# Format for ANSWERING REVIEWERS

July 01, 2014

Dear Editor,

herewith, we resubmit our manuscript (11644) entitled: "CYLD deletion triggers NF-κB-signaling and increases cell death resistance in murine hepatocytes'' to the WORLD JOURNAL OF GASTROENTEROLOGY.

We appreciate the possibility to resubmit our manuscript. Please find enclosed the revised version and detailed responses to the reviewers´ comments. We have thoroughly addressed all the editors' and reviewers' concerns.

Please find enclosed the edited manuscript in docx format (file name: 11644_Urbanik et al. CYLD in liver injury revised version.docx). Figures are provided in pptx files.

**Title: CYLD deletion triggers NF-κB-signaling and increases cell death resistance in murine hepatocytes**

**Author:**

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**Name of Journal:** *World Journal of Gastroenterology*

**ESPS Manuscript NO: 11644**

The manuscript has been improved according to the suggestions of reviewers:
1. Format has been updated

2. Revision has been made according to the suggestions of the 3 reviewers:

(1) 69015

*This study evaluated the CYLD's function in the murine hepatocytes apoptotisis network which controlled by NF-kB. The apoptosis related factors including Bcl-2, XIAP, cIAP and survivin were assessed after hepatocyte cell death in CYLD knockout mice. Subsequently, the study speculate CYLD regulate NF-kB dependent anti-apoptotic pathway. A excellent work had been done in this study. In terms of illuminate the hypothesis clearly, I recommend to make revisions:*
*The study hypothesize CYLD locate in the center position of NF-kB dependent apoptosis pathway which related to many factors. In this situation, a graph is needed to illustrate this network. Apart from cell viability, western blot, had the flow cytometry analysis already been performed in CYLD(-/-) hepatocyte cells for the effect of CYLD on apoptosis? The effect of CYLD on apoptosis was assessed directly in this assay.*

We thank the reviewer for this valuable comment. To better illustrate CYLD's role in receptor mediated apoptosis as well as in the NF-κB survival network, we added a graph to the manuscript (Figure 6). The graphical illustration provides a model of CYLD's function. The functional outcome of CYLD deletion is easier to understand within the context of this illustration.
Regarding the demanded FACS analysis of primary murine hepatocytes, we have to state that flow cytometric analysis (Annexin V or PI staining) of hepatocytes is not appropriate because of the profound spontaneous apoptosis induction through trypsin- or accutase-based detachment of the very adherent hepatocytes. So we decided to assess viability of hepatocytes in combination with the analysis of caspase activation as a direct marker for apoptosis induction (see Figure 4D). Furthermore, our study provides clear *in vivo* data about reduced apoptotic sensitivity of CYLD-/- hepatocytes (e.g. caspase activation analysis and nuclear cleaved PARP staining in Figure 2). The cleavage of PARP has been considered indicative of functional caspase activation (Koh et al., Pharmacol Res., 2005).

## (2) 8233

*The manuscript by Urbanik T et al. describes experiments performed to evaluate the role of the deubiquitinase CYLD in modulating apoptotic cell death in murine hepatocytes. They report that CYLD-/- mice could reduce sensitivity to apoptosis by increasing the anti-apoptotic NF-κB signalling. Authors demonstrate that CYLD -/- primary murine hepatocytes were less sensitive towards death receptor-mediated apoptosis by snowing increased levels of Bcl-2, XIAp, cIAP1/2, surviving and c-FLIP expression. Moreover, by inhibiting of NF-κB activation by BAY 11-7085 (Ika posphorilation inhibitor) they refer an inhibition of anti-apoptotic proteins and a re-sensitization of CYLD-/- hepatocytes towards TNF- and CD95- receptor mediated apoptosis.*

*Minor comments:*

1) *Authors should add in Figure 2 a clear title or a brief sentence to underline that both WT and CYLD-/- mice were treated with D-GALN/LPS (Fig. 2, panel A, B, C) or with Jo2 (Fig. 2, panel D,E,F) to facilitate the reader.*

We regret that we have not clearly highlighted the respective treatment procedures in Figure 2. In the revised version, we solved this misguiding.

