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Cardiomyocyte differentiation from human induced pluripotent stem cells is delayed following knockout of Bcl-2

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MS TITLE: Cardiomyocyte differentiation from iPS cells is delayed following knockout of Bcl-2

AUTHORS: Tim Vervliet, Robin Duelen, Rita La Rovere, lLewelyn H Roderick, and Maurilio Sampaolesi
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The paper shows that knockout of Bcl2 delays cardiomyocyte differentiation in hIPSC-derived cardiomyocytes. This is associated with downregulation of calcium handling gene expression. The formal establishment of that link is novel, but it is descriptive and lacks a mechanistic link.

Comments for the author

This is a reasonably interesting paper and is technically sound, with appropriate numbers of replicates and statistical validation. The methods have been applied well and the data is of high quality. The only problem is that it's a bit underwhelming in that it's descriptive and not very mechanistic. The observed loss or delay in the expression of the C2+ handling gens and c-Myc could be cause or effect. ie is cardiomyocyte differentiation delayed because of the altered expression of these genes, or is the loss/delay in expression just a consequence of delayed differentiation ?

I have no major comments regarding corrections or revisions, other than whether or not it needs an additional experiment(s) which can answer this question. I guess that's an editorial decision.

Reviewer 2

Advance summary and potential significance to field

In previous work, the authors addressed the impact of Bcl-2 on calcium signaling, especially in relation to IP3 and ryanodine receptors. Bcl-2 is regulated through a GATA4-dependent pathway indicating a role in development. In this framework, the authors therefore tested the impact of Bcl-2 in development in relation to calcium signaling events in cardiomyocytes.

The authors provide evidence that, through non-apoptotic interactions, Bcl-2 and, in relation to Bcl-2 expression, calcium signaling events appears to be required for cardiomyocyte development. The fact that Bcl-2 regulates developmental maturation of stem cells to cardiomyocytes is novel. Key events in development of induced pluripotent stem cells (iPSC) to functional cardiomyocytes were studied. The findings described here are both significant and novel.

This is an interesting article. Unlike most groups, the authors decided to perform their study in a human system by making use of human induced pluripotent stem cells (hiPSC). Following knock-out of Bcl-2 by the use of the CRISPR/Cas9 system, the authors observed a delayed maturation of these hiPSC to cardiomyocytes. In further experiments, they explored the mechanism of this non-apoptotic Bcl-2 function.

Some issues need to be addressed, however, only a few on a functional level. The other issues relate to data presentation and analysis.

Comments for the author

Major points:

1. The authors state that the observed effect of delayed cardiomyocyte differentiation is independent from apoptotic functions of Bcl-2. The evidence they provide is overall very good but to make the statement even more solid, they should show the precise percentage of cell death on a single cell level for apoptosis autophagy, and necrosis in a time course experiment. This is important as smaller changes in cell death are not necessarily reflected by e.g. Western blot analyses.

This could be solved through e.g. flow cytometric measurement of cellular DNA content or Annexin-FITC binding and propidium iodide counterstaining or through simple microscopic counting of cells with the corresponding morphology and the use of an adequate staining method. The corresponding author is a co-author of the 4th edition of the ”guidelines for the use and interpretation of assays for monitoring autophagy“ published in 2021 in the journal Autophagy and is therefore familiar with the various single cell level techniques.

2. In the results section and in the legend to figure 2, the authors make conflicting statements regarding the impact of Bcl-2 knock-out on cardiac differentiation markers. Especially the title of fig. 2 is misleading. There is definitively an impact of Bcl-2 on gene expression levels in the two k.o. hiPSC clones (green and red lines in fig. 2B) with mRNA expression levels as determined by qRT-PCR being at least transiently lower in these two clones for several of the tested genes.

3. Figure legend 2B, line 543: does ”at least 4 different“ mean quadruplicates from the same differentiation experiment or 4 independent experiments? How many replicates were done in the cell cultures and how many in the qPCR tests?

