EMBO
Molecular Medicine

Manuscript EMBO-2010-76338

T cell receptor-induced JNK activation requires proteolytic inactivation of CYLD by MALT1

Jens Staal, Yasmine Driege, Tine Bekaert, Annelies Demeyer, David Muyllaert, Petra Van Damme, Kris Gevaert, and Rudi Beyaert

Corresponding author: Rudi Beyaert, Ghent University/VIB

Review timeline:

Submission date:	26 October 2010
Editorial Decision:	07 November 2010
Revision received:	06 February 2011
Additional Editorial Correspondence:	07 February 2011
Additional Correspondence (by author):	08 February 2011
Editorial Decision:	14 February 2011
Accepted:	18 February 2011

Transaction Report:

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

1st Editorial Decision 07 November 2010

Thank you very much for submitting your research manuscript for consideration to The EMBO Journal editorial office.

As you will see from the comments enclosed, the referees express some interest in the reported Malt1 cleavage of CYLD that seems to contribute to JNK-activation. However, the comments also reveal that the functional significance and further mechanistic detail need to be established before they would support publication in The EMBO Journal. As the major shortcomings are very nicely detailed in the reports from ref#1 and #3, there is no need to repeat them. Given the overall rather positive comments on novelty, we would like to offer you the chance to expand mechanistically and provide convincing functional support (preferably in a close to physiological setting) during a single round of major revisions. I do have to urge you to take these comments serious and invest the necessary time and experimental efforts to not only convince the referee's from the significance of your findings but also to avoid later disappointments.

Finally, I do have to formerly remind you that it is EMBO_J policy to allow a single round of major revisions and that the final decision on acceptance or rejection entirely depends on the content and strength of the final version of your manuscript that will be assessed by some of the original referees!

Yours sincerely,

Editor
The EMBO Journal

© European Molecular Biology Organization

REFEREE REPORTS:

Referee #1:

In this paper Staal et al. show that CYLD is cleaved in two fragments upon TCR stimulation, demonstrate that paracapase MALT1 is responsible for this event and identify the cleavage site. In addition, they propose that this cleavage is required for JNK activation and control specific gene expression.

The first part of the study concerning the molecular analysis of CYLD cleavage upon cell stimulation is well performed and convincing. Different cell systems and modes of stimulation are used to demonstrate that CYLD represents a new target of MALT1, in addition to A20 and Bcl10. Moreover, following the identification of the cleavage site an uncleavable version of CYLD (R324A) is prepared. I have only one concern regarding the poor efficiency of the VRPR inhibitor (Fig. 2B). Is it an intrinsic property of this inhibitor or is it specific for the CYLD cleavage ? How efficient is it in inhibiting the cleavage of A20 in the same setting ?
The second part, which deals with the most important question : what is the role of this processing ?, is less convincing, based on a limited set of experiments and not always properly presented/discussed. The following critics can be made:
In several occasions the comments describing the Figs are not accurate. For instance, it is stated concerning Fig. 4A that ´ CYLD knockdown, although very efficient, only weakly affected I B degradation and JNK phosphorylation ª. Looking at the picture, an effect is clearly seen on IkBa degradation but not on JNK phosphorylation. An effect of R324A CYLD expression on Erk phophorylation is also visible in Fig. 4C (at 30 and 60 min) but presented as : ´ TCR-induced activation of NF- B, p38 and ERK MAPK (mitogen-activated protein kinase) were not affected ª. This should be modified and discussed.

