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CYLD negatively regulates cell cycle progression by inactivating HDAC6 and increasing the levels of acetylated tubulin

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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 02 July 2009

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are enclosed. As you will see, the referees vary significantly in their degree of enthusiasm for the paper. While referees 2 and 3 are broadly more positive, although still raise significant concerns with the current manuscript, referee 1 is rather negative, arguing that much of the data confirms previous reports of CYLD in regulating microtubule dynamics. We can appreciate that your data does go significantly beyond the pre-existing literature - in identifying the HDAC6 interaction, you provide an additional layer of regulation in the system. Therefore, and having discussed your submission with my colleagues and our Executive Editor, Pernille Rørth, we are prepared to overlook the negative opinion of referee 1, and to invite you to revise your manuscript according to the comments of referees 2 and 3. I will not go through all the points here, but I would in particular like to draw your attention to the comments of the referees regarding the cytokinesis defects, which would benefit from further analysis. In addition, the referees point out a number of blots where the quality needs to be improved. Finally, I would highlight the comments of referee 3 on the referencing of your manuscript. I hope you are aware of our new policy by which we have removed any reference limit (please see our website for further details), so you should be able to incorporate further relevant citations.

I would therefore like to invite you to submit a revised version of the manuscript, addressing all the comments of all three reviewers (including responding to the more technical concerns of referee 1). I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses 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

REFEREE REPORTS

Referee #1 (Remarks to the Author):

Wickstrom et al, have investigated the role of CYLD in the regulation of microtubule dynamics and cell cycle progression. The authors showed that CYLD interacts preferentially with polymerized tubulin via its two amino terminal CAP-Gly domains and stabilizes microtubules. The interaction of CYLD with microtubules was associated with increased a-tubulin acetylation and inhibition of HDAC6. The authors provide evidence that suggest a direct mechanism for the inhibition of HDAC6 by CYLD which is based on the binding of the CAP-Gly1 domain of CYLD to the catalytic domains of HDAC6. Finally, the authors provide evidence that implicate the deubiquitinating activity of CYLD in a delay in the G1/S transition of the cell cycle and the CAP-Gly1 domain of CYLD in a reduction of the rate of cytokinesis in TPA-treated primary keratinocytes or melanoma cells. This report contains a significant amount of data that confirm previous findings on the role of CYLD in microtubule dynamics (Gao et al, J Biol Chem, 283: 8802-8809, 2008), whereas the findings on the role of CYLD in cell cycle regulation are controversial and suggestive at best.
The following issues must be addressed:

A previous publication (Stegmeier et al., PNAS 104:8869-8874, 2007) has shown that downregulation of CYLD in HeLa cells causes a delay in G2/M transition of the cell cycle. How do the authors reconcile their findings with the findings of Stegmeier at al.?

The downregulation of HDAC6 in CYLD-/- keratinocytes causes an increase in the levels of total tubulin which parallels the increase in the levels of acetylated tubulin (Fig. 5D and 5E). Therefore it is not possible to conclude that HDAC6-depeleted CYLD-/- keratinocytes show an increase in alpha-tubulin acetylation.

In order to support the point that the deubiquitinating activity of CYLD is important for the delay in G1/S transition of the cell cycle the authors should show that expression of a catalytically inactive point mutant of CYLD in CYLD-/- keratinocytes or melanoma cells does not cause a delay in G1/S transition.

Similarly, in order to support the argument that CAP-Gly1 is essential for the delay in cytokinesis the authors should show that expression of a CYLD point mutant with defective CAP-Gly1 in CYLD-/- keratinocytes or melanoma cells does not reduce the rate of cytokinesis.

In figures 1A and 3D the authors should show more cells.

