

Section 5 - DETAILS OF REVISIONS / FURTHER RESEARCH REQUIRED FOR RESUBMISSION ONLY

If you have selected recommendations Bi, Bii, Biii, Cii or Ciii, you must provide a detailed statement outlining the requirements for the resubmission. You should bear in mind that the major revisions or additional research outlined in this section will be the basis of the re-examination of the revised thesis. Candidates are expected to respond to those requirements only.

If you have agreed that further research is needed then please try to outline the exact requirements. The Faculty does not normally consider additional re-analysis to be further research. Further research would be expected to comprise additional patient recruitment, further laboratory work, or other data collection.

It is helpful to both candidate and examiners if these revisions and or research requirements are stated clearly and fully. We would recommend that these are presented in a structured format wherever possible (for example, a numbered list). It may be helpful to give some indication of the scope/length of the required changes in some cases, and clearly to distinguish changes that are mandatory, from those that might be considered by the candidate but are not required.

Revisions to the thesis are written, re-analysis of data and quantitative analysis of visual data (IHC images). The required revisions are relatively substantial and would take the candidate >12 weeks to complete. This is the rationale behind category Bi which allows time for major written revisions. The candidate performed well at viva and there is no need to re-examine.

All the details of the required revisions are below AND in the annotated PDF documents supplied by both examiners:

Collated corrections – Fuhui Chen

Please see the PDFs from each supervisor as well

Comments from CD

In the first results chapter, she uses a Rac1b KO mouse to examine mammary gland development in nulliparous, pregnant and lactating dams. She then uses flow cytometry and ex vivo mammosphere assays to determine effects on mammary cell lineage. A major concern of a large part of this data is the lack of quantification of images, meaning that interpretation of data is qualitative. Next, she generated a novel transgenic reporter mouse that enables lineage tracing of Rac1b expressing mammary epithelial cells. This is a major undertaking, and involved extensive in vitro validation of the CRISPR-based approach. It is highly commendable that she managed to generate this transgenic mouse and conduct analysis within the timeframe of her PhD (and with COVID disruptions). The majority of the data around the work-up to the transgenic mouse is sound, however from the interpretation of results, I am not convinced that the candidate fully understands the approach, perhaps because a number of important schematics explaining this were too basic and essential data missing (for example, the Sanger sequencing at the CRISPR targeted sites for key clones should have been included). Her functional analysis of which cells Rac1b is expressed in was generally acceptable, although again, more could have been made of the data such as the inclusion of graphical representation of the FACS data that would show all repeats, rather than representations of the flow cytometry plots. Her final chapter involved crossing the Rac1b-RFP transgenic mouse, or the Rac1b KO mouse to the Her2 model of breast cancer, the NIC mouse. Again, more results could have been obtained for the data, such as tumour weight, grade of tumour etc. Reasons for this will be discussed in the viva. Some controls are missing, for example there is no confirmation that Rac1b is deleted (and that Rac1 is unaffected) in her Rac1b^{-/-}/NIC mouse.

The writing of these thesis was adequate, however in places it was not in enough detail to understand the approach/models and many references are omitted. For example, I am convinced that the Rac1bKO mouse is conditional as it was obtained from the Samson lab (Gudino et al 2021), however, there is no mention of the Cre-driver line in the methods or the results section. In addition, the structure of the thesis was unusual. Most of the introduction was placed at the start of each results chapter, meaning that the aims were not put into context, nor was the significance and novelty of the study. This will be discussed with the Internal examiner, but I would recommend that most of the introduction text within the body of the thesis is moved

to the main introduction section. A list of figures was missing and the choice of stats tests sometimes questionable. Given that many of the imaging data was not quantified, one could argue that some results are overstated, or that the caveats of a lack of quantification not addressed. However, data interpretation was largely correct. Titles of figure legends were not adequate but can be corrected. Some limitations of the study were identified, but no alternative approaches suggested. The discussions at the end of results chapters were insightful and displayed evidence of independent thinking and knowledge of the field, however the final discussion did not add anything, such as placing the study into the wider context of breast and other cancers. I would have liked to have seen discussion on how Rac1b can be therapeutically targeted, and if her model(s)/theories have application to other cancer types.