In order to properly study the role of CYLD cleavage by MALT1 a cleaner cell system should be used. This should be possible since a sequence working efficiently in RNAi has been identified by the authors themselves. Stable expression of the equivalent shRNA in Jurkat would allow generation of a ko/kd system which could be complemented by wt and mutant CYLD. Here, stable wt Jurkat clones expressing wt CYLD or CYLD R/A have been prepared but at no place in the paper the relative expression of wt CYLD vs mutant CYLD is shown. Moreover, the relative level of expression of the exogenous vs endogenous CYLD is also not provided. The experiment shown in Fig. 4B, CYLD R/A part, suggests a low level of expression of CYLD R/A since there is still a large amount of cleaved CYLD generated. In this respect, tagged versions of transfected CYLD would also have been helpful in distinguishing between the cleaved exogenous and the endogenous one. Finally, the data with the VRPR inhibitor (Fig. 4D and E) are quite difficult to interpret since, as said above, this inhibitor only mildly affects the cleavage of CYLD. It is also unclear why a single analysis, instead of the two distinct ones shown in Fig. 4D and E, has not been performed, especially considering the remark made about the effect of VPRP when CYLD expression is inhibited. The comparison between the effect of VRPR on JNK activation in different situations would be made easier by showing a single experiment including the effect of control siRNA vs CYLD siRNA.
It is unclear at this stage, and ambiguously presented here, how cleavage of CYLD would be necessary for activating JNK. Clearly, the cell system which is used does not help. For instance, if this cleavage indeed allows JNK activation, it is difficult to understand, looking at Fig. 4B, how JNK phosphorylation is completely inhibited when only a very limited decrease in (endogenous) CYLD cleavage occurs, especially at 15 min when JNK is already fully phosphorylated. In addition, if cleavage is required for activating JNK overexpression of the cleaved forms should be done to see if they are able, separately or together, to activate JNK.

In the final section of the paper the expression of several genes is shown to be dependent upon CYLD cleavage, providing support to the model proposed by the authors. This emphasizes the need for a comprehensive validation of the effect of CYLD cleavage on JNK activation.

In conclusion, demonstrating a cleavage of CYLD by MALT during T cell activation is a new interesting observation but the mechanism by which it impacts on TCR signaling is not fully understood because of a suboptimal experimental cell system.

Referee #2:

The paper by Staal et al reports a mechanism by which the paracaspase MALT1 regulates T-cell activation. The authors demonstrate that TCR activation stimulates MALT1-mediated cleavage of CYLD, a deubiquitinase known to negatively regulate signaling pathways leading to activation of JNK and NF-kB. Interestingly, the CYLD cleavage by MALT1 in T cells is specifically required for JNK activation and thus the induction of a subset of genes. These findings are innovative and provide new insights into the mechanism of MALT1 function and TCR signaling. The results are generally convincing and clearly presented. However, the following points should be considered by the authors in order to further strengthen the paper.

1. Does MALT1 bind CYLD in transfected cells or T cells?

2. Fig. 3B. What is the effect of CYLD-R//A on TNFa-induced JNK activation?

3. Similar to point 2, how does the CYLD-R/A affect TNFa-induced gene expression?

Referee #3:

The authors report that the paracaspase MALT1, a protein positively involved in NF- B signaling in response to TCR activation, is also involved in the activation of the JNK pathway, and they show that this activation requires cleavage and inactivation of the deubiquitinase CylD. CylD is known, among other functions, as a negative regulator of the NF- B and JNK pathways. The authors also show that the oncogenic fusion protein API2-MALT1 cleaves CylD in the absence of TCR signaling.

Although MALT1 has already been demonstrated to cleave and inactivate 2 other components of the TCR pathway, Bcl10 and A20, the identification of a new substrate is important, in particular since it affects a different pathway. These results are potentially important, but a number of additional exps and controls are required before publication can be recommended :
- the authors rather convincingly demonstrate the proteolytic activity of MALT1 (but see below), but the functional part is less convincing : Is MALT1 really required for normal JNK signaling in response to TCR ? If CylD is cleaved by MALT1 as soon as the TCR is activated, it cannot be considered a bona fide negative regulator of this pathway : have the authors identified conditions where CylD behaves as an inhibitor of the JNK pathway in response to TCR?

Additional points :

- Figure 3C : what is the purpose of this figure ? To demonstrate that MALT1 directly cleaves CylD ? In this case recombinant CylD should be used (or a fragment of it) and the exact cleavage point should be determined by sequencing. Indeed the reticulocyte lysate could be the source of the actual protease, which would require MALT1 as a cofactor. In addition Arg324 might just be an amino acid necessary for recognition by the protease.

- Figure 4D : the inhibition of JNK activation by VRPR is not convincing. A better blot should be provided. In addition, expressing exogenous CylD in the presence of the endogenous molecule could potentially complicate the interpretation of the results. How can transfected CylD R/A obscure the role of the endogenous molecule ? Is it in large excess ? Stable knockdown of endogenous CylD should be performed before introducing an exogenous molecule.

