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A structural basis for Lowe syndrome caused by mutations in the Rab binding domain of OCRL1

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

1st Editorial Decision 20 December 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments are shown below. You will be pleased to see that the referees are generally positive about your work and that they would support its ultimate publication in The EMBO Journal after appropriate revision. I would thus like to invite you to prepare a revised manuscript in which you need to address the referees' criticisms in an adequate manner. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer 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 #1 (Remarks to the Author):

This manuscript describes the crystal structure of the ASH domain of the Lowe syndrome protein OCRL1 bound to Rab8. The interaction interface in OCRL1 is comprised of residues both in a helix abutting the 5-phosphatase domain and within the β-strand structure of the ASH domain. This differs from the binding of other Rab effectors to Rabs which is mediated by helical domains with the effector. The interacting residues within Rab8 are also different to those that in other Rabs confer effector binding, likely explaining the broad specificity of Rab binding seen with OCRL1. Interestingly, a Lowe syndrome mutation is located to the Rab binding interface, indicating the in vivo importance of OCRL1 binding to Rab.

The paper is well written and the data are clear. The results are of significant interest. I have some comments that the authors should address:

1.) OCRL1 can bind to several Rabs, with Rab8 having the highest affinity. What is the molecular basis for the differences in binding affinity to different Rabs? Can the authors use sequence comparisons or model other Rabs onto their structure to answer this question?

2.) Experimental verification of the importance of the other (in addition to F668) residues mapped to the Rab binding interface is lacking. It should be easy enough to confirm these residues are required for Rab binding. It would also be interesting to show the localization of constructs in which these residues have been mutated. If Rab binding is important for membrane localization, they should be more cytosolic.

3.) The microscopy images in Figure 5 are not the most convincing. The images of the GFP-tagged mutants are saturated. Better quality images (without pixel saturation) are required.

Referee #2 (Remarks to the Author):

This excellent paper presents the crystal structure of a complex between Rab8a and the Rab-binding domain of the inositol phosphatase OCRL. This protein is mutated in Lowe syndrome, and it is also likely to play a key role in membrane organisation in combination with a closely related paralogue. There is thus much interest in OCRL and previous studies have shown that it can bind several different Rabs, but the basis of these interactions, and their biological purpose remained unclear.

In this paper the authors use biophysical methods to determine the affinities of different Rabs for OCRL in solution and to map the Rab binding site. They then solve the structure of the Rab binding site in complex with Rab8a. This reveals an atypical Rab:effector interaction ie it is not via two helices in the effector but by a beta strand. This expands our understanding of Rab:effector recognition, and may explain how other effectors that bind multiple Rabs could be recognised.

The authors confirm the interactions observed in the structure by mutagenesis and in vitro binding assays. They also explain the phenotype of a case of Lowes syndrome where the protein appeared to be folded normally. This is shown to involve a point mutant in the Rab-binding site that lowers the affinity of Rab8a binding, and also that of other Rabs, proving that all the Rabs bind to the same site. This mutation also affects membrane recruitment in vivo, thus proving the importance of Rab binding for recruitment of OCRL to membranes.

The text is clearly written and the figures present the data effectively. The work is thorough and rigorous and likely to be of great interest to the Lowe syndrome field, and also to many other labs working on Rabs and phosphoinositides. As such I am happy to reccomend publication in the EMBO Journal.

Minor comment:

a) The authors state that the structure: "reveals two Rab-binding sites in OCRL1(540-678)" This is ambiguous as it suggests that there are two sites where Rabs can bind independently, but in fact it is two regions that a single Rab contacts when it is binding. This should be reworded.

Referee #3 (Remarks to the Author):

The paper describes the structure of Rab8a in complex with an effector, OCRL1, which is linked to a hereditary disease. The structure provides insight into how several mis-sense mutations might lead to defective interactions with Rab8 and altered sub-cellular localization. The manuscript is a fine combination of structural, cellular and biophysical experiments that is an important step in understanding the molecular basis for Lowe syndrome. The nature of the Rab-effector binding interface itself provides novel conceptual insight into the structural basis for recognition - the effector binds to the switch region via a b-strand, and the conserved aromatic triad (found in all Rabs) is only peripherally associated with the effector. Consequently, the paper is suitable for publication in EMBO.