Referee #2 (Remarks to the Author):

The authors pursue their study of the role of the CylD tumor suppressor gene, which encodes a deubiquitinase and acts as an inhibitor of NF-κB activity. They previously demonstrated that CylD inhibits cell proliferation by preventing nuclear translocation of the Bcl3 protein (through deubiquitination) and thus preventing upregulation of the cyclin D1 gene. Here they show that CylD controls cell growth and mitotic division by associating with alpha-tubulin. They show that CylD binds and inhibits HDAC6, leading to increased levels of acetylated tubulin, and to a delay in G1/S transition. CylD also inhibits HDAC6 at the midbody, thus regulating cytokinesis, although this role is independent of the CylD catalytic activity.
These results are important and deserve publication, assuming the following points are adequately addressed :

figure 1A : colocalization in the +TPA conditions is difficult to assess, as basically the entire cytoplasm is labeled. Is it possible to quantify this colocalization?
figure 1B : there is almost as much CylD coprecipitating with tubulin in the IgG lane as in the lane where anti-tubulin has been used. A better experiment should be presented. What is the difference between IgG and Cont.?
co-IP's seem to use an anti-pan-tubulin. Does CylD coprecipitate with acetylated tubulin?
figure 3D, +TSA panel : the nucleus seems to be stained, and the only non-stained area seems to be the perinuclear region. I guess this is not what the authors want to demonstrate.
figure 3E : why does the amount of Bcl3 in the second panel vary under the different conditions? This casts doubt on the validity of the IP Bcl3/blot CylD experiments.
figure 4B : in the HDAC6 lanes, lane 1 and 2 seem to be fused into a single one. A better gel should be presented.
figure 5A : I am not sure to understand how this experiment has been done : is it all in vitro with recombinant proteins? What is the role of taxol? Why is a CylD band visible in lanes 1-3, if it has not been added to the mixture (if I understood correctly)? If this represents the background of the in vitro translation mix, then the experiment cannot be done under these conditions. Why don't the authors use recombinant CylD?
figure 6F : the authors write : 'These results suggest that deubiquitination of Bcl-3 but not the inhibition of HDAC6 by CYLD regulates the observed effects in G1/S transition' They should confirm this hypothesis using a mutant of CylD that no longer binds HDAC6.

Other points : page 6, bottom : the authors write : 'These results suggest that CYLD co-localizes mainly with acetylated MTs in the perinuclear region, and that this localization is constitutive in melanoma cells'. This is a bit weird as there is no CylD in melanoma cells, except when articially introduced.

Referee #3 (Remarks to the Author):

In the present work Wickstrom et al. show that the de-ubiquitinating enzyme CYLD associates with microtubules in both primary keratinocytes activated by TPA and malignant melanoma cells transfected/infected with CYLD constructs. This interaction is mediated by the first and second N-terminal CAP-Gly domains of CYLD. They show that this molecule also co-localizes with acetylated tubulin and its activation by TPA in keratinocytes or its expression in melanoma cells increase the level of acetylated tubulin through the interaction of the N-terminal domain and the catalytic domains of HDAC6. Authors show that this interaction inhibits HDAC6 mediated tubulin acetylation and claim that this inhibition allows CYLD to translocate to the perinuclear region. In this location CYLD induces a delay in the G1/S transition phase of the cell cycle via de-ubiquitination of Bcl3 independently of its interaction with HDAC6. CYLD is also found in the midbody of dividing cells where it co-localizes with HDAC6 and regulates the rate of cytokinesis in a HDAC6 dependent manner.

The work presented clearly establishes the interaction between CYLD and both microtubules, and HDAC6. It also clearly maps the domains within both molecules that mediate the interactions and shows that interaction of CYLD with HDAC6 negatively regulates its activity. Although the interaction and regulation is clear its functional relevance in the cellular system presented remains unclear. The authors make some claims that are not substantiated by the data presented and should be clarified. Major issues to be addressed are:

1) Authors state that the levels of acetylated tubulin regulate the sub-cellular localization of CYLD based on data presented in figure 3D in which they claim that in the presence of TSA CYLD translocates to the perinuclear region. The images in this figure do not show a clear perinuclear localization. In TSA treated cells CYLD staining shows an extensive area around the nucleus with no enrichment in any particular area. Since this staining is all we can see and no phase contrast images of the cells are shown, is difficult to say that this staining is perinuclear. Moreover in these images the nucleus seems to show staining. Better confocal images should be provided to claim perinuclear localization in TSA treated cells.