- Figure 5B lacks MW markers, as well as indications about the identity of the bands. What is the level of expression of the endogenous molecule when compared to the transfected one ? Why does the expression of CylD increase in the presence of CD3/28 and PMA/iono for wt CylD, but only in the presence of CD3/28 for mutant CylD ?
- the absence of MW markers in all blots makes them difficult to read.

1st Revision - Authors' Response 06 February 2011

Response to reviewers

Reviewer #1

We are happy to read that this reviewer finds the cleavage of CYLD during T cell activation a new interesting observation and considers the molecular analysis of CYLD cleavage well performed and convincing. The major criticism of this referee concerns the proposed role of CYLD cleavage. We fear that we were not clear enough and that there is some misunderstanding of the proposed mechanism. More specifically, we do not claim that CYLD cleavage results in a gain of function (e.g. activation of JNK by one of the CYLD fragments); in contrast, our data suggest that cleavage disrupts CYLD activity, abolishing its inhibitory effect on JNK activation. We apologize if our wording caused any confusion. For this reason, we also propose to change the title into "T cell receptor induced JNK activation requires proteolytic inactivation of CYLD by MALT1".

The different comments are discussed in more detail below.

1. *... regarding the poor efficiency of the VRPR inhibitor (Fig. 2B). Is it an intrinsic property of this inhibitor or is it specific for the CYLD cleavage? How efficient is it in inhibiting the cleavage of A20 in the same setting? How efficient is the VRPR inhibitor?*

   We were not able to completely prevent CYLD cleavage with the VRPR inhibitor, but in our opinion the inhibition we observe is still very significant (>50%) and is reproducible in several experiments. This inhibitor has previously been described by Rebeaud et al. (Nature Immunol. 9, 272-281, 2008) and is indeed a poor inhibitor (active in the microM range). However, this is the only available MALT1 inhibitor so far. We used a similar concentration as reported in the paper of Rebeaud et al. and could not go higher because of the solvent (DMSO), which would otherwise interfere with the assay. As requested, we have now compared its effect on A20 cleavage in the same experiment and could show that inhibition of A20 cleavage is more efficient. The more pronounced effect of z-VRPR.fmk on A20 cleavage compared to CYLD cleavage most likely reflects our observation that MALT1 cleaves A20 less efficiently than CYLD (the latter can be concluded from the many experiments we did). We have amended the text with these observations (page 6) and changed figure 2B of our manuscript, which is now also showing the A20 cleavage.

2. *It is stated concerning Fig. 4A that "CYLD knockdown, although very efficient, only weakly affected IkBa degradation and JNK phosphorylation". Looking at the picture, an effect is clearly seen on IkBa degradation but not on JNK phosphorylation.*

   We apologize if our phrasing caused some confusion. We agree with the referee that Fig. 5A (new numbering) indeed shows an effect of CYLD silencing on the level of IkBa degradation. However, we mentioned this as only 'weakly' because IkBa degradation is not sustained upon CYLD silencing (which would be expected with a very potent enhancement of NF-κB activation). Similarly, CYLD silencing also increases JNK activation (see also new Fig. 5E), but again this is not very drastic (e.g. in Fig. 5A only visible at 15 min time point). We have now made this statement clearer in the revised manuscript (page 9).

3. *An effect of R324A CYLD expression on Erk phosphorylation is also visible in Fig. 4C (at 30 and 60 min) but presented as: "TCR-induced activation of NF-kB, p38 and ERK MAPK (mitogen-activated protein kinase) were not affected". This should be modified and discussed.*

   We agree with the referee that ERK phosphorylation is less pronounced upon R324A CYLD expression at 30 and 60 min, which indicates a small difference in kinetics. However, no

© European Molecular Biology Organization

consistent differences can be seen on either p38 or ERK phosphorylation in multiple independent experiments. We have now stated this clearer in the revised manuscript (page 9).