4. For Figure 2C, evidence must be provided that the immunofluorescence images for cardiac troponin T were acquired with the same instrument settings. Means and SD or SEM should be shown for cTnT expression levels in image analyses.

5. Figure 3: molecular weights should be indicated. Vinculin is used as a loading control. But were the other proteins analysed on the same or on independent blots? In case of the former, the stripping procedure must be explained. If the latter is the case, individual vinculin blots must be provided for each of the proteins detected.

6. Figure legend 3: The title of the legend to figure 3 is misleading. The authors studied cell death-related markers but did not directly and functionally measure apoptotic or autophagic cell death, e.g. on a single cell level. Moreover, due to the descriptive nature of their Western blot analyses they cannot exclude cell death through a necrotic mechanism.

7. Figure 4 legend: What do the authors want to say when they put a question mark after ”differentiations“?. Are they sure to have provided the precise numbers of replicates or independent experiments for each of the experiments?

8. Figure 5: molecular weights should be indicated. Tubulin is used as a loading control. But were the other proteins analysed on the same or on independent blots? In case of the former, the stripping procedure must be explained. If the latter is the case, individual tubulin blots must be provided for each of the proteins detected.

9. Figure 6: molecular weights should be indicated. Vinculin is used as a loading control. But were the other proteins analysed on the same or on independent blots? In case of the former, the stripping procedure must be explained. If the latter is the case, individual vinculin blots must be provided for each of the proteins detected.

10. figure 6B, right lower panel: The authors show sharp changes for Bcl-2 in the control cells. No such change is found regarding phospho-ser70 protein expression. A look to the Western blots in the left panel of figure 6B shows, however, that the pan-Bcl-2 and phospho-ser70 Bcl-2 behave in an almost identical manner over time. How do the authors explain this? A similar problem arises when looking at p38 and the phospho-p38 MAPK where the pan-p38 shows a significant up-regulation in the quantitative analyses over time but not the phospho-p38 (the upper 2 panels of the quantitative image analyses) whereas the Western blot in the left panel of figure 6B shows such an up-regulation in both instances. This needs to be explained and re-analysed. The rationale to use two different normalisation procedures must be explained or changed.

11. The authors show error bars in several figures and these indicate either S.E.M. in fig. 2B, or S.D. in fig. 3, 4, 5, 6. The authors should justify as to why they employed these different representations of statistical errors.

Minor points:

1. Line 543: the term ”different“ is not optimal here and e.g. ”independent“ would be more to the point. See also Major point 3.

2. Figure 3: The labeling of the right part of fig. 3 is too small and will result in an illegible figure in print.

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5. Legend to Figure 5, line 584: delete ”does“.

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7. Figures 3, 5, 6: Desribe the ”L“ label in the Western blot panels.

First revision

Author response to reviewers' comments

We would like to thank both reviewers for taking the time to review our manuscript and provide useful feedback and suggestion to improve the paper. Below we have indicated our responses to all the queries posed by the reviewers in red. Changes made to the manuscript are also indicated in red. These include request from the reviewers but also textual and grammatical corrections.

Reviewer 1 Advance Summary and Potential Significance to Field.

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I have no major comments regarding corrections or revisions, other than whether or not it needs an additional experiment(s) which can answer this question. I guess that's an editorial decision.

We thank the reviewer for the positive comments on the quality of the paper. In order to improve the mechanistic aspects of the study, an additional experiment was setup to track c-Myc expression in the nucleus during the interventional study of Bcl-2 KO in human iPS cells. This experiment showed that the lack of Bcl-2 affects the nuclear localization of c-Myc that is significantly reduced compared to controls.

This aspect is now highlighted in the discussion since our data and recent literature suggest that KO of Bcl-2 leads to reduced c-Myc protein localization in the nucleus. Given that expression of cardiomyocyte specific markers, the absence of other pluripotency and/or mesodermal markers, and that c-Myc is an important regulator of the expression of these cardiomyocyte specific markers, we suggest that the reduced c-Myc is actually the cause for the reduction/delay in expression of the cardiac Ca2+ toolkit.