2) It is unclear how acetylated tubulin could cause perinuclear localization of CYLD. Figure 1 shows good binding of this molecule to tubulin in keratinocytes and melanocytes without TPA

treatment and low level of acetylation. Binding should be checked in TSA treated cells compared with untreated cells to test whether tubulin acetylation affects CYLD binding.

3) It is claimed that the so- called tubulin acetylation-mediated perinuclear localization of CYLD is responsible for Bcl3 retention in the perinuclear region. However figure 3E clearly shows that in TSA-treated cells tubulin acetylation on its own does not induce CLYD/Bcl3 association. TPA is required. It seems that TPA activation of CYLD has an effect on Bcl3 binding (Fig. 3E) and CYLD localization (Fig. 1A vs Fig. 3D) not mimicked by TSA-induced acetylation. To clarify this, both CYLD localization and binding to Bcl3 should be analyzed in keratinocytes treated with TPA and interfered for HDAC6.

4) In vitro regulation of HDAC6 activity by CYLD is analyzed by an assay in which taxol-treated purified microtubules are incubated with purified HDAC6 and in vitro translated CYLD. It was reported in the literature (EMBO J. 2002. 21: 6820-31) that, to de-acetylate tubulin, HDAC6 needs polymerized tubulin to be able to de-polymerize. Taxol-treated microtubules are "fixed" in a state in which they are not able to de-polymerize. Authors' description of the assay in Material and Methods is very brief. They should provide a more detailed explanation of the assay and clarify the discrepancy between the above mentioned published data and their clear in vitro results

Minor comments.

Description of the different construct of CYLD used in transfection and infection/transduction experiment is brief and scarce. Figure legends also give limited information about the constructs used in each case and it is difficult to know which construct they are using in each transfection, and whether they are using lentiviral o retroviral constructs in the transduction experiments. Authors should make a clearer description in Material and Methods and Figure Legends.

Authors should be more careful with the elaboration of the Reference section. Several papers quoted throughtout the text (Creppe 2009; Kawaguchy 2003; Viatour 2004) are missing in the list.

Authors should discuss and include not only a single reference for HDAC6 deacetylase activity role in cell migration. This is misleading since the HDAC6 adapter role has been reported to be critical for migration of several cell types (Cabrero et al., Mol Biol Cell 2006; Gao et al., Mol Cell Biol 2007).

The use of an HDAC6 specific chemical inhibitor such as tubacin in some of the experiments, instead of the broad-specificity TSA would be helpful in terms of specificity.

1st Revision - authors' response 16 September 2009

Reviewer #1:

1. A previous publication (Stegmeier et al., PNAS 104:8869-8874, 2007) has shown that downregulation of CYLD in HeLa cells causes a delay in G2/M transition of the cell cycle. How do the authors reconcile their findings with the findings of Stegmeier at al.?

The reason why we do not observe a difference in G2/M transition is not clear at the moment. We believe that this could be either a tissue/ cell type-specific effect or a result of the siRNA mediated downregulation used by Stegmeier et al. compared to constitutive knock out used by us. We have now included the following text into the Discussion section on p21: "This is in contrast to an earlier study where downregulation of CYLD by siRNA in HeLa cells induced a delay in G2/M transition of the cell cycle (Stegmeier et al., 2007). This discrepancy could be explained by different functions of CYLD depending on the tissue/cell type in combination with the requirement of a specific stimulus such as TPA and/or by siRNA-mediated downregulation of CYLD versus complete absence of the protein"

2. The downregulation of HDAC6 in CYLD-/- keratinocytes causes an increase in the levels of total tubulin which parallels the increase in the levels of acetylated tubulin (Fig. 5D and 5E). Therefore it is not possible to conclude that HDAC6-depeleted CYLD-/- keratinocytes show an increase in

alpha-tubulin acetylation.

We have repeated this experiment several times and do not observe an increase in total tubulin in response to HDAC6 downregulation. We have now replaced Figs. 5D and 5E with more representative experiments where the level of total tubulin is unchanged while the level of acetylated tubulin is increased (now Fig. 5E and F).