The comments and suggestions below should be considered in a revised manuscript:

- the affinity of S564P (5.2uM) is significantly weaker than WT (0.9), with respect to Rab8 binding. Presumably, it would be much weaker for Rab1/Rab6 (WT is 4uM), although this is not measured, and these findings nicely explain the cytosolic location of mutant OCRL. However, the Rab8-OCRL(S564) affinity is basically on par with Rab1/Rab6 interactions with WT OCRL, and yet, these proteins presumably are able to recruit OCRL. Perhaps the authors could comment on this - are there other issues in sub-cellular localization that are not reflected by in vitro (equilibrium) binding affinity?

- Page 6 - relative affinities of Rab8, Rab6, Rab5 and Rab1 - in addition to the different experimental conditions that the authors mention in previous work, the current study uses the 'mant' modified GTP. The derivatization could also affect the relative affinities of various Rabs, perhaps through indirect (and subtle) conformational changes transmitted through the modified sugar to switch and interswitch regions. If this were the case, similar affinities obtained from equilibrium measurements vs. kinetics assays simply reflects internal consistency of the techniques, and not evidence for the 'correctness' of the relative affinities (Rab8 > Rab6, Rab5, etc). Perhaps the authors could mention this difference in their experimental setup.

- Page 16, top, change 'destroy the ASH domain fold' to 'disrupt' or 'destabilize the ASH domain fold'

- Page 6 and Fig. 5 legend - the data reflect 'kinetics and affinity', rather than 'thermodynamics' - for example, there are no dissections of binding energies into enthalpic and entropic components. However, the difference in free energy (ddG) could be derived from the ratio of Kd (WT/mut), and so the authors could quote this value (kcal/mol) which can be attributed to the loss of hydrophobic contact surface. As the paper is for a general readership, this point is not a major issue

- Substitute 'destroy the structural conformation' with 'destabilize the conformation', page 18. Also, this description of the effects of S564P is a little too vague. Where exactly is the residue locus, with respect to the structure? Is it found in the linker between the a-helix and the Ig-like domain? It seems to be close to strand b1 (OCRL), but the ribbon model is not marked with residue numbers (Fig 3A). Perhaps the authors could clarify, and even better, indicate in one of the figures where the locus is

- OCRL1 promiscuity is discussed in the paper, in the context of the aromatic triad. However, the higher Rab8 affinity (over Rab1, Rab6, Rab5), and specificity for these subset of Rabs, is not mentioned - can the authors say something about this issue? I don't feel a sequence alignment of Rabs is necessary, affinity may be a complex contribution from sequence/conformation of switches and interswitch. However, if there is something obvious about the Rab specificity, perhaps it should be mentioned

Typos

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1st Revision - authors' response 17 January 2011

We would like thank the referees for their thoughtful comments and criticisms, which formed the basis for improving our manuscript. In the following sections we respond to the referees' remarks point by point.

Referee #1 (Remarks to the Author):

This manuscript describes the crystal structure of the ASH domain of the Lowe syndrome protein OCRL1 bound to Rab8. The interaction interface in OCRL1 is comprised of residues both in a helix abutting the 5-phosphatase domain and within the β-strand structure of the ASH domain. This differs from the binding of other Rab effectors to Rabs which is mediated by helical domains with the effector. The interacting residues within Rab8 are also different to those that in other Rabs confer effector binding, likely explaining the broad specificity of Rab binding seen with OCRL1. Interestingly, a Lowe syndrome mutation is located to the Rab binding interface, indicating the in vivo importance of OCRL1 binding to Rab.
The paper is well written and the data are clear. The results are of significant interest. I have some comments that the authors should address:

1.) OCRL1 can bind to several Rabs, with Rab8 having the highest affinity. What is the molecular basis for the differences in binding affinity to different Rabs? Can the authors use sequence comparisons or model other Rabs onto their structure to answer this question?

