s that interact with and activate the kinase activity of OspG.
2. That the E2~ubiquitin conjugate binds and activates OspG.
3. The structure of the ternary complex of OspG-UbcH5~Ub was determined, providing a mechanistic explanation for how UbcH5~Ub activates the kinase activity of OspG, by enhancing ATP affinity and OspG phosphorylation. In contrast OspG reduces the rate of Ub release from UbcH5 by promoting a more open inactivate conformation of the E2~Ub conjugate.

Overall this is a very interesting manuscript that should acceptable for publication following minor revisions.

Comments and questions.

1. Based on the structure of the OspG-UbcH5~Ub ternary complex, can the authors rationalise the specificity of OspG for specific E2s?
2. What is known about the specificity of OspG kinase (i.e. Ser/Thr, Tyr or dual?).
3. Page 6, line 13: insert 'in protein kinases' before 'include the DFG motif'.
4. The authors show that UbcH5~Ub is a better activator than unconjugated UbcH5 and Ub. However the authors also need to test UbcH5 and Ub alone. From the structure it isn't clear how Ub activates OspG other than enhancing the affinity of UbcH5 to bind and hence activate OspG.
5. The Rnf4-E2~Ub structure from Hay and colleagues indicated that the RING domain of Rnf4 orientated the catalytic Asp residue to function as a general base. How does this Asp conformation compare in the OspG-UbcH5~Ub complex?
6. The authors use ITC to determine affinities of ATP-gamma-S to either OspG or the OspG-UbcH5~Ub ternary complex. These data are quite inconclusive. A lack of an enthalpy change doesn't necessarily mean that a binding event does not occur. Binding may be driven entirely by entropy effects. The authors should consider deleting these data.
7. Supplementary Fig. 2F could be in the main text.
8. Table 1. Include Ramachandran statistics.
9. Figure labelling could be improved. The authors use a minimalistic approach that makes the manuscript hard to follow in places. Specific attention should be paid to:
  - (i) Fig. 1b. Bands should be labelled.
  - (ii) Fig. 2a either label Ub residues L8-I44-V70 of the hydrophobic patch, or generate a new figure.
  - (iii) Fig. 2a label Ub residues A46, G47, K48, S57, D58, Y59, N60.
  - (iv) Page 7. The Ub 48K-loop interactions with OspG C-terminus should be shown in a figure.

1st Revision - authors' response

28 October 2013

We have revised the manuscript entitled “E2~Ub conjugates regulate the kinase activity of the *Shigella* effector OspG during pathogenesis”, which we submit for further consideration. We were pleased with the overwhelmingly positive responses from the reviewers. However, each referee raised important points that have significantly strengthened our study. Their comments lead us to streamline the manuscript and allowed us to introduce some additional material. Below we provide a summary of changes to the manuscript as well as point-by-point responses to individual reviewer comments.

*Summary of major changes to the manuscript:*

- 1) Moved original Figure 1 to new Supplemental Figure 2 and start the Results section with a description of the structure of the OspG/UbcH5c~Ub complex.
- 2) Figure 1 has been revamped as suggested by the reviewers.
- 3) Cellular and *in vitro* binding experiments are now summarized in Table 2. This includes a synopsis of mass spectrometry analysis, the implications of which are expanded upon in the Discussion.

- 4) Additional data is presented regarding the influence of OspG and various mutants in the mouse model of Shigella infection. In particular, our analysis of a kinase-dead OspG that is competent in E2~Ub complex formation. These new results are placed in context in the discussion.
- 5) A new section of the discussion compares and contrasts the structure of UbcH5c~Ub in complex with OspG to conformations observed in RING or HECT domain E3/UbcH5~Ub complexes. These comparisons are illustrated in Supplemental Figure 9.

*Responses to individual reviewer comments.*

Reviewer #1:

*1. Supplemental Figure 2 c and d might be clearer if the structures were identical in size/orientation.*

This comment was combined with those of the other referees to reorganize the beginning of the Results section and the Supplemental materials. With editing of the text we have moved the original Supplemental Figure 2 to Supplemental Figure 1 and simplified the figure. There are now 4 panels to this Supplemental Figure. The original information content in Supplemental Figures 2C and 2D has been incorporated into the Main Text and Main Figure 1. Figure 1B is an “open book” representation of the OspG/UbcH5~Ub interface that highlights important UbcH5c and Ub structural features involved in complex formation and the locations of OspG residues that, when mutated, disrupt binding to UbcH5c~Ub.

