Participation of Tom1L1 in EGF-stimulated endocytosis of EGF receptor

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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 11 March 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. Your manuscript has now been evaluated by three referees whose comments are enclosed below. As you will see from their reports the referees express interest in the study, however, in the end they provide mixed recommendations about publication in the EMBO Journal, with referee #2 and #3 more supportive of publication than referee #1.

The referees raise a number of important concerns, both referee #1 and #3 question if you are describing a role for Tom1L1 in the clathrin mediated internalization of the EGF receptor or an alternative pathway. This is in part due to the cell line and the internalization protocol used, they find that the experiments should be repeated in another cell line such as HeLa and also the binding and internalization experiments should be performed at 37C. In addition, referee #1 requests that experiments are performed to demonstrate the requirement of SFKs in the internalization of the EGFR. Referee #2, raises a different but equally important point and asks for further analysis of the non-canonical clathrin binding motif. Based on these comments, and given the overall interest in the study and that these issues should be addressable experimentally, I would be willing to consider a revised version of your manuscript.

I would like to remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your revisions included in the next, final version of the manuscript.

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

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This is an interesting study that describes the role of Tom1L protein in EGFR internalization. This protein has been previously implicated in endocytic trafficking because it is located in endosomes, interacts with clathrin and TSG101, and in signaling because it is phosphorylated by Src family kinases and interacts with Grb2. Since Grb2 is important for EGFR internalization, the authors propose a model whereby Src (or another family member) phosphorylates Tom1L thus leading to the binding of Grb2 SH2 domain to this phosphorylation site. Subsequently, Grb2-Tom1L can bridge EGFR and clathrin heavy chain and thus mediate internalization of EGFR via clathrin coated pits. This model is well supported by the demonstration of interaction of Grb2 with Tom1L and co-immunoprecipitation of Tom1L and EGFR, as well as by functional experiments using Tom1L mutants and siRNA. Unfortunately, all experiments are performed in A-431 cells that express very high levels of EGFR and are aberrant in many aspects of EGFR regulation. It has been demonstrated in a number of studies (see for example Haigler et al., 1979, JCB; Wiley, 1982, JCB) that under experimental conditions in this manuscript (high EGF concentrations), most of the EGFRs will be internalized through clathrin-independent endocytosis pathways. Furthermore, the authors employ a biotinylation assay to monitor endocytosis and report unusually high internalization rates (see for instance Fig. 5C). Since most data in the literature are based on the use of radiolabeled EGF it is difficult to compare the present data with the numerous published data on A-431 cells. In essence, the authors need to demonstrate the role of Tom1L in another type of cells, for instance, HeLa cells that express a low level of EGFR and tend to use clathrin-mediated pathway to endocytosis EGFR.

It is counterintuitive that Tom1L needs tyrosine phosphorylation to bind to Grb2 and EGFR. This seems impossible because the SH2 domain of Grb2 can bind either to EGFR or Tom1L, and cannot bind to both simultaneously. The requirement for Src activity in this process is another major concern in the interpretation of the data and the model. Since previous studies demonstrated that Src family kinases are not necessary for EGFR endocytosis, the authors need to confirm that these kinases are required for EGFR internalization under the particular experimental conditions used in their work.

Specific comments:
1) Fig. 2C. The depletion of Grb2 and the effect of Grb2 depletion on EGFR interaction with Tom1L are not convincing. Perhaps a significantly more efficient knock-down of Grb2 is necessary to demonstrate this important point.
2) Fig. 3B. It is very difficult from the presented images to judge localization of Tom1L in coated pits and its co-localization with EGFR at the plasma membrane. This is one of the key experiments in the paper. The co-localization should be better demonstrated and quantitated.
3) Fig. 7. From the studies of Tom1L association with clathrin it is unclear why Tom1L does not constitutively bind clathrin and is not present in coated pits.

Referee #2 (Remarks to the Author):

This is a manuscript that I reviewed previously for another journal. My original review is shown below. My opinion of this manuscript remains the same: it's excellent but would benefit from revision. Of my original suggestions, the authors addressed a couple but left others unanswered. In particular, I still think that the authors need to perform a more detailed alanine scan mutagenesis of the FDPL sequence and neighboring residues and then test for interaction of the mutants with clathrin. Only this analysis will allow them to determine whether the clathrin-binding motif is indeed non-canonical. In addition, the authors need to show that a clathrin N-terminal construct comprising residues 1-363 does not bind the TOM1L1 motif but binds a canonical motif (the latter
is needed as a positive control). If the authors include this evidence, I think that this paper would be a very important contribution to the understanding of the mechanisms of EGFR downregulation.

"Binding of EGF downregulates the EGFR receptor (EGFR) through internalization and targeting to the MVB/lysosomal pathway. EGFR internalization is dependent on clathrin (in particular at low doses of EGF; see below), but the identity of the clathrin adaptor that mediates it is not known with certainty. Several studies have ruled out a role for the main endocytic adaptor, AP-2. Instead, ubiquitination of the EGFR by c-Cbl and recognition by several ubiquitin-binding, clathrin adaptors such as Epsin and Eps15 have been proposed to play key roles in endocytosis. However, conflicting evidence has been presented in the literature for the requirement of ubiquitination and Epsin/Eps15 for EGFR endocytosis.

