MCC is a centrosomal protein that relocalizes to non-centrosomal apical sites during intestinal cell differentiation

Lucian B. Tomaz, Bernard A. Liu, Meroshini M, Sheena L. M. Ong, Ee Kim Tan, Nicholas Stanislaw Tolwinski, Christopher S. Williams, Anne-Claude Gingras, Marc Leushacke and Norris Ray Dunn
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Original submission

First decision letter

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MS TITLE: Mutated in Colorectal Cancer (MCC) encodes a centrosomal protein that relocalizes to the apical membrane during intestinal cell differentiation

AUTHORS: Lucian B. Tomaz, Bernard A Liu, Sheena L. M. Ong, Ee Kim Tan, Meroshini M, Nicholas Stanislaw Tolwinski, Christopher S. Williams, Anne-Claude Gingras, Marc Leushacke, and Norris Ray Dunn
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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 substantial 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.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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 Manuscript by Tomaz et al, describes the cellular localization of the human colon cancer-related protein (MCC). Their main findings are that MCC localizes to the centrosome in proliferating crypt cells and to the MTOC in differentiated villus cells. Their study is based on in-situ stainings and on proteomics data obtained in HEK293 cells, showing the association of MCC with centrosomal genes and on co-localization between MCC and centrosomal genes in HEK cells. They further show that MCC is phosphorylated by a specific CK1 kinase and claim that this phosphorylation is important for its localization at the MTOC.
While I find the topic of this study of potential interest, the data provided are mostly descriptive, lack a temporal dimension, and are not sufficient to support the conclusions drawn by the authors.

Comments for the author

Below please find a list of the main limitations of the study:
1. The authors claim that MCC migrates from the Centrosome to the MTOC. This is not supported by data for several reasons:
a) The dynamics of MCC in cells are not shown.
b) The localization of MCC to the apical membrane does not necessarily mean localization to the MTOC, and no MTOC marker is used in the study.
c) The MTOC and the centrosome share many proteins; hence the localization of MCC to the centrosomes and the MTOC may be via the same protein-protein interactions.
2. The main reason for the authors to suggest centrosomal localization of NCC is based on results obtained in HEK cells, which (as indicated by the authors) do not endogenously express MCC. In figure 3, the authors use IF to substantiate these findings in physiologically relevant cells. However, no centrosomal/ MTOC markers were used, and hence this remained questionable.
3. The conclusion that MCC phosphorylation affects its localization in 3D cyst cells is also problematic. The entire morphology of the cyst is disrupted, and given the many roles of kinases, the documented effect on MCC localization can be indirect.
4. Last, the role of MCC in centrosomes is not addressed. While a model is proposed in the discussion, no experiments were performed to support the model.
To conclude, live imaging, in-situ co-localization with centrosomal and MTOC markers, and performing experiments that will allow suggesting a feasible model for the role of MCC in centrosomes will considerably improve the manuscript.

Reviewer 2

Advance summary and potential significance to field

Tomaz et al. report that there is confusion in the field regarding the cellular and subcellular localization of MCC transcript and protein. In this study they first identify the crypt cells of the small intestine as the location for MCC transcription and then they use the identification of associating partners by mass spec to lead them to localize protein to the centrosome in the Crypt cells and they produce evidence that the protein moves to the apical surface when cells become villi cells. The authors identify CK1 epsilon and delta as a kinase that phosphorylates MCC and likely influences its affinity for associating proteins at the centrosome.

This work employs strategic use of proteomics. biochemistry, and imagining strategies to investigate the expression, location, and regulation of MCC. It provides important foundational information for future discoveries regarding how MCC functions and how disfunction causes disease. This work will likely be a contribution to the field suitable for publication after concerns discussed below are addressed.