3. In order to support the point that the deubiquitinating activity of CYLD is important for the delay in G1/S transition of the cell cycle the authors should show that expression of a catalytically inactive point mutant of CYLD in CYLD-/- keratinocytes or melanoma cells does not cause a delay in G1/S transition.

We analysed the duration in the cell cycle in melanoma cells expressing full length CYLD, the catalytically inactive CYLD-C/S as well as an N-terminal truncation mutant which lacks the first CAP-Gly domain and is thus incapable of binding HDAC6. The results, presented in the new Fig. 6G, show that full length CYLD but neither of the mutants induces a delay in G1/S transition. This suggests that both the catalytical activity and the perinuclear localization of CYLD are required for this effect.

4. Similarly, in order to support the argument that CAP-Gly1 is essential for the delay in cytokinesis the authors should show that expression of a CYLD point mutant with defective CAP-Gly1 in CYLD-/- keratinocytes or melanoma cells does not reduce the rate of cytokinesis.

We thank the reviewer for the suggestion. A triple mutation in the CAP-Gly domain of Alf1p, which is a CLIP-170 domain-containing protein was shown to abolish tubulin binding (Feierbach et al., J Cell Biol. 1999 144:113-24). We constructed similar mutations within the CAP-Gly1 domain of CYLD by mutating the GFTDG sequence into AFADA and analysed the duration of cytokinesis in melanoma cells transfected with this mutant. The results of these experiments, shown in the new Fig. 7C, confirm that the first CAP-Gly domain of CYLD is required for its effect on cytokinesis.

5. In figures 1A and 3D the authors should show more cells.

Showing single cells with high magnification (100x) is necessary to clearly visualize microtubular structures, which are lost in lower magnification.

Reviewer #2:

These results are important and deserve publication, assuming the following points are adequately addressed

The authors thank the reviewer for the suggestions which helped us to better highlight the molecular mechanism underlying the function of CYLD.

1. figure 1A : colocalization in the +TPA conditions is difficult to assess, as basically the entire cytoplasm is labeled. Is it possible to quantify this colocalization?

We have measured the co-localization between CYLD and acetylated tubulin by linescan analyses using the Metamorph image analysis software. These analyses confirm that CYLD and acetylated -tubulin extensively co-localize in TPA-treated cells (Figure 1A right panel). In addition, immunofluorescent stainings of CYLD together with either tyrosinated tubulin (Supplementary figure 1 lower panels) or actin (see Massoumi et al., Cell 2006) in TPA-treated cells demonstrate that CYLD translocates from the peripheral cytoplasm to the perinuclear area in response to TPA treatment.

2. figure 1B : there is almost as much CylD coprecipitating with tubulin in the IgG lane as in the lane where anti-tubulin has been used. A better experiment should be presented. What is the difference between IgG and Cont.? co-IP's seem to use an anti-pan-tubulin. Does CylD coprecipitate with acetylated tubulin?

- We have replaced the anti-tubulin co-immunoprecipitation blot with a higher quality blot (Fig 1B,

right panels). In addition, we have followed the suggestion of the reviewer and analysed the interaction between CYLD and acetylated tubulin. The results of these experiments show that CYLD associates with acetylated tubulin upon TPA treatment (new Fig. 2B).

- The "IgG" label corresponds to the immunoprecipitation performed with pre-immune serum while "control" corresponds to immunoprecipitation using anti-CYLD or anti-tubulin antibody. To prevent confusion we have replaced the "control" labelling in Fig. 1B, 1C, 1D, and 2B with "anti-CYLD", "anti-tubulin" or "anti-acetylated tubulin".

3. Figure 3D, +TSA panel : the nucleus seems to be stained, and the only non-stained area seems to be the perinuclear region. I guess this is not what the authors want to demonstrate.

We apologize for the quality of the immunofluorescence image. We have now replaced this panel with a better image (new Fig. 3F, lower panels).

4. figure 3E : why does the amount of Bcl3 in the second panel vary under the different conditions? This casts doubt on the validity of the IP Bcl3/blot CylD experiments.

The reviewer is correct in that there was variation in the amount of Bcl-3 in the experiment presented in the previous version of the manuscript. We have now replaced this experiment with a new set of experiments where the level of Bcl-3 immunoprecipitation is unchanged under different conditions (new Fig. 3G).