The present manuscript proposes an alternative mechanism in which the VHS-GAT-containing protein, Tom1L1, functions as a clathrin adaptor for Grb2 and, through it, for the activated EGFR. This is consistent with the requirement of Grb2 for EGFR internalization shown in previous studies. Specifically, the authors show that Tom1L1 binds to the EGFR activated through phosphorylation by Src family kinases. Tom1L1 binding and EGFR phosphorylation are transient and occur with similar kinetics, unlike EGFR ubiquitination, which is more sustained. Upon stimulation with EGF, Tom1L1 is first recruited from the cytosol to the plasma membrane and then redistributed to endosomes. Dominant-negative interference or RNAi of Tom1L1 inhibits EGFR endocytosis. In addition, the authors show that Tom1L1 contains a non-canonical clathrin-binding motif in its C-terminal domain, which is also required for EGFR endocytosis.

Overall, I find the evidence presented in this paper very compelling. The experimental data are of extraordinarily high quality, crisp and thoroughly-controlled. The writing is also excellent for its logic and clarity, despite an occasional grammar slip that can be easily corrected on copyediting. What I like best about this study is that it provides a simpler, more convincing mechanism for the endocytic step of downregulation. Despite the focus on ubiquitination as a trigger for internalization, there is just too much negative or conflicting evidence in the literature to support an essential role in EGFR endocytosis (as opposed to some post-endocytic role). Nevertheless, I think that the paper would benefit from revision as follows:

1. Perhaps the least convincing set of experiments concern the identification of the clathrin-binding motif in Tom1L1 and its cognate binding site on clathrin (Fig. 7). The authors propose that the sequence FDPL constitutes a non-canonical clathrin-binding motif. However, they did not perform a detailed alanine scan mutagenesis to analyze the requirement of each FDPL residue or neighboring residues for clathrin binding. In fact, I see a resemblance of the VMEFD (or LLEFD in the frog) sequence in Tom1L1 to canonical clathrin-binding motifs (e.g., LLDDL, LIEFE, LILED, etc.). Single alanine substitutions at each of the VMEFDPL residues would resolve this issue. I am also not convinced by the evidence that the Tom1L1 motif binds to the C-terminus of clathrin, unlike the canonical motifs, which bind to the N-terminal domain. A problem I see is that the authors did not test the entire clathrin N-terminal domain (residues 1-363) but a fragment of it (residues 1-336) in their pulldown experiments (Fig. 1E). These experiments should be redone with a construct comprising the N-terminal domain.

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3. Did the authors test whether RNAi of Tom1 and Tom1L2 does not affect EGFR endocytosis? I don't think it's essential for the story but it would underscore the specific role of Tom1L1 in this process. Another issue I am curious about is whether the ubiquitin-binding activity of Tom1L is dispensable for internalization of EGFR. Again, this goes beyond the message of the paper, but it would be interesting to know.

4. On page 24, reference to Michael and Sylvie, should be to Clague and UrbÈ.

5. Page 24. Although I like the way that the authors deal with the "low-dose vs. high-dose" EGF problem (I never understood this dichotomy myself), I think that the discussion could be shortened."
Referee #3 (Remarks to the Author):

The paper by Liu and colleagues entitled "Participation of Tom1L1 in EGF-stimulated endocytosis of EGF receptor" uncovers the interesting observation that Tom1L1 is required for the endocytosis of activated EGF receptor.

The general problem addressed here focuses on how activated EGFR is internalized. A detailed answer remains evasive, in part due to the complexity of interactions between the activated EGFR and several endocytic machineries that are engaged depending on the extent of EGFR activation. One recognized endocytic route is clathrin mediated, often referred to as the high affinity/low capacity (elicited with low concentrations of EGF); a second route, less defined, is referred to as the low affinity/high capacity (elicited by high concentration of EGF, e.g. 100 ng/ml as used in this work). Engagement of these pathways seems to revolve on the extent of receptor activation. Activated (e.g. Y-phosphorylated) EGFR binds to a number of proteins that act as adaptors for signaling and/or targeting to a ubiquitin-recognition system required for delivery of the internalized EGFR to multivesicular endosomes for its ultimate degradation. EGFR also binds to the clathrin adaptor AP-2, required for assembly of endocytic clathrin coated pits and for targeting of EGFR to the forming pits. Liu and colleagues have now found that Tom1L1 is also a partner of activated EGFR, and that its functionality/presence is required for endocytosis of activated EGFR. This new observation is obviously important, as it expands the catalog of molecules involved in EGFR traffic and regulation.

The authors have shown that Tom1L1, a protein containing a VHS domain, interacts with Grb2 (itself a partner of activated EGFR), and that it also has the apparent potential to bind to clathrin heavy chain at a region towards the hub of the clathrin triskelion. They mapped regions on Tom1L1 required for these interactions, and found that their presence is required for the endocytosis and ultimate degradation of activated EGFR. The authors also describe the transfer of cytosolic Tom1L1 to the plasma membrane upon activation of EGFR, and their traffic from the cell surface to endosomal structures that are positive for clathrin. Based on these observations, Liu et al conclude that Tom1L1 is an endocytic adaptor for activated EGFR required for capture of EGFR in endocytic clathrin coated pits/vesicles.