4. *In order to properly study the role of CYLD cleavage by MALT1 a cleaner cell system should be used. This should be possible since a sequence working efficiently in RNAi has been identified by the authors themselves. Stable expression of the equivalent shRNA in Jurkat would allow generation of a ko/kd system which could be complemented by wt and mutant CYLD.*

Because of technical problems with stable expression of CYLD shRNA, we have followed a slightly different approach to perform the suggested complementation experiments. More specifically, we have used the cell clones that stably express wild type and non-cleavable CYLD, respectively, and performed knockdown of endogenous CYLD by transfecting the cells with a morpholino targeting the CYLD 5'UTR. This shows that CYLD silenced cells complemented with wild type CYLD do activate JNK in response to stimulation, whereas cells complemented with non-cleavable CYLD do not respond. These findings are completely in line with the experiments that were already shown in Fig. 5B and are included as an additional supplementary Fig. S2 in the revised paper (also mentioned on page 9).

5. *Here, stable wt Jurkat clones expressing wt CYLD or CYLD R/A have been prepared but at no place in the paper the relative expression of wt CYLD vs mutant CYLD is shown. Moreover, the relative level of expression of the exogenous vs endogenous CYLD is also not provided. The experiment shown in Fig. 4B, CYLD R/A part, suggests a low level of expression of CYLD R/A since there is still a large amount of cleaved CYLD generated. In this respect, tagged versions of transfected CYLD would also have been helpful in distinguishing between the cleaved exogenous and the endogenous one.*

The transfected CYLD contains a Flag-tag. However, anti-Flag immunoblotting did not allow us to detect expression of the CYLD clones in total lysates, indicative of rather low expression. In order to specifically detect transfected CYLD, we therefore first did enrichment by immunoprecipitation with anti-Flag and then detected expression via immunoblotting with anti-CYLD. This revealed approximately equal expression of the transfected wild type and mutant CYLD (Suppl. Fig. S1C).

To compare expression levels of endogenous versus transfected CYLD, we included an experiment also showing CYLD expression (detected with anti-CYLD) in total lysates of non-transfected Jurkat cells (suppl. Figure S1A and S1B). Quantification of CYLD shows that the transfected CYLD is expressed at about 4-6 fold higher levels compared to endogenous CYLD. This is now mentioned in the revised manuscript (page 9).

6. *Fig. 4D and E are quite difficult to interpret since, as said above, this inhibitor only mildly affects the cleavage of CYLD. It is also unclear why a single analysis, instead of the two distinct ones shown in Fig. 4D and E, has not been performed, especially considering the remark made about the effect of VPRP when CYLD expression is inhibited. The comparison between the effects of VRPR on JNK activation in different situations would be made easier by showing a single experiment including the effect of control siRNA vs CYLD siRNA.*

We have repeated this experiment and now show a single gel from an experiment that includes all different setups (Fig. 5E). As before, z-VRPR.fmk inhibits JNK-P significantly. However, the effect is only partial (in contrast with the much stronger inhibition obtained in the case of non-cleavable CYLD expression), most likely reflecting our observation that z-VRPR.fmk only partially prevents CYLD cleavage (as also pointed out by the referee and discussed in point 1).

© European Molecular Biology Organization

7. *It is unclear at this stage, and ambiguously presented here, how cleavage of CYLD would be necessary for activating JNK. Clearly, the cell system which is used does not help. For instance, if this cleavage indeed allows JNK activation, it is difficult to understand, looking at Fig. 4B, how JNK phosphorylation is completely inhibited when only a very limited decrease in (endogenous) CYLD cleavage occurs, especially at 15 min when JNK is already fully phosphorylated. In addition, if cleavage is required for activating JNK, over expression of the cleaved forms should be done to see if they are able, separately or together, to activate JNK.*

We fear that there is some misunderstanding of the proposed mechanism. We do not propose that CYLD cleavage results in a gain of function (e.g. activation of JNK by one of the CYLD fragments), and we apologize if our wording somehow suggested this. In contrast, our data suggest that cleavage disrupts CYLD activity, abolishing its inhibitory effect on JNK activation. This model is consistent with our observation that JNK cannot be activated in cells expressing a non-cleavable CYLD mutant. In fact, the absence of JNK activation in CYLD-R324A expressing Jurkat cells, despite the presence of the endogenous CYLD cleavage fragment (Fig. 5B), excludes a gain of function effect of CYLD cleavage. We mention this again in the discussion of our manuscript (page 14) and have included a new figure (Figure 8) illustrating the model that we propose. Also the title of our manuscript was changed to avoid any confusion.