The work with the two mouse models is novel and suitable for publication if the candidate can explain the data in Figure 37 (this will be discussed in the viva).

If the Internal examiner agrees that further quantification of image data is not required, then a predict pass with minor corrections. If more data needs to be generated, I recommend major corrections.

a. Identify any corrections required to the thesis.

General comments:

1. Move introduction text from the start of results chapters to the main introduction
2. Include list of figures
3. Change figure legends so that they describe the result rather than the approach
4. Include references where indicated on PDF

Introduction:

End of section 1.3: Include a section on the mechanism by which RhoGTPase (focusing on Rac1) lead to changes in cellular morphology that lead to the phenotypes described above (i.e. cell migration and EMT).

Address all comments on Thesis PDF file entitled "CDMOD_Fuhui CHEN Thesis".

Methods:

1. All concentrations should be final concentrations, not "1ul of 10uM"
2. **Address all comments on Thesis PDF file entitled "CDMOD_Fuhui CHEN Thesis".**

Results:

1. In figure legend, make it clear which data is obtained on a C57B6 background, and which on FVB.
2. p.46 – please include a paragraph of which transcription factors are thought to be expressed in the various cell types during lineage commitment, and what is known molecularly about this process.
3. p.79 – major comments about explaining the targeting strategy of the RFP transgenic reported line (please see PDF).
4. p.86 - major comments about explaining the targeting strategy of the RFP transgenic reported line (please see PDF)
5. In all data that is presented as representative FACS plot, please include a graph showing n=3 data so SD can be plotted and statistical analysis conducted.
6. **Address all comments on Thesis PDF file entitled "CDMOD_Fuhui CHEN Thesis"**

General discussion

1. Critically analyse your approach – was it suitable, what could have been done better? What other experiments could you do to further support your hypothesis? What are the next big questions in the field of Rac1b and breast cancer? Mechanistically how do you think Rac1b functions in BC? What can you draw upon from other research in the field (including outside the BC space?)
2. Expand to include the bigger picture. For example, how can you drug target Rac1b specifically? What utility does your Rac1b RFP report mice have for research into other diseases?

Comments from KF

Sections are a little shorter than would be expected. In particular, the main introduction is only 12 pages long and lacks the clarity and detail expected for a thesis. Many terms and concepts are not fully explained and there is little compare and contrast or critical analysis of the literature in the field – something that must be demonstrated for the award of PhD. This can be tested at viva however.

At the end of the introduction there is limited rationale for the work stated, there are no aims and objectives listed or any hypotheses put forward. These can be discussed at viva.

Discussion is limited in each sub-chapter and sometimes integrated into results making it hard to interpret what has been found and if the student understands the limitations or impact of their work. This can be assessed at viva.

Discussion is provided at the end of chapter 3, but this is hard to map back to the data as it is not provided for each subsection of chapter 3 – which in my opinion represents discrete results “chapters”.

Need to split this up to discussions at end of each subsection in chapter 3.

Overall conclusion to thesis is very limited and needs to be expanded.

Future work needs to be added as a discrete section.