The idea of identifying structural and sequential determinants by using amino acid sequence alignments in order to explain the difference in binding affinities of OCRL1 to different Rabs is admittedly tempting. But, as we have laid out in our response to referee #3, the affinity of an effector to a Rab protein is a result of complex contributions of the amino acid sequences and the conformational flexibility of the switch and interswitch regions. We are unable to identify obvious amino acid determinants for the higher affinity of Rab8a to OCRL1 compared to Rab1b, Rab5a or Rab6a. Thus, it is conceivable that conformational flexibility of the switch regions influences the affinity of Rab-OCRL1 interaction. However, a detailed biophysical analysis addressing this phenomenon is beyond the scope of our manuscript.

2.) Experimental verification of the importance of the other (in addition to F668) residues mapped to the Rab binding interface is lacking. It should be easy enough to confirm these residues are required for Rab binding. It would also be interesting to show the localization of constructs in which these residues have been mutated. If Rab binding is important for membrane localization, they should be more cytosolic.

Actually, the significance of other amino acids of OCRL1 in addition to F668 has been demonstrated to be important for Rab-binding in our manuscript (page 11 and Figure S3): We have shown, that amino acids 555-559 (which constitute binding site I) contribute to Rab binding since their absence in a OCRL1 truncation construct ($OCRL1_{560-678}$) abolished Rab-OCRL1 complex formation (Figure S3). The significance of binding site II of OCRL1 for Rab-binding has been demonstrated by the disease relevant mutation $F668A_{OCRL1}$ (a central element of site II), which leads to decreased Rab interaction. To the best of our knowledge, no other amino acid of OCRL1 involved directly in Rab-OCRL1 interaction has been identified to be responsible for disease development.

As we have already shown the contributions of the OCRL1 binding sites to complex formation with Rab proteins, we feel that mutational studies do not add to the further understanding of Rab-OCRL1 interaction and its relevance in health and disease.

3.) The microscopy images in Figure 5 are not the most convincing. The images of the GFP-tagged mutants are saturated. Better quality images (without pixel saturation) are required.

The conversion and compression of the original data for the PDF-file used for the review process resulted in a loss of resolution and led to the wrong impression that images might be pixel saturated. We now provide the original data in higher resolution.

Referee #2 (Remarks to the Author):

This excellent paper presents the crystal structure of a complex between Rab8a and the Rab-binding domain of the inositol phosphatase OCRL. This protein is mutated in Lowe syndrome, and it is also likely to play a key role in membrane organisation in combination with a closely related paralogue. There is thus much interest in OCRL and previous studies have shown that it can bind several different Rabs, but the basis of these interactions, and their biological purpose remained unclear.

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The authors confirm the interactions observed in the structure by mutagenesis and in vitro binding assays. They also explain the phenotype of a case of Lowes syndrome where the protein appeared to be folded normally. This is shown to involve a point mutant in the Rab-binding site that lowers the affinity of Rab8a binding, and also that of other Rabs, proving that all the Rabs bind to the same site. This mutation also affects membrane recruitment in vivo, thus proving the importance of Rab binding for recruitment of OCRL to membranes.

The text is clearly written and the figures present the data effectively. The work is thorough and rigorous and likely to be of great interest to the Lowe syndrome field, and also to many other labs working on Rabs and phosphoinositides. As such I am happy to reccomend publication in the EMBO Journal.

Minor comment:

a) The authors state that the structure: "reveals two Rab-binding sites in OCRL1(540-678)" This is ambiguous as it suggests that there are two sites where Rabs can bind independently, but in fact it is two regions that a single Rab contacts when it is binding. This should be reworded.

We agree with the referee on the possible ambiguity of our statement. Therefore, we have rephrased the sentence in question into “The complex crystal structure reveals a Rab-binding site in OCRL1540-678 that consists of two separate elements:…”.

Referee #3 (Remarks to the Author):

The paper describes the structure of Rab8a in complex with an effector, OCRL1, which is linked to a hereditary disease. The structure provides insight into how several mis-sense mutations might lead to defective interactions with Rab8 and altered sub-cellular localization. The manuscript is a fine combination of structural, cellular and biophysical experiments that is an important step in understanding the molecular basis for Lowe syndrome. The nature of the Rab-effector binding interface itself provides novel conceptual insight into the structural basis for recognition - the effector binds to the switch region via a b-strand, and the conserved aromatic triad (found in all Rabs) is only peripherally associated with the effector. Consequently, the paper is suitable for publication in EMBO.