I praise the authors for the execution of their experiments and for the description of their results. I believe that they make a convincing argument for the role of Tom1L1 in the endocytic traffic of activated EGFR. For the following reasons, however, I am not convinced that they have data in support of a possible role of Tom1L1 in the clathrin-based entry pathway. Instead, I would suggest that Tom1L1 has an important role on the traffic of EGFR through the low affinity/high capacity route.

1. All of the internalization experiments described in this paper are based on the activation of EGFR by incubation of A431 cells with EGF at 4°C (when endocytosis is minimal), followed by transfer to 37°C (to turn on endocytosis). This experimental design, although used widely (as described in this paper), has a fundamental flaw: internalization of activated EGFR bypasses the need for AP-2 (depleted using RNAi approaches); this result led a number of groups to the incorrect conclusion that endocytosis of activated EGFR does not require AP-2, and hence AP-2 is not its endocytic adaptor. Sorkin’s group (Huang et al JBC 2004; not quoted in this paper) has clearly shown that this is certainly not the case: presence of AP-2 is essential for the entry of activated EGFR. Their experiment was carried by always keeping the cells at 37°C, both during EGFR activation and endocytosis. Thus, I surmise that Liu et al are following the entry mediated by the low affinity/high capacity route.

2. The experiments are carried in A431 cells. These cells have ~106 EGF receptors. About 50% of the receptors are degraded within 30-40 min or so upon receptor activation. The number of coated pits forming on the surface of these cells is simply not sufficient to transport the internalized receptors (amply discussed by Wiley et al in the late 90's, and corroborated by current data describing the number of clathrin pits that form per time and surface area). In fact, McNiven's group has time-lapse images showing massive entry of activated EGFR in a process linked to the formation of large surface ruffles. These observations, again, indicate that the large-scale entry of
activated EGFR bypasses the clathrin route. Inspection of the movies provided in this study is consistent with this view. I would like to add that the movies do not show the traffic of EGFR in tiny coated pits/vesicles; instead they show accumulation of EGFR in fairly large structures that often are positive for clathrin. I would suggest that these structures are endosomes.

3. The authors did pull down experiments to identify a possible interaction between the c-terminus of clathrin and Tom1L1. They also suggest that, upon EGFR activation, Tom1L1 colocalizes with clathrin on the cell surface. I agree that Tom1L1 colocalizes with large patches of endosomal clathrin; I do not believe, however, that the data, as presented, shows specific colocalization of Tom1L1 and clathrin at the cell surface. An alternative interpretation is that Tom1L1 is recruited in large amounts to activated EGFR located on the cell surface, but that the optical resolution of the fluorescence microscope precludes the conclusion derived by the authors. In fact, close inspection of their images does not show concentration of EGFR and Tom1L1 on the clathrin spots. Indeed such result is highly unlikely, if one considers that only a few copies of EGFR can be captured at any given moment by a single coated pit/vesicle.

To conclude, I would suggest that the authors reconsider their entry model, and place Tom1L1 in the context of the low affinity/high capacity route. I would suggest checking whether the role of Tom1L1 is maintained in cells always kept at 37°C, and I would also consider verifying their results with even more stringent (e.g. lower) EGF concentrations, to ensure that they are functionally dissecting both entry pathways.

The point-to-point responses to the reviewers’ comments

Referee #1 (Remarks to the Author):

This is an interesting study that describes the role of Tom1L protein in EGFR internalization. This protein has been previously implicated in endocytic trafficking because it is located in endosomes, interacts with clathrin and TSG101, and in signaling because it is phosphorylated by Src family kinases and interacts with Grb2. Since Grb2 is important for EGFR internalization, the authors propose a model whereby Src (or another family member) phosphorylates Tom1L thus leading to the binding of Grb2 SH2 domain to this phosphorylation site. Subsequently, Grb2-Tom1L can bridge EGFR and clathrin heavy chain and thus mediate internalization of EGFR via clathrin coated pits. This model is well supported by the demonstration of interaction of Grb2 with Tom1L and co-immunoprecipitation of Tom1L and EGFR, as well as by functional experiments using Tom1L mutants and siRNA. Unfortunately, all experiments are performed in A-431 cells that express very high levels of EGFR and are aberrant in many aspects of EGFR regulation. It has been demonstrated in a number of studies (see for example Haigler et al., 1979, JCB; Wiley, 1982, JCB) that under experimental conditions in this manuscript (high EGF concentrations), most of the EGFRs will be internalized through clathrin-independent endocytosis pathways. Furthermore, the authors employ a biotinylation assay to monitor endocytosis and report unusually high internalization rates (see for instance Fig. 5C). Since most data in the literature are based on the use of radiolabeled EGF it is difficult to compare the present data with the numerous published data on A-431 cells. In essence, the authors need to demonstrate the role of Tom1L in another type of cells, for instance, HeLa cells that express a low level of EGFR and tend to use clathrin-mediated pathway to endocytosis EGFR.