Reviewer 2 Advance Summary and Potential Significance to Field...

In previous work, the authors addressed the impact of Bcl-2 on calcium signaling, especially in relation to IP3 and ryanodine receptors. Bcl-2 is regulated through a GATA4- dependent pathway indicating a role in development. In this framework, the authors therefore tested the impact of Bcl-2 in development in relation to calcium signaling events in cardiomyocytes.

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This is an interesting article. Unlike most groups, the authors decided to perform their study in a human system by making use of human induced pluripotent stem cells (hiPSC). Following knock-out of Bcl-2 by the use of the CRISPR/Cas9 system, the authors observed a delayed maturation of these hiPSC to cardiomyocytes. In further experiments, they explored the mechanism of this non-apoptotic Bcl-2 function.

Some issues need to be addressed, however, only a few on a functional level. The other issues relate to data presentation and analysis.

Reviewer 2 Comments for the Author.

Major points:

1. The authors state that the observed effect of delayed cardiomyocyte differentiation is independent from apoptotic functions of Bcl-2. The evidence they provide is overall very good but to make the statement even more solid, they should show the precise percentage of cell death on a single cell level for apoptosis, autophagy, and necrosis in a time course experiment. This is important as smaller changes in cell death are not necessarily reflected by e.g. Western blot analyses. This could be solved through e.g. flow cytometric measurement of cellular DNA content or Annexin-FITC binding and propidium iodide counterstaining or through simple microscopic counting of cells with the corresponding morphology and the use of an adequate staining method. The corresponding author is a co-author of the 4th edition of the ”guidelines for the use and interpretation of assays for monitoring autophagy“ published in 2021 in the journal Autophagy and is therefore familiar with the various single cell level techniques.

We thank the reviewer for this comment. In order to address the percentage of apoptotic and necrotic cells we have performed on day 0, 7, 14 and 21 co-staining with a cell permeable Hoechst, propidium iodide (PI) and NuncView®488 (to study caspase 3 activity) and quantified this relative to the total cell count obtained by the Hoechst stain. The quantification was added to figure 3 and representative images are added as supplemental data.

2. In the results section and in the legend to figure 2, the authors make conflicting statements regarding the impact of Bcl-2 knock-out on cardiac differentiation markers. Especially the title of fig. 2 is misleading. There is definitively an impact of Bcl-2 on gene expression levels in the two k.o. hiPSC clones (green and red lines in fig. 2B) with mRNA expression levels as determined by qRT-PCR being at least transiently lower in these two clones for several of the tested genes.

We agree wit the reviewer that this is indeed misleading. The title of the figure was changed to **Bcl-2 KO delays the expression of certain cardiac differentiation markers**

3. Figure legend 2B, line 543: does ”at least 4 different“ mean quadruplicates from the same differentiation experiment or 4 independent experiments? How many replicates were done in the cell cultures and how many in the qPCR tests?

We apologize for the confusion here. At least 4 independent differentiation experiments were performed of which an independent qPCR analysis was performed. Within each test, each sample was taken with in duplicate.

4. For Figure 2C, evidence must be provided that the immunofluorescence images for cardiac troponin T were acquired with the same instrument settings. Means and SD or SEM should be shown for cTnT expression levels in image analyses.

We would like to stress that all the images were taken using the same settings. As requested we performed a quantification and added this to the figure. Additionally, we have included below this document screenshots taken from the instrument settings from the confocal software showing that

these were identical. A statement was added in the materials and methods that all settings were identical for these images.

5. Figure 3: molecular weights should be indicated. Vinculin is used as a loading control. But were the other proteins analysed on the same or on independent blots? In case of the former, the stripping procedure must be explained. If the latter is the case, individual vinculin blots must be provided for each of the proteins detected.

We thank the reviewer for this comment. These were in fact ran on separate immunoblots. This is now also indicated by the black line were necessary. We have included the method of stripping, when this was required, in the materials and methods.