The fact that only a small fraction of the total CYLD is cleaved is probably reflecting cleavage of a sub-population of CYLD that is part of specific signalling complexes regulating JNK (now mentioned in the discussion on page 12). Such a partial cleavage was previously also demonstrated for the two other known MALT1 substrates, A20 and Bcl10.

Reviewer #2

We are happy to read that this referee appreciates our findings as innovative, generally convincing and clearly presented. The suggested experiments are well taken and are discussed below.

1. *Does MALT1 bind CYLD in transfected cells or T cells?*

We have attempted to show this interaction via co-immunoprecipitation without any success. Binding between MALT1 and Bcl10, or CYLD and TAK1, were used as positive controls and nicely worked. This is now briefly mentioned in the discussion of the revised paper (page 12). There might be multiple explanations for this. We hypothesize that MALT1 only comes into proximity of its CYLD substrate via a weakly expressed intermediate interacting partner, lowering the sensitivity of detection. Alternatively, we cannot exclude direct binding that escapes detection because of its transient nature (typical for enzyme/substrate interactions) and weak affinity, or because only a very small fraction of CYLD binds MALT1 (consistent with our observation that only a small fraction of the total CYLD is cleaved; see also our answer to reviewer 1, point 7).

2. *What is the effect of CYLD-R324A on TNF-induced JNK activation?*

   How does CYLD-R324A influence TNF-induced gene expression?

We were not able to observe JNK activation in TNF-treated Jurkat cells (Suppl. figure 3A), and consistent with this we also did not observe AP-1 dependent gene expression (c-jun, IL-2) in response to TNF (data not shown). In contrast, TNF activates NF-κB and increases IκBα expression (which is largely AP-1 independent but NF-κB dependent) in Jurkat cells, but this

TNF effect was comparable in non-transfected cells or cells transfected with wild-type CYLD or CYLD-R324A (Suppl. figure 3B; the more sustained expression in CYLD-R324A expressing cells at later time points is not significant). These data are now mentioned in the discussion of the revised paper.

Reviewer #3

We are happy to read that this reviewer considers the finding of CYLD as a new MALT1 substrate important. The request for a number of additional experiments and controls is addressed below.

1. *Is MALT1 really required for normal JNK signaling in response to TCR? If CylD is cleaved by MALT1 as soon as the TCR is activated, it cannot be considered a bona fide negative regulator of this pathway: have the authors identified conditions where CylD behaves as an inhibitor of the JNK pathway in response to TCR?*

   Evidence in literature for a requirement of MALT1 (based on MALT1 knockout experiments) for normal JNK signalling is rather controversial. According to the work of Ruefli-Brasse and colleagues (Ruefli-Brasse et al., 2003), MALT1 deficient primary T cells can still activate JNK in response to TCR stimulation. In contrast, Ruland and colleagues showed that MALT1 deficient T cells are unable to activate JNK (Ruland et al., 2003). The reason for these differences is still unclear but might reflect differences in mouse background, gene targeting strategy or experimental settings. Furthermore, MALT1 might regulate specific JNK isoforms, as suggested by the finding that different JNK isoforms are regulated by CARMA1-Bcl10-dependent and -independent mechanisms (Blonska et al., 2007). The JNK antibody we used recognizes both JNK1 and 2, and the activation of both is defective in CYLD-R324A expressing cells (Fig. 5B). This clearly indicates that MALT1 proteolytic activity is required for JNK activation. Whether MALT1 also has protease-independent functions (e.g. as adaptor protein) in JNK signalling remains to be investigated. We briefly discuss the role of MALT1 in TCR-induced JNK activation in the introduction of the revised manuscript.

   Regarding the question if we have determined conditions where CYLD behaves as an inhibitor of the JNK pathway in response to TCR, we can refer to the amended Fig. 5A and 5D, where we can see a slightly increased JNK activation in CYLD silenced cells.