*2. This paper seems aimed at experts in kinases, and the analyses of OspG mechanism are fascinating. However, OspG may well have a major role influencing ubiquitin conjugation, and a figure comparing OspG/UbcH5c~Ub and other E2~Ub conjugate structures may enhance the influence of the paper.*

We appreciate the reviewer’s suggestion to broaden the context of our findings. Accordingly, we have modified the text to provide more balanced dialog on the implications of the structure regarding the kinase function of OspG and the parallel role of E2~Ub conjugates in Ub transfer reactions. To this end we have streamlined the comparison of OspG with eukaryotic kinases and have included a new Figure (Supplemental Figure 9) that compares the OspG/E2~Ub complex with RING/E2~Ub and HECT/E2~Ub complexes and expanded the Discussion section regarding the mechanistic implications of these comparisons (see Discussion, page 14).

Reviewer #2:

*(1) The authors attempt to determine which E2-Ub complexes bind to OspG. They identify 10 interacting complexes, all by two of which are found using two different approaches. However, these studies do not address binding specificity since it is possible that these specific E2-Ub complexes are highly abundant in multiple cell types. The authors should perform control experiments testing if other E2-Ub complexes, not identified by mass spec, interact with OspG.*

*We have responded to these comments in four ways:*

1a) In the original version of the manuscript, we discussed common elements shared by E2s that interact with OspG that were identified by mass spectrometry. These common elements predicted that an additional two E2s (UbcH5a and Ubc13) would also interact, which we then confirmed by *in vitro* pulldown. As the reviewer correctly points out, we did not identify any E2 structural features that might prevent binding to OspG. This latter issue led us to re-examine the structure of the complex.

1b) We now note there are extensive side chain interactions that involve UbcH5c Phe62 (and likely the analogous residues in other OspG interacting E2s). In order to emphasize this point we have included a new Figure 3 which illustrates the interactions of UbcH5 F62 with OspG. This additional figure also shows that mutation of this residue disrupts complex formation. In addition, Ube2S, which shares many E2 features needed to bind OspG but encodes an Alanine at the position structurally analogous to UbcH5c F62, fails to form a complex with OspG.

1c) The results of cellular and *in vitro* E2~Ub/OspG interaction experiments are combined into new Table 2. The original Figure 1, which shows the widespread distribution of OspG in transfected HeLa cells and *in vitro* binding experiments has now been moved to the new Supplemental Figure 2.

1d) We have also changed the labeling and figure legend for the new Supplemental Figure 2 to address additional issues raised by Reviewer #3 (see below). These changes allowed us to significantly streamline the manuscript.

(2) *In the pulldown assays shown in Figure 1B and S1B, all of the interactions appear to be sub-stoichiometric. While this is not a problem per se, there are no negative controls demonstrating the GST-alone or beads do not pellet the E2~ub complex. The experiments without ATP are nice, but they do not answer the question if the complex is non-specifically associating with GST.*

This control is part of Supplemental Figure 5B, panel 2. Instead of showing this panel again, we modified the main text to refer to this figure and to clearly address this point (Page 7 line 21).

(3) *The survival curves in Figure 6A are difficult to evaluate. These should be presented in a higher quality graph with colors that are more distinct or do not perfectly overlap.*

We have amended Figure 6A and its caption to increase clarity of the results.

(4) *There are not experiments demonstrating that the complemented strains express or translocate equal amounts OspG (and mutant).*

Unfortunately we do not possess an antibody to OspG. Furthermore we cannot make use of an epitope tag as there is reason to believe that it will interfere with normal translocation through the Type 3 Secretion System. Repeated attempts to identify OspG using mass spectrometry have been unsuccessful. In wild type cultures, we have examined the effector profile secreted into the culture supernatant many times and we have never detected OspG peptides. We suspect that OspG may be poorly expressed during *in vitro* conditions. We have analyzed the effector secretion profile from the DospG mutant and that of our complemented strains and find that they are similar, providing evidence that our genetic manipulations have not grossly altered the normal function of the Type 3 Secretion System, these data are presented as Supplemental Figure 8.

Our strongest data for production of OspG and the OspG variants in the DospG mutant is that the phenotypes observed in the DospG mutant are abolished when the strains harbor wild type OspG. In addition, the catalytically dead K53M allele also restores some phenotypes observed in the wild type strain. These data argue that the OspG translocation of the complemented DospG bearing a wild type copy is similar to that of the wild type and argues that our complementation strategy is sound. The other OspG variants were introduced into the DospG mutant in the exact same manner as was the wild type allele.