6. Figure legend 3: The title of the legend to figure 3 is misleading. The authors studied cell death-related markers but did not directly and functionally measure apoptotic or autophagic cell death, e.g. on a single cell level. Moreover, due to the descriptive nature of their Western blot analyses they cannot exclude cell death through a necrotic mechanism.

We agree with the reviewer on this point. As suggested by the reviewer a more in-depth single cell study was implemented in order to address the levels of apoptosis and necrosis. As for autophagy we did not study or make claims with regards to autophagic cell death as Bcl-2 has mainly been described to modulate the induction of autophagy, which we also study here with the immunoblots. In order to fit with the data presented the figure title was changed to “Basal levels of apoptosis, necrosis or autophagy induction are unaltered upon KO of Bcl-2”.

7. Figure 4 legend: What do the authors want to say when they put a question mark after ”differentiations“?. Are they sure to have provided the precise numbers of replicates or independent experiments for each of the experiments?

We apologize for this mistake. The “?” was part of a query a co-author had with the initial manuscript. This should have been removed during the final correction. The figure legend was amended as follows: The experiment was performed at least 9 times independently for each condition (n≥9).

8. Figure 5: molecular weights should be indicated. Tubulin is used as a loading control. But were the other proteins analysed on the same or on independent blots? In case of the former, the stripping procedure must be explained. If the latter is the case, individual tubulin blots must be provided for each of the proteins detected.

We agree with the comment of the referee and amended this as requested and performed similar corrections as in reviewer comment 5.

9. Figure 6: molecular weights should be indicated. Vinculin is used as a loading control. But were the other proteins analysed on the same or on independent blots? In case of the former, the stripping procedure must be explained. If the latter is the case, individual vinculin blots must be provided for each of the proteins detected.

We agree with the comment of the referee and amended this as requested and performed similar corrections as in reviewer comment 5. Figure A and B are two separate blots on which all the indicated immunostainings were performed.

10. figure 6B, right lower panel: The authors show sharp changes for Bcl-2 in the control cells. No such change is found regarding phospho-ser70 protein expression. A look to the Western blots in the left panel of figure 6B shows, however, that the pan-Bcl-2 and phospho-ser70 Bcl-2 behave in an almost identical manner over time. How do the authors explain this? A similar problem arises when looking at p38 and the phospho-p38 MAPK where the pan-p38 shows a significant up-regulation in the quantitative analyses over time but not the phospho-p38 (the upper 2 panels of the quantitative image analyses) whereas the Western blot in the left panel of figure 6B shows such an up-regulation in both instances. This needs to be explained and re- analysed. The rationale to use two different normalisation procedures must be explained or changed.

We agree that the use of both quantification methods may seem confusing and will clarify why we opted for this. Both methods show different components of the observation.
Phosphorylation status of a protein is usually depicted relative to the total protein level (depicted by the left panels of the p-Bcl-2 and p-p38). As in our case both phosphorylated and total protein levels increase similarly this shows as no change in phosphorylated protein relative to its total protein levels. However, as mentioned by the reviewer it is clear from the blots that the total protein levels in fact do show significant increases over time in the control conditions, making this a relevant observation.
Combining the two observations, we indicate that the absolute amount of phosphorylated protein does in fact increase.

11. The authors show error bars in several figures and these indicate either S.E.M. in fig. 2B, or S.D. in fig. 3, 4, 5, 6. The authors should justify as to why they employed these different representations of statistical errors.

We thank the reviewer for this comment and have changed the error bars in fig 2B to S.D. in order to be consistent.

Minor points:

1. Line 543: the term ”different“ is not optimal here and e.g. ”independent“ would be more to the point.
See also Major point 3.

This was amended as suggested by the reviewer

2. Figure 3: The labeling of the right part of fig. 3 is too small and will result in an illegible figure in print.

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Labeling, size of y-axis and symbols should be identical for all panels of the quantitative analyses.

Answer to comment 2, 3, 4 and 6: we thank the reviewer for the above comments and have amended the figures as suggested. This greatly improved the readability of the figures.