   Finally, we are not really sure what the referee means by saying that CYLD cannot be considered as bona fide negative regulator of the JNK pathway if CYLD is cleaved by MALT1 as soon as the TCR is activated. CYLD cleavage can be observed as early as 15 min after TCR stimulation (Fig. 5B) and coincides with the time of JNK activation. We propose a model in which constitutively expressed CYLD prevents JNK activation by de-ubiquitinating an upstream protein that is involved in JNK activation. As soon as MALT1 is activated, this results in the proteolytic inactivation of CYLD, allowing JNK activation by the upstream ubiquitinated signalling protein. To make this clearer in the text, we have included a new figure (Fig. 8) depicting the proposed model in the revised manuscript.

2. *Figure 3C: what is the purpose of this figure? To demonstrate that MALT1 directly cleaves CylD? In this case recombinant CylD should be used (or a fragment of it) and the exact cleavage point should be determined by sequencing. Indeed the reticulocyte lysate could be the source of the actual protease, which would require MALT1 as a cofactor. In addition Arg324 might just be an amino acid necessary for recognition by the protease. Does MALT1 directly cleave CYLD?*

This is a valid point and we agree with the referee that it is important to clarify whether MALT1 can directly cleave CYLD and whether Arg 324 is indeed the cleavage site.

It can indeed not be excluded that the reticulocyte lysate contains a protease that is cleaving CYLD, and that MALT1 functions as a cofactor for this protease. In fact, we did this experiment using both reticulocyte cell extracts and wheat germ extracts, with similar results (the data shown in Fig. 3C are from the wheat germ extract, as indicated in the materials and methods section). Although this still not proves that the effect is direct, it lowers the chance of the MALT1 protease being present in both types of extracts.

To directly prove that MALT1 is the protease that cleaves CYLD, we made use of recombinant CYLD (expressed and purified from bacteria) that was incubated with recombinant MALT1. Coomassie Blue staining clearly shows that in vitro co-incubation of CYLD and MALT1 results in the generation of two new bands corresponding to the N- and C-terminal fragments of CYLD (amended Fig. 3D). To determine the exact cleavage site, the generated neo-C-terminus of the N-terminal CYLD was determined by mass spectrometry. This confirmed that CYLD is cleaved at Arg 324. These data are now included in the revised manuscript as a new figure 4.

3. *Figure 4D: the inhibition of JNK activation by VRPR is not convincing. A better blot should be provided. In addition, expressing exogenous CylD in the presence of the endogenous molecule could potentially complicate the interpretation of the results. How can transfected CylD R/A obscure the role of the endogenous molecule? Is it in large excess? Stable knockdown of endogenous CylD should be performed before introducing an exogenous molecule.*

As this issue was also addressed by reviewer 1, we refer to the detailed answer to reviewer 1, points 4, 5 and 6.

4. *Figure 5B lacks MW markers, as well as indications about the identity of the bands. What is the level of expression of the endogenous molecule when compared to the transfected one? Why does the expression of CylD increase in the presence of CD3/28 and PMA/iono for wt CylD, but only in the presence of CD3/28 for mutant CylD?*

The figure has been updated with MW markers and identify labels of the bands.

As also pointed out in our answer to reviewer 1 (point 5), expression levels of transfected CYLD are about 4-6 times the level of endogenous CYLD. In the present experiment, however, endogenous CYLD has been silenced (see also suppl. fig. S2B for morpholino mediated CYLD silencing efficiency).

In several experiments we can observe some up-regulation of CYLD upon TCR or PMA stimulation (Fig. 1A, 2C and S2A) and also after TNF (Fig. S3A). Because this can be observed for both endogenous as well as exogenously expressed CYLD, and as early as 15 min after stimulation (e.g. Fig. S2A), we hypothesize a role for stabilization of CYLD at the mRNA or protein level (this is now mentioned in the revised paper). Why CYLD-R/A expression does not increase after PMA/I treatment is not known, but could be related to our observation that CYLD-R/A gets ubiquitinated after PMA stimulation (results not shown). Elucidation of the underlying mechanism is beyond the scope of the present paper but will be the topic of future research.

© European Molecular Biology Organization

5. *The absence of MW markers in all blots makes them difficult to read.*

All figures have been updated with MW markers.