We agree with the reviewer and have now provided results of experiments using HeLa cells. Both Tom1L1 knockdown and overexpression of dominant-negative Tom1L1/Y460F mutant inhibited EGF-induced endocytosis of EGFR in HeLa cells, suggesting that Tom1L1 does play a general role in EGF-induced endocytosis of EGFR. These results are presented in Supplementary Figures S8B, S8C and S8D.
The basis for the use of surface biotinylation is that this method allows us to directly measure the internalization of EGFR itself rather than through an indirect approach using radio-labeled EGF as a surrogate marker for EGFR. Measuring EGFR endocytosis indirectly through monitoring the uptake of EGF has intrinsic problems as the uptake of EGF could potentially be contributed by other routes of endocytosis (such as non-receptor mediated pinocytosis at high concentrations), in addition to endocytosis of the EGF-EGFR complex. Since surface biotinylation is a well established procedure and has been widely used to mark only surface exposed proteins, this approach eliminates potentially non-specific contributions of EGF uptake by other routes of endocytosis. This approach also minimizes the complication of non-surface localized EGFR (such as endosomal or newly synthesized EGFR in the biosynthetic pathway). As such, we believe that monitoring the endocytosis of surface-biotinylated EGFR in response to EGF is a much more direct and specific procedure.

It is counterintuitive that Tom1L needs tyrosine phosphorylation to bind to Grb2 and EGFR. This seems impossible because the SH2 domain of Grb2 can bind either to EGFR or Tom1L, and cannot bind to both simultaneously. The requirement for Src activity in this process is another major concern in the interpretation of the data and the model. Since previous studies demonstrated that Src family kinases are not necessary for EGFR endocytosis, the authors need to confirm that these kinases are required for EGFR internalization under the particular experimental conditions used in their work.

We have now provided insightful evidence indicating that the function of Tom1L1 in EGFR endocytosis is likely mediated by interaction of Tyr-phosphorylated Tom1L1 with EGFR through Grb2 and Shc adaptors. In this scenario, Shc adaptor serves to bridge the Tom1L1-Grb2 complex with the tyrosine-phosphorylated EGFR. An earlier study has shown that Grb2 can interact with EGFR indirectly through Shc (Rozakis-Adcock, M., et al. Nature, 1992, 360, 689-692), enabling the SH2 domain of Grb2 to interact with other proteins such as Tom1L1. We have now shown that knockdown of Shc also impaired the interaction of Tom1L1 with EGFR, and simultaneous knockdown of Grb2 and Shc led to robust inhibition of Tom1L1-EGFR interaction. Furthermore, Shc knockdown also reduced the endocytosis of EGFR and simultaneous knockdown of both Grb2 and Shc resulted in more severe inhibition on EGFR endocytosis. These results are now included in new Fig 2D and Supplementary Figure S7C.

We conducted additional experiments employing the Src kinase inhibitor, PP1, and these results indicate that Src family kinases play a role in EGF-induced endocytosis of EGFR. The results are shown in Supplementary Figure S7B. Our conclusion is also consistent with a previous study demonstrating a role of Src kinase in EGFR endocytosis (Wilde A, et al., Cell, 1999, 96,677-87).

Specific comments:
1) Fig. 2C. The depletion of Grb2 and the effect of Grb2 depletion on EGFR interaction with Tom1L are not convincing. Perhaps a significantly more efficient knock-down of Grb2 is necessary to demonstrate this important point.

We conducted additional experiments and the results indicate that knockdown of either Grb2 or Shc2 significantly reduced the association of Tom1L1 with EGFR and that simultaneous knockdown of both Grb2 and Shc led to robust abrogation of Tom1L1-EGFR interaction. The new results are used to replace the original Fig. 2D.

2) Fig. 3B. It is very difficult from the presented images to judge localization of Tom1L in coated pits and its co-localization with EGFR at the plasma membrane. This is one of the key experiments in the paper. The co-localization should be better demonstrated and quantitated.

We have now provided better images showing that Tom1L1 is recruited to clathrin-coated structures and co-localized with EGFR (new Figure 3B). Similar results were obtained in HeLa cells and were shown in Supplementary Figure S4D.

3) Fig. 7. From the studies of Tom1L association with clathrin it is unclear why Tom1L does not constitutively bind clathrin and is not present in coated pits.
We agree with the reviewer this is an interesting point. Since the majority of Tom1L1 is present in the cytoplasm and its tyrosine phosphorylation and association with EGFR is tightly regulated, its role in endocytosis is thus regulated. One possibility that needs future experiments to address is whether interaction of Tom1L1 with clathrin is also regulated in vivo. We hope the reviewer will agree that this point does not affect the significance of the current study and is beyond the scope of this manuscript.

Referee #2 (Remarks to the Author):

This is a manuscript that I reviewed previously for another journal. My original review is shown below. My opinion of this manuscript remains the same: it's excellent but would benefit from revision. Of my original suggestions, the authors addressed a couple but left others unanswered. In particular, I still think that the authors need to perform a more detailed alanine scan mutagenesis of the FDPL sequence and neighboring residues and then test for interaction of the mutants with clathrin. Only this analysis will allow them to determine whether the clathrin-binding motif is indeed non-canonical. In addition, the authors need to show that a clathrin N-terminal construct comprising residues 1-363 does not bind the TOM1L1 motif but binds a canonical motif (the latter is needed as a positive control). If the authors include this evidence, I think that this paper would be a very important contribution to the understanding of the mechanisms of EGFR downregulation.

As advised, we have performed additional mutagenesis analysis of the FDPL motif and its neighboring residues (namely the VMEFDPL stretch). The results showed again that Tom1L1 bind to C-terminal region of clathrin but not the N-terminal domain. Mutation of individual residue of the VMEFDPL stretch by Ala-scanning mutagenesis did not affect the interaction with clathrin (Supplementary Figure S5B). This property is different from the canonical clathrin-binding box. For example, mutation of each of the first four residues of Ack1’s canonical clathrin box (LIDFG) into Ala abolished clathrin interaction (Teo et al., JBC, 2001, 276, 18392-18398). Although the detailed structural basis for this motif to interact with clathrin remains to be further investigated, these additional results indeed strengthen the conclusion that it is part of a novel clathrin-binding module.