The comments and suggestions below should be considered in a revised manuscript:

-the affinity of S564P (5.2uM) is significantly weaker than WT (0.9), with respect to Rab8 binding. Presumably, it would be much weaker for Rab1/Rab6 (WT is 4uM), although this is not measured, and these findings nicely explain the cytosolic location of mutant OCRL. However, the Rab8-OCRL(S564) affinity is basically on par with Rab1/Rab6 interactions with WT OCRL, and yet, these proteins presumably are able to recruit OCRL. Perhaps the authors could comment on this - are there other issues in sub-cellular localization that are not reflected by in vitro (equilibrium) binding affinity?

We believe the reviewer actually refers to point mutant F668V, since we did not determine the binding affinity of point mutant S564P.

In fact, the binding affinity of F668V to Rab8 appears to be in the range of Rab1/Rab6 and we agree that binding affinity to Rab1/Rab6 is likely to be much weaker. It has been shown before that Rab1/Rab6 are major determinants for Golgi recruitment of OCRL1. Thus our data that mutant F668V shows increased cytosolic localization is in line with these published data. We do not have any evidence for issues in subcellular localization that are not reflected by in vitro equilibrium binding affinity.

-Page 6 - relative affinities of Rab8, Rab6, Rab5 and Rab1 - in addition to the different experimental conditions that the authors mention in previous work, the current study uses the 'mant' modified GTP. The derivatization could also affect the relative affinities of various Rabs, perhaps through indirect (and subtle) conformational changes transmitted through the modified sugar to switch and interswitch regions. If this were the case, similar affinities obtained from equilibrium measurements vs. kinetics assays simply reflects internal consistency of the techniques, and not evidence for the 'correctness' of the relative affinities (Rab8 > Rab6, Rab5, etc). Perhaps the authors could mention this difference in their experimental setup.

The presence of the mant-group of derivatized nucleotides on the interaction of Rabs with its binding partners could indeed be a possible reason for the differences in binding affinities among different Rab-proteins. We concur with the point brought up by the referee and have added an additional sentence to the manuscript: “Also, an indirect influence of the mant-group at the ribose moiety of mantGppNHp cannot be excluded and might have a minor effect on the binding of the derivatized Rab-protein to OCRL1.” (page 8, bottom; page 9. Top)

-Page 16, top, change 'destroy the ASH domain fold' to 'disrupt' or 'destabilize the ASH domain fold'

According to the referee’s suggestion, we have rephrased the sentence into “Mutation L634P is likely to destabilize the ASH domain fold, which is in line with complete loss of binding to APPL1 and Ses proteins”.

-Page 6 and Fig. 5 legend - the data reflect 'kinetics and affinity', rather than 'thermodynamics' - for example, there are no dissections of binding energies into enthalpic and entropic components. However, the difference in free energy (ddG) could be derived from the ratio of Kd (WT/mut), and so the authors could quote this value (kcal/mol) which can be attributed to the loss of hydrophobic contact surface. As the paper is for a general readership, this point is not a major issue.

In order to prevent any confusion, we have followed the suggestion of the referee by changing “kinetics and thermodynamics” into “kinetics and affinities”. We prefer not to go into the details of thermodynamics here and hence do not provide additional discussion on the change in free energy ($\Delta\Delta G$) in agreement with the referee that this point is not of major significance for the general message of our manuscript.

-Substitute 'destroy the structural conformation' with 'destabilize the conformation', page 18. Also, this description of the effects of S564P is a little too vague. Where exactly is the residue locus, with respect to the structure? Is it found in the linker between the a-helix and the Ig-like domain? It seems to be close to strand b1 (OCRL), but the ribbon model is not marked with residue numbers

(Fig 3A). Perhaps the authors could clarify, and even better, indicate in one of the figures where the locus is.