2) *By performing western blotting analysis, authors demonstrate that both in vivo (D-GALN/LPS or Jo2 injury model in CYLD-/- mice, Fig. 2 B, E) that in vitro (CYLD-/- hepatocytes, Fig. 4 D) there was a reduction of caspase 8, 9 and 3 activation. To better characterize this data, since caspases are very important in apoptosis network, it should be better if authors provide a caspase activity assay, at least of caspase 3.*

We additionally analysed caspase-3 activation via a caspase activity assay. The obtained caspase-3 substrate turnover kinetic assessed by fluorometric analysis indicated significantly reduced caspase-3 activation levels in CYLD-/- livers after D- GalN/LPS and Jo2 treatment. We added the results of this experiment to the appropriate section (results part, page 9-10, Supplementary Figure S2).

3) *In the Result session (page 10-11) by referring to Figure 3 B, Authors report that "p105 and p50*

*were expressed equally compared to WT"; by referring to Figure 3 D, upper panel, Authors state that: "…IκB-α levels were not significantly different"; by referring to Figure 3 F, left panel, Authors state that: "...liver lysates of D-GalN/LPS treated CYLD-/- mice showed increased expression levels of the NF-κB subunits p50…". Authors should provide densitometric analysis for these western blotting or show other images more convincing to allow reader to note the really states of proteins.*

*Some of Western blotting analysis showed in Figure 5 A (both panels) are not quite convincing: please Authors to provide densitometric analysis also for these experiments.*

We thank for the comment. In the revised version we provided densitometric analysis of the requested Western blots. The band density was measured relative to the corresponding controls (set to 1) and then adjusted to tubulin as loading control. The results of our analysis led to corrections of the statements about expression levels of IκB-α in Figure 3D, upper panel ("IκB-α phosphorylation was not substantially increased in *CYLD*$^{-/-}$ but total IκB-α levels were slightly reduced.", page 10); and about expression levels of the NF-κB subunits after D-GalN/LPS treatment in Figure 3 F, left panel ("Additionally, liver lysates of D-GalN/LPS treated *CYLD*$^{-/-}$ mice showed increased expression levels of the NF-κB precursor proteins p105 and p100. However, only the active subunit p52 showed increased expression levels. RelA and RelB expression levels were increased compared to *WT.*", page 11).

3) *Figure 5 C: Authors show Western blotting concerning the pro-caspase 3 and the caspase 3 cleaved form, but they do not cite this data on text: I suggest to add a short sentence in the test or to eliminate the image if it is not so significative.*

We regret that we neglected to cite the pro-caspase 3 and cl. Caspase 3 Western blot of Figure 5C in the text of our manuscript. In addition to the data of viability assay of primary murine hepatocytes after YM-155 treatment, this Caspase Western blot should indicate no apoptosis induction in PMH by YM-155 in concentrations up to 50 μM. We cited this figure in the revised manuscript (page 14) as follows:

"24 h YM155 treatment alone did not induce caspase-3 activation and did not reduce of viability in concentrations up to 50 μM, in both *WT* and *CYLD*$^{-/-}$ PMH (Fig. 5C, Supplementary Fig. S3, right panel)".

## (3) 227449

*This manuscript used gene knockout model to determine the role of Cyld in the liver under basal condition (fig 1-3) and with chemical-induced stress (fig 3-4). The results of extensive measurements are consistent with previous observations in other tissues that in the absence of Cyld, there are more growth and more responses to stress. The authors then used chemical inhibitors to determine the pathway behind the enhanced resistance to death in the absence of Clyd (fig 5). The inhibitor experiments have their limits and thus the conclusion is an oversimplified one. Perhaps the more appropriate title should be "Deletion of Cyld increases liver growth and the resistance toward stress response in murine hepatocytes*"

To follow the reviewer comments we changed to title of our manuscript to ''CYLD deletion triggers NF-κB-signaling and increases cell death resistance in murine hepatocytes''. This

new title considers the concerns of the reviewer regarding limits of the used inhibitor experiments.

3. References and typesetting were corrected

Thank you again for publishing our manuscript in the *World Journal of Gastroenterology*.

Sincerely yours,

Henning Schulze-Bergkamen

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