We have also performed interaction assays using the N-terminal domain of clathrin consisting of residues 1-363. As such, the results of experiments using this fragment were presented in the revised Figure 7E. Similar results were obtained showing that Tom1L1 binds to the C-terminal but not N-terminal region of clathrin. As advised by this reviewer, we have used a 19-residue region (residues 564-582) of Ack1 that contains a canonical clathrin-box (LIDFG) (Teo et al., JBC, 2001, 276, 18392-18398) as a control. When fused to GST, the GST-Ack1564-582 interacted with the N-terminal but not the C-terminal region of clathrin. The results are now shown as Figure 7G. The original Figure 7G and 7H are now 7H and 7I, respectively in the revised Figure 7.

"Binding of EGF downregulates the EGF receptor (EGFR) through internalization and targeting to the MVB/lysosomal pathway. EGFR internalization is dependent on clathrin (in particular at low doses of EGF; see below), but the identity of the clathrin adaptor that mediates it is not known with certainty. Several studies have ruled out a role for the main endocytic adaptor, AP-2. Instead, ubiquitination of the EGFR by c-Cbl and recognition by several ubiquitin-binding, clathrin adaptors such as Epsin and Eps15 have been proposed to play key roles in endocytosis. However, conflicting evidence has been presented in the literature for the requirement of ubiquitination and Epsin/Eps15 for EGFR endocytosis.

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This important point has been addressed. Please see above regarding the mutagenesis analysis of the VMEFDPL motif.

2. I would like to see higher resolution fluorescence microscopy showing recruitment of Tom1L1 not just to the plasma membrane but to clathrin-coated pits. In the same way that one can visualize AP-2 or Epsin within clathrin-coated pits it should be possible to see Tom1L co-localizing with EGFR within such structures.

We agree with the reviewer and have provided better images showing recruitment of Tom1L1 to the plasma membrane as well as clathrin-coated structures (new Fig 3B, and Supplementary Figure S4D).

3. Did the authors test whether RNAi of Tom1 and Tom1L2 does not affect EGFR endocytosis? I don’t think it’s essential for the story but it would underscore the specific role of Tom1L1 in this process. Another issue I am curious about is whether the ubiquitin-binding activity of Tom1L is dispensable for internalization of EGFR. Again, this goes beyond the message of the paper, but it would be interesting to know.

We have shown that, unlike knockdown of Tom1L1, knockdown of Tom1 or Tom1L2 did not affect EGFR endocytosis and the results are now presented in Figure 6G and 6H. This is consistent with the observation that endocytosis defects due to Tom1L1 knockdown can be rescued by Tom1L1 but not by Tom1 (Figure 6E and 6F for A431 cells, Supplementary Figure S8D for HeLa cells). Many thanks to this reviewer for his/her understanding regarding other interesting issues that are beyond the scope of this current manuscript.

4. On page 24, reference to Michael and Sylvie, should be to Clague and Urbé.

Many thanks and we have made the change.

5. Page 24. Although I like the way that the authors deal with the “low-dose vs. high-dose” EGF problem (I never understood this dichotomy myself), I think that the discussion could be shortened.

We have tried our best to shorten the discussion from 1759 to 1419 words.
Referee #3 (Remarks to the Author):

The paper by Liu and colleagues entitled "Participation of Tom1L1 in EGF-stimulated endocytosis of EGF receptor" uncovers the interesting observation that Tom1L1 is required for the endocytosis of activated EGF receptor.

The general problem addressed here focuses on how activated EGFR is internalized. A detailed answer remains evasive, in part due to the complexity of interactions between the activated EGFR and several endocytic machineries that are engaged depending on the extent of EGFR activation. One recognized endocytic route is clathrin mediated, often referred to as the high affinity/low capacity (elicited with low concentrations of EGF); a second route, less defined, is referred to as the low affinity/high capacity (elicited by high concentration of EGF, e.g. 100 ng/ml as used in this work). Engagement of these pathways seems to revolve on the extent of receptor activation. Activated (e.g. Y-phosphorylated) EGFR binds to a number of proteins that act as adaptors for signaling and/or targeting to a ubiquitin-recognition system required for delivery of the internalized EGFR to multivesicular endosomes for its ultimate degradation. EGFR also binds to the clathrin adaptor AP-2, required for assembly of endocytic clathrin coated pits and for targeting of EGFR to the forming pits. Liu and colleagues have now found that Tom1L1 is also a partner of activated EGFR, and that its functionality/presence is required for endocytosis of activated EGFR. This new observation is obviously important, as it expands the catalog of molecules involved in EGFR traffic and regulation.

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I praise the authors for the execution of their experiments and for the description of their results. I believe that they make a convincing argument for the role of Tom1L1 in the endocytic traffic of activated EGFR. For the following reasons, however, I am not convinced that they have data in support of a possible role of Tom1L1 in the clathrin-based entry pathway. Instead, I would suggest that Tom1L1 has an important role on the traffic of EGFR through the low affinity/high capacity route.