As suggested, we have replaced "destroy" by "destabilize". Additionally, we agree with the reviewer that the position of S564 in the OCRL1 structure needs clarification in order to illustrate the potential detrimental influence of the proline mutation of this residue on the conformation of the OCRL1 ASH-domain. Thus, we have modified Figure 3A to contain a sphere at the position of Ser564. This residue is located at the end of the linker region between α-helix α1 and the ASH-domain. Hence, it can possibly affect the correct conformation of the ASH-domain and additionally impair the conformational flexibility between the α-helix α1 and the ASH-domain. This loss of conformational freedom could hinder the optimal relative arrangement of the two OCRL1 subdomains for Rab binding. We have acknowledged these possibilities in the final version of the manuscript and speculated on the conformational consequences of the proline mutation (S564P): "Based on our complex structure, the substitution S564P is expected to destabilize the conformation of binding site II as this residue appears to be an integral part of the beginning of the ASH-domain, leading to complete loss of Rab-binding activity. Additionally, the S564P mutation could impair the correct relative arrangement of the α1 helix and the ASH-domain since proline has reduced conformational freedom compared to serine. Thus, since S564 is at the C-terminal end of the α1-ASH linker region, its mutation to proline possibly affects Rab binding by impairing the orientation of helix α1 (Rab binding site 1) and the ASH domain (Rab binding site 2)."

-OCRL1 promiscuity is discussed in the paper, in the context of the aromatic triad. However, the higher Rab8 affinity (over Rab1, Rab6, Rab5), and specificity for these subset of Rabs, is not mentioned - can the authors say something about this issue? I don't feel a sequence alignment of Rabs is necessary, affinity may be a complex contribution from sequence/conformation of switches and interswitch. However, if there is something obvious about the Rab specificity, perhaps it should be mentioned.

It is indeed tempting to compare the amino acid sequences of Rab proteins to identify residues that determine specificity and affinity towards Rab binding proteins such as OCRL1. However, when comparing the Rab8a:OCRL1-interface amino acid residues with the homologous residues in Rab1b, Rab5a, and Rab6a, we cannot identify any obvious determinants for the higher affinity of OCRL1 to Rab8a. The affinity and differences in affinity are not only a function of the amino acid sequences, but also of the conformational flexibilities of the switch and interswitch regions of a Rab-protein (as suggested also by the referee). As we are unable to identify obvious sequence determinants for the higher Rab8a-OCRL1 affinity and a detailed conformational study of the switch regions of the different Rab proteins is beyond the scope of our manuscript, we prefer not to present a detailed discussion here.

Typos

-Page 13, change 'is not as central requirement' to 'may not be as critical a requirement' or something similar

The suggestion of the referee has been implemented.

-There is something odd about Fig5B, F668 colour is strange, it appears to be both sphere and stick models, this should be revised. Also, the I41-G42 (blue) sticks are overlapping at this angle, perhaps a slight tilt would help.

The color saturation of the amino acid residue $F668_O$ is indeed somewhat reduced in Figure 5B when compared to Figure 5A. This is a result of depicting $F668_O$ in a semi-transparent mode in Figure 5B. We needed to add transparency to the sphere depiction of the residue in order to visualize $Y77_R$, which is positioned behind $F668_O$ in this view. We prefer to keep the design of the figure and the transparency of the spheres representing $F668_O$ in order to show the encompassment of $F668_O$ by hydrophobic amino acid residues of the Rab protein.

Furthermore, we prefer to keep the orientation of the Figure 5B, even if the amino acids $I41_R$ and $G42_R$ are slightly difficult to spot. We have attempted to find a better view by tilting the structure slightly, but this procedure leads to an impaired visualization of $Y77_R$ (the depiction of the

ring structure vanishes) and moves $I43_R$ and $G42_R$ behind $F668_O$. Hence, the current view of the $F668_O$ binding site on Rab8, although not perfect, is the best possible compromise.

2nd Editorial Decision 25 January 2011

Thank you for sending us your revised manuscript. Our original referees 1 and 3 have now seen it again. In general, the referees are now positive about publication of your paper. Still, referee 1 is not fully satisfied with the revisions made and feels that there are a few remaining issues that need to be addressed (see below) before we can ultimately accept your manuscript. I would therefore like to ask you to deal with the issues raised. Please let us have a suitably amended manuscript as soon as possible.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The authors have addressed my original points to some degree, but I have further comments below.

Original point 2: Experimental verification of interactions in crystal structure. It would have been good to see further point mutagenesis of residues identified in the binding sites I and II (D555, R556, N559) and (R570, E571, G664, D666, T670). This may not add further mechanistic understanding, as the authors maintain, but would help verify the crystal structure. Studying the localization of these mutants would also more rigorously test whether Rab binding is required for OCRL targeting.