5. figure 4B : in the HDAC6 lanes, lane 1 and 2 seem to be fused into a single one. A better gel should be presented.

We have now replaced this panel with an experiment of higher quality.

6. figure 5A : I am not sure to understand how this experiment has been done : is it all in vitro with recombinant proteins? What is the role of taxol? Why is a CylD band visible in lanes 1-3, if it has not been added to the mixture (if I understood correctly)? If this represents the background of the in vitro translation mix, then the experiment cannot be done under these conditions. Why don't the authors use recombinant CylD?

The reviewer is correct and has understood the experiment correctly. In this experiment taxol was used to prevent depolymerisation of microtubules. The CYLD band in lanes 1-3 was a background band derived from the reticulocyte lysate (in vitro translation mix). We agree with the reviewer that using purified recombinant CYLD protein from bacteria/insect cells would be the best choice in our assay. However, so far no research group including ours has reported successful production of recombinant full length CYLD protein. To overcome the problem with the background from the reticulocyte lysate, we have taken another approach for the in vitro tubulin deacetylation assay:

In the new experiment microtubule-associated protein (MAP)-enriched tubulin (cytoskeleton) was polymerised into microtubules in the absence of taxol or glycerol by incubation for 30 min at 35^{o}C. The polymerised microtubules were subsequently incubated with HDAC6 and/or CYLD immunoprecipitates in the absence or presence of TSA at 37^{o}C for 2 hours. Samples were then placed on ice for 15 minutes to depolymerise the microtubules and the supernatant was collected by centrifugation and analysed by immunoblotting. The results of these experiments show that the presence of TSA or CYLD protects tubulin from HDAC6-mediated deacetylation (new Fig. 5A).

To confirm that the presence of CYLD is critical for HDAC6 inhibition, we transfected COS cells with increasing concentrations of FLAG-tagged full length CYLD (0.5, 1.0 and 3,0 μg) or HA-tagged full length HDAC6 (1.0 μg). The polymerised microtubules were then incubated in the presence of FLAG-CYLD and/or HA-HDAC6 immunoprecipitates (after elution using FLAG or HA-peptide) at 37^{o}C for 2 hours. Samples were placed on ice for 15 minutes and the supernatant was collected by centrifugation and analysed by immunoblotting. The results of these experiments show that HDAC6-mediated deacetylation of tubulin is inhibited by CYLD in a dose-dependent manner (new Fig. 5B).

Finally, to determine the precise domain of CYLD which is responsible for this effect, we transfected

COS cells with wild type or truncation mutants of FLAG-CYLD or HA-HDAC6 constructs. Polymerised microtubules were then incubated in the presence of FLAG-CYLD and/or HA-HDAC6 immunoprecipitates (after elution using FLAG or HA-peptide) at 37^{o}C for 2 hours, after which tubulin acetylation was analysed as described above (new Fig. 5C).

These results collectively confirm that CYLD prevents deacetylation of microtubules in vitro by inhibiting HDAC6 activity.

7. figure 6F : the authors write : 'These results suggest that deubiquitination of Bcl-3 but not the inhibition of HDAC6 by CYLD regulates the observed effects in G1/S transition' They should confirm this hypothesis using a mutant of CylD that no longer binds HDAC6.

We analysed the duration of the cell cycle in melanoma cells expressing full length CYLD, the catalytically inactive CYLD-C/S as well as an N-terminal truncation mutant which lacks the first CAP-Gly domain and is thus incapable of binding HDAC6. The results, presented in the new Fig. 6G, show that full-length CYLD but neither of the mutants induces a delay in G1/S transition. This suggests that although inhibition of HDAC6 is not sufficient to induce a delay in the cell cycle, it facilitates the localization of CYLD to the perinuclear region of the cell. This further enables the interaction of CYLD and Bcl-3, which leads to the deubiquitination of Bcl-3 by CYLD and a subsequent delay in the cell cycle.