1. All of the internalization experiments described in this paper are based on the activation of EGFR by incubation of A431 cells with EGF at 4°C (when endocytosis is minimal), followed by transfer to 37°C (to turn on endocytosis). This experimental design, although used widely (as described in this paper), has a fundamental flaw: internalization of activated EGFR bypasses the need for AP-2 (depleted using RNAi approaches); this result led a number of groups to the incorrect conclusion that endocytosis of activated EGFR does not require AP-2, and hence AP-2 is not its endocytic adaptor. Sorkin's group (Huang et al JBC 2004; not quoted in this paper) has clearly shown that this is certainly not the case: presence of AP-2 is essential for the entry of activated EGFR. Their experiment was carried by always keeping the cells at 37°C, both during EGFR activation and endocytosis. Thus, I surmise that Liu et al are following the entry mediated by the low affinity/high capacity route.

My apology for not citing the JBC paper from Sorkin’s group and we have done so in the revised manuscript. As advised, we have now performed additional experiments in which both binding and endocytosis were done at 37 °C. The results from these new experiments also support the importance of Tom1L1 in endocytosis of EGFR in response to EGF. The results are now presented as supplementary Figure S8C, S8D. Moreover, according to Sorkin’s latest review, he supports the notion that the function of AP2 in EGFR endocytosis is still not clear, consistent with our model (Sorkin and Goh, Exp Cell Res. 2009, 315, 683-696).

2. The experiments are carried in A431 cells. These cells have ~106 EGF receptors. About 50% of
the receptors are degraded within 30-40 min or so upon receptor activation. The number of coated pits forming on the surface of these cells is simply not sufficient to transport the internalized receptors (amplel discussed by Wiley et al in the late 90's, and corroborated by current data describing the number of clathrin pits that form per time and surface area). In fact, McNiven's group has time-lapse images showing massive entry of activated EGFR in a process linked to the formation of large surface ruffles. These observations, again, indicate that the large-scale entry of activated EGFR bypasses the clathrin route. Inspection of the movies provided in this study is consistent with this view. I would like to add that the movies do not show the traffic of EGFR in tiny coated pits/vesicles; instead they show accumulation of EGFR in fairly large structures that often are positive for clathrin. I would suggest that these structures are endosomes.

Besides A431 cells, we have also performed experiments in HeLa cells and similar results were obtained supporting a role of Tom1L1 in EGF-stimulated endocytosis of EGFR (Supplementary Figures S4D, S8B, S8C, S8D). Since EGFR endocytosis defect due to Tom1L1 knockdown can be rescued by wildtype Tom1L1 but not Tom1L1 mutant defective in interaction with clathrin (Figure 7I and 7J), the role of Tom1L1 in mediating EGFR endocytosis is coupled to its ability to interact with clathrin. As such, we favor the working model that Tom1L1 mediates clathrin-dependent endocytosis of EGFR. We agree that the movies presented are not having resolution that is high enough to resolve the detailed fine structures involved in endocytosis and the fairly large structures may be endosomes. As such, we have deleted these two movies and the associated supplementary Figure 9. We hope the reviewer will agree, with the additional data in Hela cells, that Tom1L1 does play a more general role in EGF-stimulated clathrin-mediated endocytosis of EGFR.

3. The authors did pull down experiments to identify a possible interaction between the c-terminus of clathrin and Tom1L1. They also suggest that, upon EGFR activation, Tom1L1 colocalizes with clathrin on the cell surface. I agree that Tom1L1 colocalizes with large patches of endosomal clathrin; I do notbelieve, however, that the data, as presented, shows specific colocalization of Tom1L1 and clathrin at the cell surface. An alternative interpretation is that Tom1L1 is recruited in large amounts to activated EGFR located on the cell surface, but that the optical resolution of the fluorescence microscope precludes the conclusion derived by the authors. In fact, close inspection of their images does not show concentration of EGFR and Tom1L1 on the clathrin spots. Indeed such result is highly unlikely, if one considers that only a few copies of EGFR can be captured at any given moment by a single coated pit/vesicle.

To conclude, I would suggest that the authors reconsider their entry model, and place Tom1L1 in the context of the low affinity/high capacity route. I would suggest checking whether the role of Tom1L1 is maintained in cells always kept at 37°C, and I would also consider verifying their results with even more stringent (e.g. lower) EGF concentrations, to ensure that they are functionally dissecting both entry pathways.

We have performed experiments in HeLa cells (Supplementary Figures S4D, S8B, S8C, S8D) and also using lower concentrations of EGF (Supplementary Figures S7A, S8A) and these results are supportive of a role of Tom1L1 in mediating endocytosis of EGFR. Again, the experiments performed only at 37°C (Supplementary Figures S8C, S8D) are consistent with the conclusion. Other independent studies reported recently have also shown that endocytosis of EGFR is still primarily mediated by clathrin-dependent pathway, even in the presence of high EGF dose (up to 200 ng/ml) (Kazazic et al., Traffic, 2006, 7, 1518-1527; Lakadamyali et al., Cell, 2006, 124, 997-1009). As such, our results are more consistent with the model that Tom1L1 mediates endocytosis of activated EGFR via clathrin-dependent endocytosis.