(5) *In general, the mouse data is not convincing. The phenotypes observed with the OspG mutants are poorly characterized. There are no differences between bacterial count or histology between WT and ospG- infected mice. Perhaps competitive index measurements would reveal differences in growth or survival that are not observed in the current experimental set up. Also, the analysis of the mouse colons are concerning. The authors show increased inflammation in colon (Figure 6B) in OspG mutants, but this is not readily apparent in the histology sections. For Fig 6 D: representative images for complemented strains are missing. To show differences in inflammatory responses to the Shigella strains, blinded scoring for histopathological changes should be performed on the sections.*

We agree that the infected mouse phenotype is not particularly remarkable when assessed histologically. However, our data is consistent with the oral model of infection described in BALB/c mice by Bernadini and coworkers (Martino, et. al., (2005)). Those investigators also reported a lack of remarkable pathology in the colons including when comparing pathogenic versus non-pathogenic strains of Shigella. This included the period when bacterial recoveries varied, roughly 21 days of infection and beyond. On the other hand we show for the first time, a lethal infection using Shigella in adult mice (with the DospG mutant). While this is exciting, we cannot explain the mechanism behind the mortality.

As the referee points out, we do not observe differences in bacterial burden between the strains in the feces of infected mice. We investigated the idea that the mutant may be escaping to other organs and causing sepsis. However we do not observe an increase in bacterial burden in other organs and we do not observe differences in pro-inflammatory cytokines in the serum of infected mice that would indicate a heightened cytokine storm. We note that our results are consistent with the results of Kim and coworkers (2005) that reported that infection with DospG mutant results in an increase in the inflammatory response in the rabbit ligated ileal loop model of infection when compared to infection with the parental wild-type.

We did not include images for the histopathology of all of the complemented strains. This is because there is little difference between the histopathology of colons infected with wild-type or DospG

strains. We note that we did use a blinded scoring system for the analysis of the histopathology and this is now indicated in the Supplementary Experimental Methods.

*6) While the mouse studies suggest a role of E2-ub binding for in vivo function of OpsG, it is unclear if this is mediated by kinase activation, or alternatively, by regulation of E2-Ub interaction with E3s (as predicted by the crystal structure). The authors should conduct experiments with kinase-dead version of OspG, which should give a similar phenotype to loss of function E2-Ub binding.*

We thank the referee for this insightful comment. Indeed our complementation studies using the kinase-dead allele of OspG provides a phenotype in-between that of the DospG mutant and wild type. There is no statistical difference between the wild type and the DospG mutant complemented with the kinase-dead allele in mortality. However the gross histology of the colons from the DospG mutant complemented with the kinase-dead allele more closely resembles those from the DospG mutant than the wild-type. These data are congruent with data presented by Kim et al (2005) who show K53 is required for kinase function but is only partially required for OspG function in downregulating NF-KB promoter activity (for example Kim et al Figure 4). We have modified the discussion to indicate the possibility of a dual-regulatory mechanism in which E2~Ub activated OspG kinase function while OspG inhibits E2~Ub transfer activity.

Minor points:

*(1) In the introduction, the authors list the contributions of Kim Et al. The authors should also mention the contributions of Zhao et al. (Plos One) that recently demonstrate that OspG can be activated by Ub. Additional discussion about the discrepancy between these findings and those presented here would be helpful.*

We now refer to the manuscript by Zhao et. al. (2013) in both the Introduction and the Discussion Sections. We feel that there is not so much a discrepancy with our findings, but instead they are complementary. We show that Ub does indeed stimulate OspG activity, but complex formation with an E2~Ub conjugate results in significantly greater activity. Therefore, this paper presents a much more complete picture of activation that is consistent with the original observations of Kim et. al. (2005). A question also raised by Reviewer #3, is how Ub can achieve partial activation. We now address this point in the Results and Discussion sections by describing how the Ub subunit binds to the C-terminal lobe of OspG helping to stabilize the orientation of critical catalytic residues. The presence of the E2 further aligns the N- and C-terminal lobes, providing a significantly greater level of catalytic enhancement.