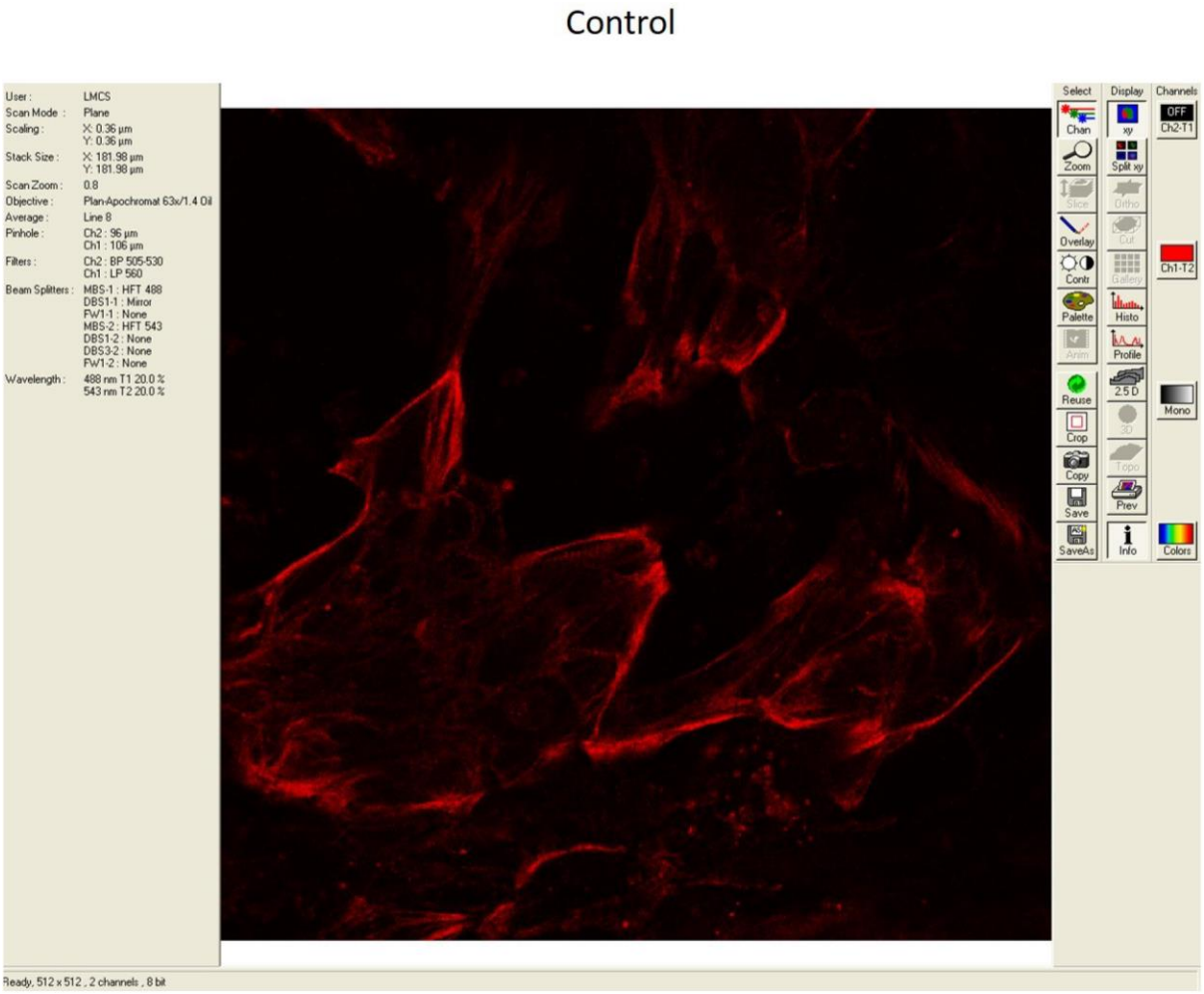
5. Legend to Figure 5, line 584: delete ”does“.