Additional Editorial Correspondence 07 February 2011

I am in the process seeking further referee input on your revised version. In the meantime, I noticed that in Fig.5D not all of the phospho-JNK and JNK blots seem to be derived from a single gel and particularly the JNK-lanes are cut in a way that impinges correct assesment of the blot. I would thus kindly ask to prepare a more informative figure or minimally clearly indicate whether and where separate lanes had been compiled. Also, as you mention three independent experiments in the legend, a graph of quantified data would offer stronger support for these results.

Yours sincerely,

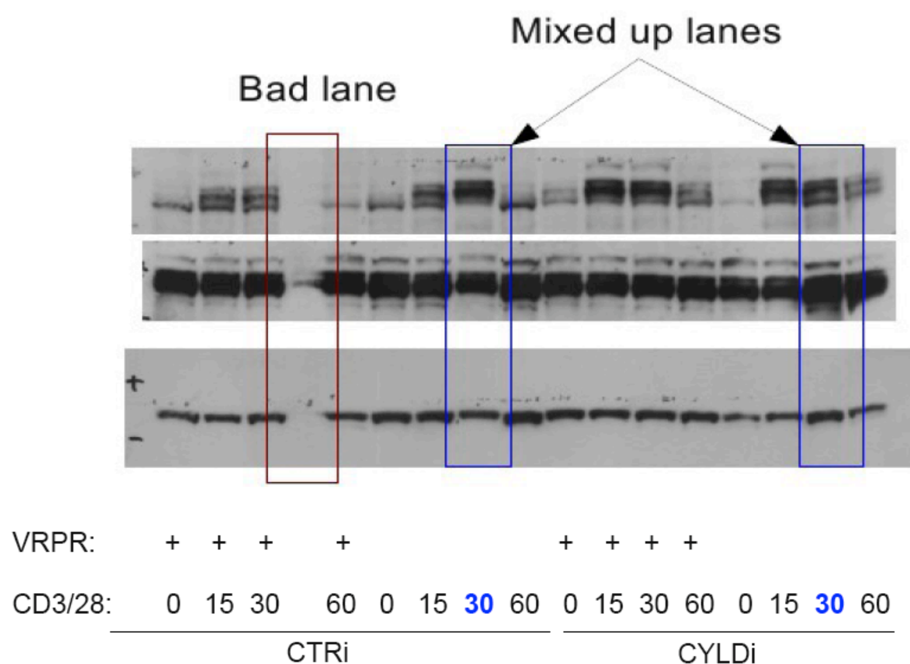
Editor
The EMBO Journal

Additional Correspondence (by author) 08 February 2011

I am sorry for the confusion.

Fig. 5D is from a single gel, but samples in lanes 7 and 15 were misloaded and the picture was corrected for this by recompiling the corresponding lanes. The cut between lanes 3 and 4 is due to an empty lane (because of a bad loading slot) that we removed.

I propose to provide you with a new figure in which we keep the empty lane, but where lanes 7 and 15 are still recompiled for clarity reasons (we will add a sentence to the figure legend mentioning this).
For your information, I enclose the original picture of the gel (where the lanes are not yet recompiled).



Unfortunately, we cannot run a new gel because we have no samples left anymore.
The other experiments we did gave similar results (already shown in the original manuscript). However, in these experiments the samples from CYLD silenced cells were not loaded on the same gel as the samples from the non-silenced cells. Since it is most important to compare VRPR treated versus untreated cells within each group (silenced versus non-silenced), we thought loading them on a single gel was not essential (we usually run small gels that do not contain that many lanes). Because one of the referees asked us to show all samples on a single gel, we now only repeated this once on a bigger gel (also because the inhibitor is
very expensive and we need a whole batch for one experiment).

Since panel 5D is only confirming our conclusions that were already drawn from the results depicted in Fig. 5B, we hope this will be acceptable.

Quantification is difficult to perform correctly in this case due to the low dynamic range of the X-ray film (the black signal of the band is rapidly saturated). Because, this would cause false quantified data, we prefer to leave it as such.

2nd Editorial Decision 14 February 2011

I did receive comments from one of the original referees that is essentially satisfied with the provided revisions.

The editorial office will soon be in touch with you related to further necessary paperwork.

I like to congratulate you to your study.

Yours sincerely,

Editor
The EMBO Journal

© European Molecular Biology Organization