Original point 3: The images of the mutant still look pixel-saturated, and as such are not acceptable. They also have overall higher brightness than the WT image suggesting the proteins are expressed at different levels. Better images are required.

Referee #3 (Remarks to the Author):

The authors have addressed all of my concerns regarding the manuscript. Figure 3 and its corresponding discussion are clarified with respect to the Ser-Pro mutation, and the possible effects of mant-GTP on binding affinities is indicated.

Therefore, the paper is suitable for publication in EMBO.

2nd Revision - authors' response 02 February 2011

We would like to thank the referees for their positive reception of our manuscript and the improvements that have been implemented. Please find below our response to the specific points raised by the reviewer.

The authors have addressed my original points to some degree, but I have further comments below.

Original point 2: Experimental verification of interactions in crystal structure. It would have been good to see further point mutagenesis of residues identified in the binding sites I and II (D555, R556, N559) and (R570, E571, G664, D666, T670). This may not add further mechanistic understanding, as the authors maintain, but would help verify the crystal structure. Studying the localization of these mutants would also more rigorously test whether Rab binding is required for OCRL targeting.

We would like to take the opportunity to lay out in detail the arguments that verify the correctness of the observed crystal structure and the observed Rab8-OCRL1-interface and would like to apologize for the lack of such detail in our previous response to the referee comments.

There are several strong indications for the correctness of the OCRL1 amino acids suggested by our crystal structure to be involved in OCRL1-Rab8-complex formation:

1. OCRL1 F668A: The amino acid residue F668 is a central element of binding site II of OCRL1. Its mutation to alanine strongly affects the affinity of OCRL1 for Rab8a (Figure 5A-C). Additionally, OCRL1 F668A shows a more cytosolic localization than the wildtype protein (Figure 5E+F), thus demonstrating the significance of F668 for Rab-binding and membrane localization.
2. Truncation mutant OCRL1 555-678 vs. OCRL1 560-678: Among the OCRL1 truncation mutants tested, OCRL1 555-678 is the shortest construct that is still able to bind to Rab8a with measurable affinity. Truncating 5 amino acids more from the N-terminus (OCRL1 560-678) abolishes Rab8a binding in our assays (Figure S3). This is apparent from the structure, since the amino acids D555, R556, and N559 are involved in establishing the Rab8a binding interface. This agreement is a strong indication that the contact seen in the crystal is important for the interaction in solution.
3. Amino acid point mutants analyzed by Hyvola et al. (Hyvola et al, 2006) (D555E, S568G, S564P, G664D, N606K): Previously, Hyvola et al. generated amino acid point mutants of OCRL1 and analyzed their significance for Rab-binding and Golgi membrane localization. All mutants have been shown to be active in binding to clathrin, indicating correct folding of the mutant proteins. Only S568G and N606K were able to still bind to Rab proteins and localize to the Golgi, whereas D555E, S564P, and G664D failed to bind Rabs in vitro and to localize to Golgi membranes in vivo (for a summary see below Table I from (Hyvola et al, 2006).

Table I Summary of the OCRL1 point mutant rab binding and localisation results

GFP-OCRL1	Rab6Q72L interaction	Rab5Q79L interaction	Golgi targeting	Endosomal targeting
WT	+ + +	+ + +	+ + +	+ + +
P526H	+ + + + +	+ + + +	+ + +	ND
D555E	+/–	+/–	+/–	+/–
S564P	–	–	–	–
S568G	+ + +	+ + + +	+ + +	ND
N606K	+ + + +	+ + + +	+ + +	+ + +
G664D	+/–	+	+/–	+/–

ND, not determined.

These previous findings make perfect sense in the light of our observed crystal structure: D555 and G664 are implicated in Rab8a-OCRL1 complex formation, and S564 connects binding sites I and II. Mutation of these amino acids would be expected to have a negative effect on complex formation either by impairing the binding with cognate Rab8a amino acids (D555, G664) or by impairing the relative arrangement of binding sites I and II to each other (S564), which is exactly what is observed.