2nd Editorial Decision
24 June 2009

Your manuscript has been reviewed by two of the original referees. The overall decision is that we are able to accept publication in the EMBO Journal pending satisfactory minor revision. Referee #1 raises a number of valid issues that should be addressed, the use of different labeling techniques for monitoring EGFR internalization should be discussed in the manuscript as should an expansion of
the model regarding the role of the SH2 domain.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The revised manuscript contains many new experiments that support the role of Tom1L in EGFR endocytosis. Overall, when analyzed apart from the EGFR literature, this study is indeed a convincing demonstration of the function of Tom1L in EGFR internalization. However, the major points raised in the previous critique have not been addressed.

1) Measurement of endocytosis of occupied EGFR (using radiolabeled EGF) has been utilized in more than 300 papers. It is very well established that there is no uptake of radioEGF in the absence of the receptor (as the authors suggest) and there is very minimal internalization if the receptor kinase activity is blocked. Although the authors argue that biotinylation is the appropriate technique to measure the EGFR endocytic rates, this protocol involves chemical modification of EGFR and other membrane proteins prior to receptor stimulation and endocytosis, and a temperature shift from 4°C to 37°C. It is unclear how these conditions would affect EGF binding and endocytosis. Also, reverse biotinylation measures the internalization of the total surface receptor pool rather than the endocytosis of EGF-occupied, activated receptors. Thus, while reverse surface biotinylation is a legitimate internalization assay, the main problem is that it is difficult to compare the biotinylation data with most published quantitative data on the EGFR endocytosis that were obtained using radiolabeled EGF.

2) The involvement of Shc demonstrated in new experiments does not help to address the dilemma of the model. Even if Shc is involved, Grb2 would bind to Shc using its SH2 domain and, therefore, would not be able to use the same SH2 domain to bind phosphorylated Tom1L. Here again, it has been previously shown in several papers that Shc binding sites in the EGFR are not important for internalization, and inability to compare the data using the same assay would result in the situation that the question about the involvement of Shc endocytosis will remain open. In fact, the effect of Shc siRNA is evident in Fig. 2 but not apparent in the supplemental figure. Likewise, the effect of PP1 (inhibitor of Src) is not apparent in the supplemental figure. The authors refer to one paper that used high concentrations of PP1 (inhibiting EGFR kinase as well) but do not cite the studies where cells lacking all Srk family kinases normally internalized EGF.

3) The revised images do not demonstrate that Tom1L is recruited into clathrin coated pits. The immunofluorescence staining is not typical of clathrin coated pits and, probably, mostly shows intracellular clathrin. There is some overlap but no clear co-localization of the pattern of dots in images of both A431 and Hela cells.

2nd Revision - authors’ response 16 August 2009

Point-to-point responses to reviewer 1’s comments

The revised manuscript contains many new experiments that support the role of Tom1L in EGFR endocytosis. Overall, when analyzed apart from the EGFR literature, this study is indeed a convincing demonstration of the function of Tom1L in EGFR internalization. However, the major points raised in the previous critique have not been addressed.

We are happy that the reviewer found our study cohesive and convincing enough in demonstrating a role of Tom1L1 in ligand-stimulated endocytosis of EGFR. Since the literature in the field of EGFR
endocytosis has been rather confusing and sometimes controversial due to the presence of many papers reporting the role of various proteins in this process, the clear demonstration of Tom1L1 by our study is thus significant and impactful. Despite the controversy in the literature, the most recent review by Sorkin and Goh (Exp Cell Res. 2009, 315, 683-696) offers rather up-to-date summary and informed perspective of the field and our conclusions fit well with the balanced discussions in this review.

1) Measurement of endocytosis of occupied EGFR (using radiolabeled EGF) has been utilized in more than 300 papers. It is very well established that there is no uptake of radioEGF in the absence of the receptor (as the authors suggest) and there is very minimal internalization if the receptor kinase activity is blocked. Although the authors argue that biotinylation is the appropriate technique to measure the EGFR endocytic rates, this protocol involves chemical modification of EGF and other membrane proteins prior to receptor stimulation and endocytosis, and a temperature shift from 4oC to 37oC. It is unclear how these conditions would affect EGF binding and endocytosis. Also, reverse biotinylation measures the internalization of the total surface receptor pool rather than the endocytosis of EGF-occupied, activated receptors. Thus, while reverse surface biotinylation is a legitimate internalization assay, the main problem is that it is difficult to compare the biotinylation data with most published quantitative data on the EGFR endocytosis that were obtained using radiolabeled EGF.

Thanks for raising this point again. We believe the results obtained using surface biotinylation are valid for several reasons. Firstly, as mentioned previously, this approach is widely used to selectively mark surface-exposed proteins and no alterations of trafficking or function have been reported, as biotin is a small (244 Da) naturally occurring vitamin and has been conjugated to many proteins without altering their biological activities (Elia G. Proteomics 2008, 8, 4012-4024).

Secondary, a similar approach was used to monitor EGF-induced endocytosis of EGFR (Choi et al., J. Cell Sci. 2004, 117, 4209-4218). We have cited these two manuscripts in the revised text of our manuscript by adding the following sentence "---as biotinylation is widely used to selectively mark surface-exposed proteins including EGFR without reported alterations of their trafficking or function (Choi et al, 2004; Elia, 2008)". Furthermore, the results obtained using surface-biotinylation (Fig. 5B and 5C) were supported by an independent method: immunofluorescence microscopy using anti-EGFR antibody, which does not involve surface biotinylation (Fig.5A).