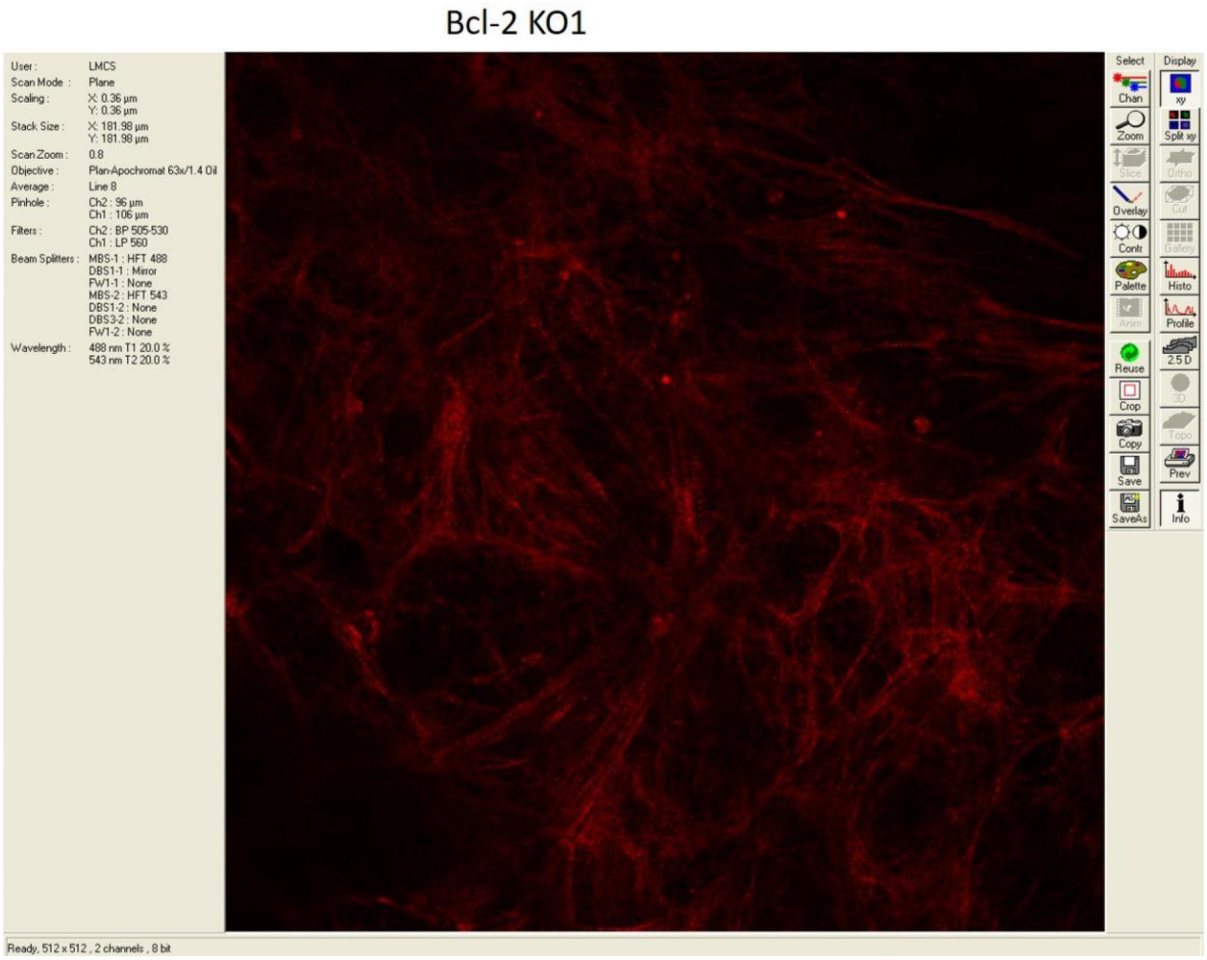
Sentence now reads as **Figure 5: Bcl-2 KO impairs the expression of the cardiac Ca^{2+} toolkit**