- a) Identify any preliminary corrections required to the thesis

See PDF file with corrections as annotations

1. Introduction needs to be expanded to include the detailed information needed and a critical analysis of the state of the art in the field.
2. Diagrams are missing from introduction to show signalling networks of the target pathway
3. Need a section on the therapeutic targeting of the RhoGTPases to contextualise if the thesis findings have therapeutic application and how far we are from achieving that
4. Aims/objectives and hypotheses need to be included, following on from a clearly stated rationale for the project
5. Results in chapter 3 on analysis of phenotype of Rab1b are limited in areas. Add discussion of what is already known and possible compensation mechanisms
6. Results in section 3 contain limited, integrated discussion. Discussion section needs to be included at the end of each section of results: Contextualizing the data with the current literature and critical appraisal of the possible meaning and impact of the data and discuss work that could have further substantiated the conclusions, culminating in future research direction - as per the layout of the discussion section in a peer reviewed manuscript in their research area.
7. Figure legends lack full information in places – add this based on specific comments in the PDF
8. Discussion sections needed at the end of each sub-section (results “chapter”) of section 3.
9. Discussion lack depth and contextualisation of results to the field. Instead, much of the discussion is re-stating of results. This must be heavily added in the revised version, should the student pass oral viva.
10. N number is not always made clear as to what this is from esp. in chapter 4 with MFE and FACS. Is this mice or technical replicates or a combination of both. This will need to be made clear in all the figure legends.
11. There is no section on future work – this needs to be added.

Additional corrections highlighted at viva

- A full diagram of Rac1 and Rac1b signalling is needed. This should show stimulators/inhibitors of the pathway e.g. integrins, upstream activators (specific), downstream effectors (specific) and how these converge on specific genes to elicit the global cellular effects you stated in the thesis e.g. cell proliferation or changes in actin cytoskeleton.
- Full information on everything we know on Rac1b needs to be in the main introduction so we know the context of the state of the art and what is novel for your thesis.
- Including especially in the context of breast cancer and the work done previously or in parallel in the lab e.g. the mRNA levels of Rac1b are higher in breast cancer (Mcf7) versus

normal tissues. This is vital information that contextualises your results and highlights why this could be a good drug target.

- A discussion of the effect of Rac1b on anything other than BCSC is absent – you must consider its function beyond this compartment, not least because it is clearly expressed beyond this compartment in cancer – ie most MCF7 seem to express it, but most MCF7 are not BCSC. Discuss the impact, effect, function Rac1b may have on the tumour microenvironment. Discussion needs to be broader than the narrow focus of the thesis (BCSC). Please amend this issue throughout all discussions in the thesis
- Phenotype and genotype of RFP/Rac mouse combinations not always made clear. Please address.
 - E.g what is the phenotype of the het? I think you said no phenotype in terms of development etc. But what about in stress e.g. cancer? Perhaps the het then has a differential phenotype to the WT.
 - E.g give more information on the mouse you were provided with by the collaborator – what is it, how was it made, what is the phenotype – how have they used and tested this – development? Disease? All this information is needed to contextualise your work.
- Add information on the crystal structure of Rac1b to the thesis
- More information on the spliceosome and how the Rac1 v Rac1b mRNA are produced is needed. What controls this in development, normal adult tissue and in cancer – is it dysregulated – how? What is the impact on Rac1b expression in this instance.
- NSC23766 inhibitor not used to contextualise your data and your assertion that targeting Rac1b only is better therapeutically. Discussion needs to add this drug in and compare and contrast your data and come to a conclusion on what is the best therapeutic strategy moving forward. - ADD
- All reagents in methods should be at final concentration - modify in the thesis to reflect this
- Information needed throughout on the experimental design assistant (or similar software) that you used to power your animal experiments. Power calculations need to be included, mouse numbers per experiments added to text and figure legend and fully justified ie DEMONSTRATE that n=4 mice enough to see statistically significant effects on the parameter you are studying
- Data on histology needs to be quantified, the sample size/effect size justified (i.e. that this was enough mice to see change at the histological level – power calculation) and the statistical analysis done. This applies to all data with histology, but particularly Figure 56, where Ki67 staining needs to be quantified from a range of fields from the full n number of mice in the experiments and blind counted by a colleague.
- For all histology quantification graphically represent the data and add to the figures alongside the “representative” image of the whole cohort.
- HER staining needs to be added to Figure 56A.