The OCRL1 amino acid residues S568 and N606, however, are not involved in the Rab8a-OCLR1 complex interface in our crystal structure. Consequently, their mutations (S568G and N606K) neither abrogate Rab binding nor Golgi membrane localization.

We have summarized the positions of the already characterized relevant mutations in the Rab8a:OCRL1-complex structure in the following figure (Figure R1).

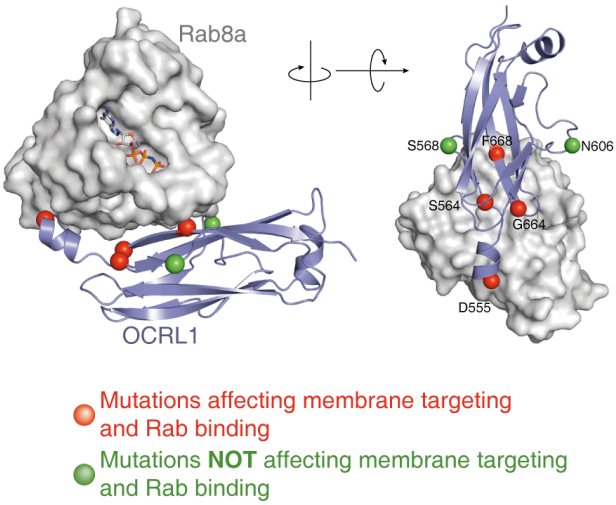

Figure R1 (now Figure S5): Positions of previously analyzed OCLR1 point mutations in the Rab8a:OCRL1 complex structure. The positions of amino acid point mutations analyzed by us (F668) or Hyvola et al. (D555, S564, S568, N606, G664) (Hyvola et al, 2006) are indicated by red and green spheres. Only amino acid point mutants implicated in the formation of the binding interface (D555E, S564P, G664D, F668A) affect Rab-interaction and membrane targeting of OCRL1 (red spheres), but not mutations located outside of the interface (S568G, N606K; green spheres).

In summary, we are convinced that our results together with data published previously by others (Hyvola et al, 2006) strongly support the correctness of the observed Rab8a-OCRL1-binding interface in our crystal structure. In order to emphasize this point raised by the referee we have extended our arguments in the final version of the manuscript and have provided the supplementary information with an additional figure (Figure S5) to give the positions of the previously analyzed amino acid substitutions.

In addition, we have moved the discussion of the amino acid mutants analyzed by Hyvola et al. from the paragraph “A structural basis for Lowe syndrome” to “The Rab8a:OCRL1 complex interface”. Since this paragraph concerns validity of the observed complex interface, we believe it to be more appropriately discussed at an earlier point of in the manuscript. We have implemented this modification into the manuscript on the basis of the reviewer’s comment. This leads to a significant improvement on the logic of the paper.

The discussion of the amino acids point mutants in the paragraph “The Rab8a:OCRL1 complex interface” now reads as follows: “The molecular interactions seen in the Rab8a:OCRL1 complex also provide a structural understanding of previous Rab-binding studies (D555E, S568G, S564P, G664D, N606K; Figure S5) (Hyvola et al, 2006). According to the complex structure, D555 and G664 are responsible for the interaction with the interswitch region by forming polar and hydrophobic interactions, respectively. Consequently, the D555E and G664D substitutions generated by Hyvola et al. reduced the Rab-binding activity of OCRL1 in vitro and impaired OCRL1 membrane localization in vivo (Hyvola et al, 2006), hence supporting their implication in Rab8a-OCRL1 complex formation. The amino acids S568 and N606 are not involved in establishing the complex interface and their substitutions (S568G and N606K) do not influence Rab-binding or OCRL1 membrane recruitment (Hyvola et al, 2006). Variant S564P had the strongest effect on Rab binding, abolishing it completely (Hyvola et al, 2006). Based on our complex structure, the substitution S564P is expected to destabilize the conformation of binding site II, since this residue appears to be an integral part of the beginning of the ASH-domain, leading to complete loss of Rab-binding activity. Additionally, the S564P mutation could impair the correct relative arrangement of the α1 helix and the ASH-domain, since proline has reduced conformational freedom compared to

serine. Since S564 is at the C-terminal end of the α1-ASH linker region, its mutation to proline possibly affects Rab binding by impairing the orientation of helix α1 (Rab binding site I) and the ASH domain (Rab binding site II). Thus, the in vivo and in vitro effects of OCRL1 point mutations reported previously perfectly correlate with their relevance for Rab8a:OCRL1-complex formation as suggested by the complex crystal structure."