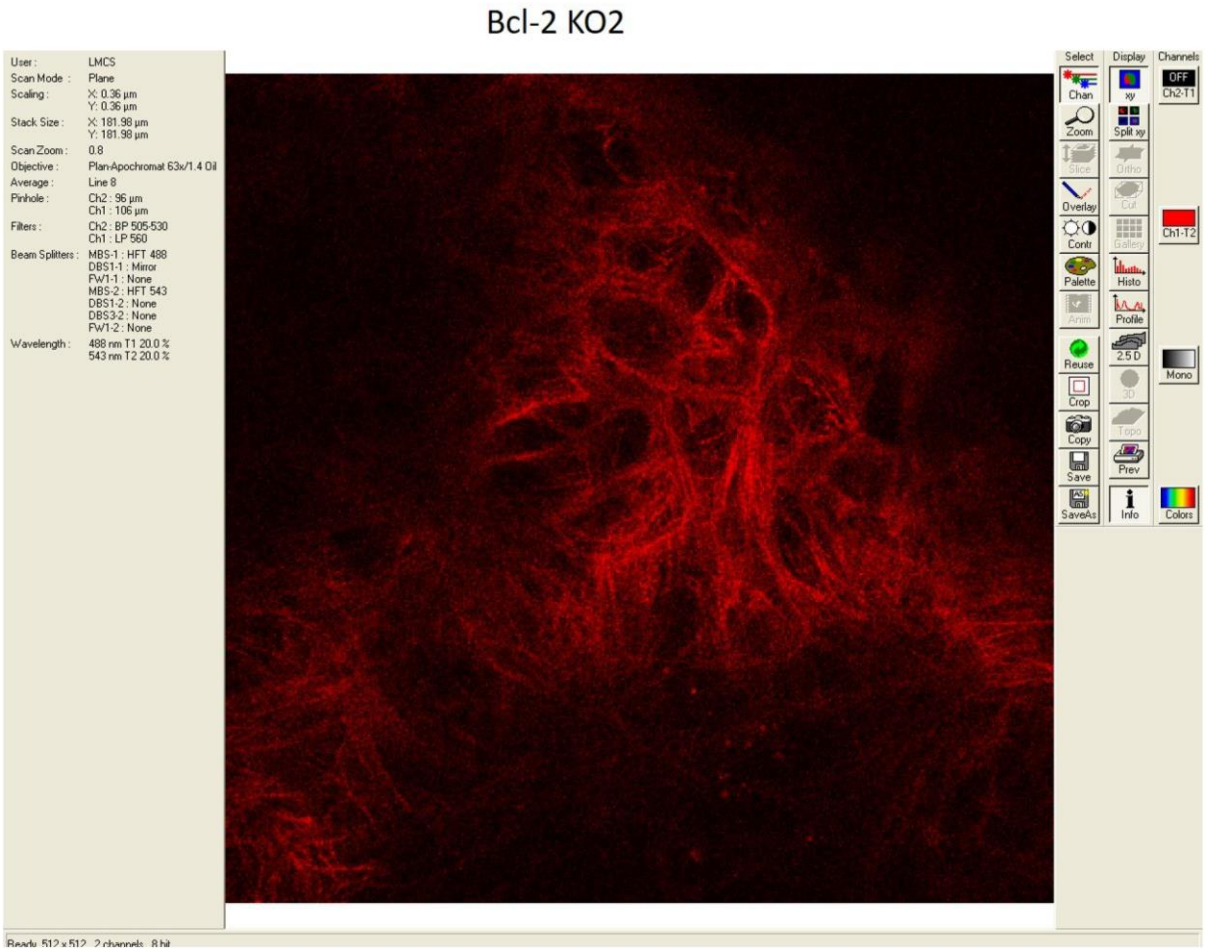
7. Figures 3, 5, 6: Desribe the ”L“ label in the Western blot panels.

“L” was described as being the lane in which molecular weight ladder was loaded

Confocal images fig 2 settings:







Second decision letter

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ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.