8. Other points : page 6, bottom : the authors write : 'These results suggest that CYLD co-localizes mainly with acetylated MTs in the perinuclear region, and that this localization is constitutive in melanoma cells'. This is a bit weird as there is no CylD in melanoma cells, except when articially introduced.

The reviewer is correct. We now changed the text on p. 7 to clarify this point: "... and this localization is constitutive in CYLD transduced melanoma cells,"

Reviewer #3:

The work presented clearly establishes the interaction between CYLD and both microtubules, and HDAC6. It also clearly maps the domains within both molecules that mediate the interactions and shows that interaction of CYLD with HDAC6 negatively regulates its activity. Although the interaction and regulation is clear its functional relevance in the cellular system presented remains unclear. The authors make some claims that are not substantiated by the data presented and should be clarified. Major issues to be addressed are:

The reviewer is raising important points, which we have addressed in the revised version of our manuscript. We have performed several additional experiments and also changed the text of the manuscript in order to more precisely highlight the molecular mechanism underlying the function of CYLD in HDAC6 inhibition and subsequent tubulin acetylation.

1. Authors state that the levels of acetylated tubulin regulate the sub-cellular localization of CYLD based on data presented in figure 3D in which they claim that in the presence of TSA CYLD translocates to the perinuclear region. The images in this figure do not show a clear perinuclear localization. In TSA treated cells CYLD staining shows an extensive area around the nucleus with no enrichment in any particular area. Since this staining is all we can see and no phase contrast images of the cells are shown, is difficult to say that this staining is perinuclear. Moreover in these images the nucleus seems to show staining. Better confocal images should be provided to claim perinuclear localization in TSA treated cells.

We apologize for the quality of the immunofluorescence image. We have repeated and replaced this experiment with a new image (Fig. 3F, lower panels).

2. It is unclear how acetylated tubulin could cause perinuclear localization of CYLD. Figure 1 shows good binding of this molecule to tubulin in keratinocytes and melanocytes without TPA treatment and low level of acetylation. Binding should be checked in TSA treated cells compared with untreated cells to test whether tubulin acetylation affects CYLD binding.

We thank the reviewer for the suggested experiment which is presented as the new Supplementary Fig. 3E of the revised manuscript. We found that TSA treatment of keratinocytes caused an increase in the interaction of CYLD with tubulin compared to untreated cells. This result suggests that tubulin acetylation induced by TSA enhances the association of CYLD with microtubules.

3) It is claimed that the so- called tubulin acetylation-mediated perinuclear localization of CYLD is responsible for Bcl3 retention in the perinuclear region. However figure 3E clearly shows that in TSA-treated cells tubulin acetylation on its own does not induce CLYD/Bcl3 association. TPA is required. It seems that TPA activation of CYLD has an effect on Bcl3 binding (Fig. 3E) and CYLD localization (Fig. 1A vs Fig. 3D) not mimicked by TSA-induced acetylation. To clarify this, both CYLD localization and binding to Bcl3 should be analyzed in keratinocytes treated with TPA and interfered for HDAC6.

The reviewer is correct in that tubulin acetylation alone does not seem to be sufficient to induce the interaction between CYLD and Bcl-3, whereas an intact microtubule network is required both for the perinuclear localization as well as Bcl-3 binding (Fig. 3G). This is in agreement with the result that depletion of HDAC6 alone does not induce a delay in the cell cycle (Fig. 6F). To further clarify this issue we have carried out the experiments suggested by the reviewer and analysed the interaction between CYLD and Bcl-3 as well as the localization of CYLD in HDAC6-depleted cells. The results, presented in the new Fig. 3H and Supplementary Fig. 3D, respectively, show that HDAC6 depletion induces co-localization of CYLD with acetylated microtubules both with and without TPA, but does not induce an interaction between CYLD and Bcl-3. We have changed the discussion section on p. 21 to more accurately describe our findings.