Original point 3: The images of the mutant still look pixel-saturated, and as such are not acceptable. They also have overall higher brightness than the WT image suggesting the proteins are expressed at different levels. Better images are required.

As requested by the reviewer we have completely revised figure 5E and F. We have now introduced, as we believe, better images. The green channel exposure time was identical for all pictures. In addition, we did not further process the pictures in the green channel (no change of contrast or brightness). Furthermore, we provide (for for the review process only) a figure quantifying pixel intensity within the green channel showing that the pixel intensity is below the maximum (gray value 250), ruling out overexposure of the figures presented (Figure R2).

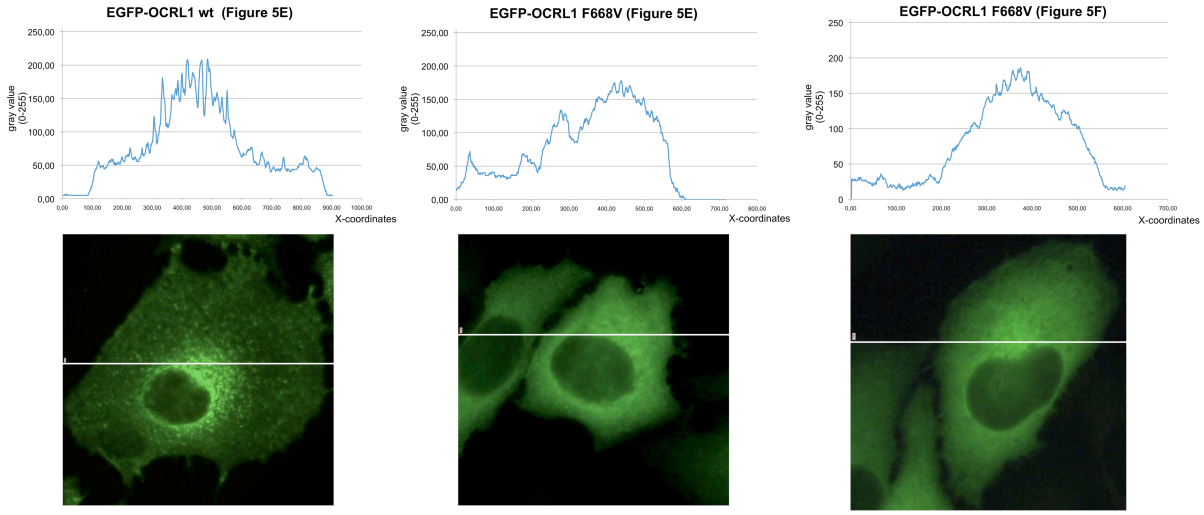

Figure R2: Intensity profile of EGFP-derived fluorescence images in Figure E and F. Original images were analyzed for the gray value intensity (Y-axis, range from 0-255) with AnalysisB software in the marked region (white line) For better comparison, images are shown below in the same scale as represented in the X-axis of the diagram above.

Referee #3 (Remarks to the Author):

The authors have addressed all of my concerns regarding the manuscript. Figure 3 and its corresponding discussion are clarified with respect to the Ser-Pro mutation, and the possible effects of mant-GTP on binding affinities is indicated.

Therefore, the paper is suitable for publication in EMBO.

The referee appears to be satisfied with the manuscript.

References

Hyvola N, Diao A, McKenzie E, Skippen A, Cockcroft S, Lowe M (2006) Membrane targeting and activation of the Lowe syndrome protein OCRL1 by rab GTPases. *EMBO J* **25**(16)**:** 3750-3761

3rd Editorial Decision 04 February 2011

Thank you for sending us your revised manuscript. Referee 1 has now seen it again, and you will be pleased to learn that in his/her view you have addressed all criticisms in a satisfactory manner. The paper will now be publishable in The EMBO Journal and you will receive a formal acceptance letter shortly.

Thank you very much again for considering our journal for publication of your work.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The referees have addressed my concerns and I recommend publication.