*(2) Figure 1A does not provide much information could be moved to supplemental data. More importantly, the gel stain or a table representing the mass spec analysis would be much more pertinent to the study.*

As suggested by the reviewer, we moved the original Figure 1 to the supplement (new Supplemental Figure 2) and tabulated the mass spectrometry analysis and binding data into a new Table 2.

*(3) The "clinical illness" metric used in the study is poorly defined in the materials and methods. The Materials and Methods Section has been revised to include more description of the clinical score.*

Reviewer #3:

1. *Based on the structure of the OspG-UbcH5~Ub ternary complex, can the authors rationalise the specificity of OspG for specific E2s?*

As described above (see response 1 to Reviewer #2) this turned out to be a very important issue in revising the manuscript. We added a new Figure 3 to address this point.

2. *What is known about the specificity of OspG kinase (i.e. Ser/Thr, Tyr or dual?).*

Previous studies suggest that OspG can modify Ser/Thr residues. We now mention these findings in the results (Page 9, line 11), but we hesitate to make definitive statements about specificity until an actual target has been confirmed and characterized.

3. *Page 6, line 13: insert 'in protein kinases' before 'include the DFG motif'.*

This section has been re-written (Page 6, paragraph 1) making this change unnecessary.

4. *The authors show that UbcH5~Ub is a better activator than unconjugated UbcH5 and Ub. However the authors also need to test UbcH5 and Ub alone. From the structure it isn't clear how Ub activates OspG other than enhancing the affinity of UbcH5 to bind and hence activate OspG.*

*We have responded to these issues in two ways.*

4a) We have modified the text in the Results and Discussion section to address this point. In Supplementary Figure 5C we compare the activity of Ub alone relative to other Ub-like proteins.

4b) In Figure 4A, we demonstrate that free Ub has higher affinity for OspG than free UbcH5c and in Figure 5B, we show that the activity of the OspG/UbcH5c~Ub complex is substantially greater than addition of free Ub alone. Based on the OspG/UbcH5c~Ub structure (Fig. 1), it appears that Ub helps to stabilize the conformation of the OspG C-terminal lobe and, therefore, the position of critical catalytic residues (Main text Page 6 line 22). Thus, binding of free Ub to the C-terminal lobe can enhance activity. Simultaneous interaction with both subunits of the E2~Ub conjugate further positions both the N- and C-terminal lobes of OspG providing a much, much greater enhancement than Ub alone. In the Discussion, we combine these findings and attempt to present this interpretation more clearly.

5. *The Rnf4-E2~Ub structure from Hay and colleagues indicated that the RING domain of Rnf4 orientated the catalytic Asp residue to function as a general base. How does this Asp conformation compare in the OspG-UbcH5~Ub complex?*

This point also relates to comments posed by Reviewer #1. We address this by adding Supplementary Figure 9 which compares the conformation of E2~Ub in the OspG complex to E2~Ub conjugates in RING and HECT complexes. This new figure highlights differences and similarities in the relative configuration of E2 and Ub subunits and in the conformation of E2 active site residues and the covalent linkage between the E2 active site and the C-terminus of Ub.

6. *The authors use ITC to determine affinities of ATP-gamma-S to either OspG or the OspG-UbcH5~Ub ternary complex. These data are quite inconclusive. A lack of an enthalpy change doesn't necessarily mean that a binding event does not occur. Binding may be driven entirely by entropy effects. The authors should consider deleting these data.*

The reviewer correctly points out that a lack of enthalpy change doesn't necessarily mean a binding event does not occur. In this case, we are comparing ATP-gamma-S binding to OspG either free or complexed with UbcH5~Ub. Binding of ATP to free OspG could be entropically driven whereas binding to OspG/UbcH5~Ub may realize a larger component due to enthalpy changes. Since ATP binds to the same protein site in either case (ie. the OspG active site), the data show a change in affinity for ATP, a change in the mechanism of binding, or some combination of both which is dependent upon complex formation with E2~Ub. In any case, formation of an OspG/E2~Ub has a significant impact on ligand binding. Therefore, we have elected to keep the data, but change the text in the Results section to acknowledge the different possibilities (Page 9 line 6).



7. *Supplementary Fig. 2F could be in the main text.*

Supplementary Fig. 2F is now Fig. 1C

8. *Table 1. Include Ramachandran statistics.*

Ramachandran statistics are now included in Table 1.