4) In vitro regulation of HDAC6 activity by CYLD is analyzed by an assay in which taxol-treated purified microtubules are incubated with purified HDAC6 and in vitro translated CYLD. It was reported in the literature (EMBO J. 2002. 21: 6820-31) that, to de-acetylate tubulin, HDAC6 needs polymerized tubulin to be able to de-polymerize. Taxol-treated microtubules are "fixed" in a state in which they are not able to de-polymerize. Authors' description of the assay in Material and Methods is very brief. They should provide a more detailed explanation of the assay and clarify the discrepancy between the above mentioned published data and their clear in vitro results

The reviewer is correct. It was reported earlier (EMBO J. 2002. 21: 6820-31), that taxol-treated microtubules are "fixed" in a state in which they are not able to depolymerise. However, in the same study a more active HDAC6 protein isolated from mouse testis was found to induce some degree of deacetylation even in the presence of taxol.
Based on the criticism from the reviewer as well as from reviewer #2 (see above), we have removed this experiment from the manuscript, and used an alternative assay to analyse tubulin acetylation in vitro:

In the new experiment microtubule-associated protein (MAP)-enriched tubulin (cytoskeleton) was polymerised into microtubules in the absence of taxol or glycerol by incubation for 30 min at 35^{o}C. The polymerised microtubules were subsequently incubated with HDAC6 and/or CYLD immunoprecipitates in the absence or presence of TSA at 37^{o}C for 2 hours. Samples were then placed on ice for 15 minutes to depolymerise the microtubules and the supernatant was collected by centrifugation and analysed by immunoblotting. The results of these experiments show that the presence of TSA or CYLD protects tubulin from HDAC6-mediated deacetylation (new Fig. 5A).

To confirm that the presence of CYLD is critical for HDAC6 inhibition, we transfected COS cells with increasing concentrations of FLAG-tagged full length CYLD (0.5, 1.0 and 3,0 μg) or HA-tagged full length HDAC6 (1.0 μg). The polymerised microtubules were then incubated in the presence of FLAG-CYLD and/or HA-HDAC6 immunoprecipitates (after elution using FLAG or HA-peptide) at 37^{o}C for 2 hours. Samples were placed on ice for 15 minutes and the supernatant was collected by centrifugation and analysed by immunoblotting. The results of these experiments show that HDAC6-mediated deacetylation of tubulin is inhibited by CYLD in a dose-dependent manner (new Fig. 5B).

Finally, to determine the precise domain of CYLD which is responsible for this effect, we transfected COS cells with wild type or truncation mutants of FLAG-CYLD or HA-HDAC6 constructs. Polymerised microtubules were then incubated in the presence of FLAG-CYLD and/or HA-HDAC6

immunoprecipitates (after elution using FLAG or HA-peptide) at 37°C for 2 hours, after which tubulin acetylation was analysed as described above (new Fig. 5C).

These results collectively confirm that CYLD prevents deacetylation of microtubules in vitro by inhibiting HDAC6 activity.

We apologize for the brief description of the assay in the Material and Methods section. We have now re-written this part to provide more details.

Minor comments.

5. Description of the different construct of CYLD used in transfection and infection/transduction experiment is brief and scarce. Figure legends also give limited information about the constructs used in each case and it is difficult to know which construct they are using in each transfection, and whether they are using lentiviral o retroviral constructs in the transduction experiments. Authors should make a clearer description in Material and Methods and Figure Legends.

We have extensively edited the Figure legends as well as the Materials and methods sections to provide more details on the transfection and infection/transduction experiments.

6. Authors should be more careful with the elaboration of the Reference section. Several papers quoted throughtout the text (Creppe 2009; Kawaguchy 2003; Viatour 2004) are missing in the list.

We apologize for these mistakes which have now been corrected.

7. Authors should discuss and include not only a single reference for HDAC6 deacetylase activity role in cell migration. This is misleading since the HDAC6 adapter role has been reported to be critical for migration of several cell types (Cabrero et al., Mol Biol Cell 2006; Gao et al., Mol Cell Biol 2007).

We have included these critical references.

8. The use of an HDAC6 specific chemical inhibitor such as tubacin in some of the experiments, instead of the broad-specificity TSA would be helpful in terms of specificity.