After stimulation with EGF for 20 min, the majority of EGFR was detected in intracellular vesicular structures, with some being detected on the surface in A431 cells (Figure 5A, panel d). In contrast, EGFR was predominantly detected on the cell surface in cells expressing Tom1L1/Y460F mutant (panel p). Furthermore, the analysis of the effect of the various Tom1L1 mutants on the degradation of Texas-red EGF, presented in Fig 4A, also showed that Tom1L1 mutant defective in Tyr-phosphorylation and/or interaction with Grb2 inhibited endocytosis/degradation of the ligand. The results on ligand-induced degradation of EGF without being biotinylated (Fig 4B and 4C) also support the conclusions reached by surface biotinylation. Therefore, results acquired from several independent assays without biotinylating EGFR are consistent with the results obtained using surface-biotinylation to directly monitor the internalization of EGFR.

2) The involvement of Shc demonstrated in new experiments does not help to address the dilemma of the model. Even if Shc is involved, Grb2 would bind to Shc using its SH2 domain and, therefore, would not be able to use the same SH2 domain to bind phosphorylated Tom1L. Here again, it has been previously shown in several papers that Shc binding sites in the EGFR are not important for internalization, and inability to compare the data using the same assay would result in the situation that the question about the involvement of Shc endocytosis will remain open. In fact, the effect of Shc siRNA is evident in Fig. 2 but not apparent in the supplemental figure. Likewise, the effect of PP1 (inhibitor of Src) is not apparent in the supplemental figure. The authors refer to one paper that used high concentrations of PP1 (inhibiting EGFR kinase as well) but do not cite the studies where cells lacking all Src family kinases normally internalized EGF.

In our study, Grb2 and Shc have additive role in mediating interaction of Tom1L1 with EGFR. Our results are most consistent with a role of Grb2-Shc complex in mediating an interaction of Tom1L1 with EGFR. Beside the well-known interaction between Shc phosphotyrosine and Grb2 SH2 domain, Shc, via its NH2-terminal proline-rich collagen homology 2 (CH2), can also bind to the C-terminal SH3 domain of Grb2 (Khendant et al, 2006, 172, 817-822), leaving the SH2 domain of Grb2 available for interacting with other proteins such as Tom1L1. We have cited this paper by...
adding the sentence in the discussion "---and the interaction between Grb2 SH3 domain and N-terminal pro-rich region of Shc (Khanday et al, 2006) also supports our hypothesis". Although our results are most consistent with the role of Src-phosphorylated Tom1L1 in the context of Tom1L1-Grb2-Shc-EGFR complex in endocytosis of EGFR, it remains possible that endocytosis of EGFR may also occur independent of Shc or Src kinases under some conditions. Future studies are needed to really define the various routes and underlying molecular machinery involved in EGFR endocytosis under various experimental or physiological conditions.

In our study, the importance of Grb2 and Shc in mediating the interaction of endogenous Tom1L1 with EGFR was supported by the observation that EGF-induced co-immunoprecipitation of EGFR with Tom1L1 was significantly compromised in cells transfected with siRNA to knockdown the expression of Grb2 (Figure 2D, Lane 4) or Shc (Figure 2D, Lane 5). Simultaneous knockdown of both Grb2 and Shc led to robust inhibition of Tom1L1-EGFR interaction (Figure 2D, Lane 6), as compared to cells transfected with non-targeting siRNA (Figure 2D, Lane 3). Moreover, in Supp Fig 7C, it is clearly demonstrated that knockdown of Grb2 or Shc reduced the endocytosis of EGFR to some extent, while knockdown of both Grb2 and Shc reduced EGFR endocytosis dramatically. These results indicate that Shc and Grb2 are both involved in Tom1L1-mediated endocytosis of EGFR and our working model is most consistent with these results. Quantitative densitometric analysis was performed on immunoblots shown in Supp Fig. 7B, and the percentage of EGFR endocytosed with (without) PP1 treatment, is 33 (61)%, 56 (76)% 79 (90)% at 2, 5, 20 min, respectively. Therefore, in our system, treatment with PP1, a Src family kinase inhibitor, clearly reduced the endocytosis of EGFR.

3). The revised images do not demonstrate that Tom1L is recruited into clathrin coated pits. The immunofluorescence staining is not typical of clathrin coated pits and, probably, mostly shows intracellular clathrin. There is some overlap but no clear co-localization of the pattern of dots in images of both A431 and Hela cells.

We have repeated the co-localization experiments several times (the basis for the slight delay in submitting this revised manuscript) and similar results were obtained. Although we are not able to provide better images than those presented in Fig 3B and S4D, it is our opinion that these images do show noticeable co-localization Tom1L1 with clathrin and EGFR. These results are supportive to the conclusions derived from biochemical results. Since clathrin is distributed in many cellular structures such as the TGN and endosomes in addition to clathrin-coated pits/vesicles, noticeable but not complete co-localization is expected. We have softened our description in the text by modifying the sentence "Tom1L1 was initially detected in the cytoplasm but was observed to be enriched on the plasma membrane after a 2-5 min treatment with EGF (Figure 3A) and co-localized with EGFR and clathrin in A431 cells (Figure 3B)." into "---co-localized noticeably with EGFR and clathrin in A431 cells (Figure 3B)."