9. *Figure labeling could be improved. The authors use a minimalistic approach that makes the manuscript hard to follow in places. Specific attention should be paid to:*

(i) *Fig. 1b. Bands should be labeled.*

Original Figure 1b has been moved to Supplemental Figure 2B. The bands have been labeled and the appropriate species, either E2~Ub conjugates in non-reducing gels or free E2s in reducing gels, have been highlighted.

(ii) *Fig. 2a; either label Ub residues L8-I44-V70 of the hydrophobic patch, or generate a new figure.*

(iii) *Fig. 2a; label Ub residues A46, G47, K48, S57, D58, Y59, N60.*

(iii) *Page 7. The Ub 48K-loop interactions with OspG C-terminus should be shown in a figure.*

We have generated a new Figure 1B which presents an “open book” representation of the protein interfaces involved in complex formation. This highlights important UbcH5c and Ub structural features, including the Ub L8-I44-V70 hydrophobic patch and the Ub K-48 loop, that are necessary for complex formation. We have also included a new Supplemental Figure 1B that shows details of the Ub/OspG interface. Additionally, our new Figure 3A shows details of UbcH5c-F62 interactions in the UbcH5c/OspG interface.

2nd Editorial Decision

11 November 2013

Thank you for submitting your revised manuscript on OspG/E2-Ub interaction and its consequences for our consideration. It has now been seen once more by the original referees 1 and 2, and I am pleased to inform you that both of them consider the original concerns well-addressed and the study now suitable for publication in The EMBO Journal, pending a few remaining minor modifications requested by referee 2 (please see below). After these final revisions and incorporation of the following editorial points, we shall be happy to proceed with final acceptance and publication of this work!

Editorial points:

- please modify text/figures to incorporate the remaining referee points.

- we encourage the inclusion of figure source data for gels, blots and autoradiographs such as those in Figures 3 and 5, in order to make the primary data more accessible. This should be in the form of a single PDF/JPG/GIF file per figure comprising the original, uncropped and unprocessed blot scans/photographs, labelled with the appropriate figure/panel number and molecular weight markers; further annotation would clearly be useful but is not essential.

I hope you will be able to return your this ultimate revision to us as early as possible, should you have any questions in this regard please don't hesitate to let me know. I look forward to receiving your final version!

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Referee #1 (Report):

This is yet another outstanding paper from Pruneda, Klevit, Brzovic, and colleagues. The work is very high quality, it is extremely novel, and the paper was a joy to read. I anticipate that the work will be highly cited in many fields not limited to but including microbiology, kinase field, and the ubiquitin field. I recommend rapid publication in EMBO Journal.

Referee #2 (Report):

The authors have addressed my concerns through new figures and experiments. This work will add significantly to the field. I have only a few minor concerns that should be addressed prior to publication.

1) A sequence alignment of selected E2s (including both that bind OspG and those that are predicted not to bind) would help convey the specificity information in Figure 3. The argument is that the large aromatic residue at equivalent to F62 plays a major role in E2 selection by OspG. The alanine substitution is a nice experiment, and this would be strongly supported by a sequence alignment. A concluding sentence at the end of the description of Figure 3 would also be nice.

2) The very last sentence in the results is somewhat overstated. "..., primarily for regulation of its kinase function" has not been directly tested but rather relies on correlation from very nice *in vitro* work and some relatively preliminary *in vivo* analysis. The authors could make some minor changes that would be accurate and still convey the significance of the finding: "We conclude that OspG is required for decreasing the severity of Shigella infection in mice and that this activity depends upon interaction with host E2~Ub conjugates. In addition, our data support E2~Ub mediated OspG kinase regulation *in vivo*." Because the kinase dead mutant shows an intermediate phenotype, it is important to leave some room for other interpretations that may result from further studies.

3) Similarly, in the last sentence of the abstract, the oral infections do not "confirm" the requirement of E2~Ub activation of kinase activity *in vivo*, but rather support the authors hypothesis. Simply replace the word "confirm" with "indicate" and it will be an accurate representation of the data. I think it is important not to overstate the mouse data since there are many more experiments needed to validate these findings (outlined in my original review). Nevertheless, if stated properly the mouse data indeed supports the structural and biochemical data that provide the major new insights of the work.

2nd Revision - authors' response

16 November 2013

We have modified the manuscript as requested by referee #2 and addressed the editorial points. These changes are summarized below:

1) The sequence alignment of interacting E2s (Supplemental Figure 3) has been modified to include Ube2S, which does not form a complex with OspG.

2) We have changed the last sentence in the Abstract and the last sentence of the Results Section as suggested by the reviewer.