We thank the reviewer for the suggestion. In our new Fig. 3C we show the effect of tubacin in the absence or presence of TPA using primary keratinocytes. In the absence of TPA, tubacin increases the level of acetylated tubulin to the same extent as TPA. In addition, we carried out similar experiments using EGFP and EGFP-CYLD expressing melanoma cells (new Fig. 3E), where tubacin induced an increase in tubulin acetylation of EGFP-expressing cells comparable to the levels induced by EGFP-CYLD.

Additional Correspondence 05 October 2009

Many thanks for submitting the revised version of your manuscript EMBOJ-2009- 71504R. It has now been seen again by referee 3, who finds that the revision addresses most of the concerns raised in the initial round of review, and who is now supportive of publication. However, as you will see from the comments attached below, he/she raises a couple of minor concerns to be addressed before we can accept the manuscript for publication.

In terms of the referee's request that you show HDAC6 expression and tubulin acetylation in panel 3H, I do recognise that you address the effects of HDAC6 knockdown on tubulin acetylation status in figure 5, and would not insist on your including these data here. However, I can see that showing the efficiency of HDAC6 knockdown in this panel might be useful, and would therefore like to give you the option of including these data here, should you have the blots or be able to produce them.

Clearly the labelling of Supplementary Figure 3 needs to be corrected as highlighted by the referee. Again, I can recognise the value of showing the colocalisation of CYLD and Bcl3 in this figure, but

do not think it essential.

I therefore leave it up to you whether you wish to include these additional data, but would minimally ask that you correct the labelling as mentioned above. I suggest that the easiest way for you to proceed would be for you to modify the supplementary material PDF, and to send it to us by e-mail. We can then upload it in place of the previous version. If you do want to make changes to figure 3 (and to the figure legends), you can also send me the updated versions by email. This way, you will not have to go through a formal revision process and it should make things easier at your end.

Referee 3 (remarks to author):

In the present form of the revised manuscript Wickstrom et al. have performed new experiments to reply to the criticisms raised by the reviewers, and they include substantial new data that address all their major concerns about the work. Therefore the manuscript is suitable for publication providing some minor issues about some of the figures are addressed:

Figure 3. Panel H. It should include western blot data from cell lysates showing HDAC6 expression, and tubulin acetylation.

Supplementary figure 3. Panel D. The two pictures in the middle panel are labelled as HDAC6, whereas the figure legends indicate that the cells have been stained for acetylated tubulin. The pictures should be correctly labelled. This panel would be greatly improved if the authors could also show colocalization of CYLD and Bcl-3.

Additional Correspondence 06 October 2009

I would like to thank you in advance for your time and for handling of our manuscript as well as your positive decision.

We have now included necessary changes regarding the minor concerns raised by referee 3 which include:
1. The effects of HDAC6 knockdown in Fig. 3H: You are absolutely correct that we show the efficiency of the effects of HDAC6 knockdown on tubulin acetylation status in keratinocytes in figure 5. However we decided to show HDAC6 knockdown efficiency in this figure as well. We did not have blot from this experiment but we had samples left. After running those samples on the gel we could show knockdown efficiency of HDAC6. This new blot is included in Fig. 3H lower panel. Please see attached our new Fig. 3 (PDF). Wikstrom_Fig3_revised2.

2. We have included "and knockdown efficiency of HDAC6" in the ending of the figure legend 3H (see below). Please see attached our modified manuscript (word). Manus_Wikstrom_revised2. (H) Cyld+/+keratinocytes were transiently transfected with control or HDAC6 siRNA (24 hours) in the absence or presence of TPA (100 nM for 30 minutes), followed by immunoprecipitation of endogenous Bcl-3 and immunoblotting against CYLD. Lysate shows equal amount of protein used for immunoprecipitation and knockdown efficiency of HDAC6.

3. The labelling of Supplementary Figure 3 has been corrected now. Please see attached our new Supplementary Figure 3 D (PDF) with the correct labelling. Wikstrom_SupFig3_revised2.

4. Colocalization of CYLD and Bcl-3: We agree with you that it is not essential to show colocalisation of CYLD and Bcl3 in Supplementary Figure 3 D since we already demonstrate in Fig. 3H that HDAC6 depletion induces co-localization of CYLD with acetylated microtubules both with and without TPA, but does not induce an interaction between CYLD and Bcl-3.