Comments for the author

Below are specific comments about the text and figures. In broad strokes, the biggest concerns I have are that the data is not all presented in a way that supports the conclusions that are being drawn. In addition, in an effort to define a mechanism, the authors have extended their conclusions beyond the data to create a model. The current work does not need this model in order to be valuable.
Essential 1)Discuss the mass spec strategy in the text. Why did you use a gel-digest and a gel-free approach? Why are there differences between the results of each method?
Does that tell you anything?
2) Figure 1H - Please explain why no MCC transcript is detected in the Mcc lacZ/lacZ crypts. I must be missing something because I thought this animal expressed LacZ under the promoter of MCC and that the animals would have wild type levels of MCC.
3) Figure 2E - The shape of the MCC signal in the two panels is different. In the cell with Pcnt the MCC appears to be a few internal Puncta, but in the cell with Nenien, MCC appears elongated and more dispersed. Additional angles and or additional examples could resolve this.
4) Figure 3A and 3B and 3F - The punctate signal in 3A and 3B is consistent with the hypothesis that MCC is localized to the centrosome in tissue. However demonstrating that MCC is centrosome localized is only accomplished by the co-localization with an established marker, such as pericentrin in 3F. 3F is not conclusive because we cannot see the Pcnt signal clearly in the crypt without single color channels.
5) Figure 3C and 3D - co-stain with a centrosome marker and determine if any MCC remains associated with the centrosome.
6) Figure 3C and 3D - Please include panels (or insets of panels) showing a single channel.
7) Figure 4D - Quantitation of replicate experiments in addition to the displayed panel is necessary to support the conclusions regarding changes in MCC association with RASL2 and NDE1 upon phosphorylation 8) Beta-catenin was not detected in the proteomic analysis. Although the manuscript says that MCC "overlaps with beta-catenin specifically at the apico-lateral junction" there is little evidence of contact or interaction. the junction complexes create boundaries between the apical and basolateral surfaces. It seems likely that at high resolution beta catenin and MCC would be on different sides of the junctions. In addition, the fraction of the total pool of each of these proteins at the interface is very small. These data cannot be used to support the 9) Figure 4 - E, F, G and H - I have several questions related to the organoids.
They are cool but I am not sure they are conclusive.
a) Where are the centrosomes? Can you find MCC at the centrosomes?
b) Are the organoids representative of both villa and crypts? if so, which portions are imaged in G and H? The WT signal looks like the crypts in Fig. 3.
It would be good to show that there are differences in MCC localization in both crypt and villus like regions of organoids.
c) The P-670462 influences overall organoid development (likely through cell division defects). Are the drug treated organoids polarized? Is there an apical membrane that MCC could go to?
Recommended 1) Provide full mass spec dataset as a supplemental file. Include information that can provide information about the confidence or frequency of individual hit discovery.
2) Revise images using a different coloring scheme that is accessible for more readers (see the journal instructions regarding manuscript preparation - last paragraph of section 4.1 3) Provide higher magnification inset of centrosomes in each channel for figure 2C, 2D 4) Wherever possible include the single channels images in addition to the merged image (not needed for Hoechst). In some images like Sup Fig. 3B, it would be sufficient to show the single channel of only the zoomed images.
5) Strengthen the discussion of the difference in location of MCC transcription (Crypt only) and MCC protein (crypts and villi). This is interesting and it is only highlighted in the last sentence of the 1st paragraph of the discussion.
Opportunities 1) It would be interesting to know if you could detect Ezrin association with MCC using a pulldown assay. Could you do the pulldown on organoid lysates or villa tissue lysate?

2) There is additional MCC signal in some of the images that suggest that MCC might also localize to centriolar satellites. It would be interesting to stain with a centriolar satellite marker and look and see if MCC is in both.
3) If you generate a movie from the data presented in Fig. 2E, others could more fully appreciate the 3D information in the SIM data.
4) I don't see MCC listed as identified in a proteomic study that identified binding partners of CK1 delta and epsilon (Guillen et al Sci Reports 2020).
There are several proteomic studies of centrosomes. Can you find MCC included in any of those?
5) The closing paragraph of the discussion is interesting and could be strengthened by incorporating more ideas related to the data presented. For example, can centrosome localization or movement of MCC be related to the concepts discussed?

Important Details
1) In the pdf, but not the Word file, the text in many figures is unclear. There may have been a conversion error.
2) Good you have negative controls for anti-MCC antibody. The centrosome can be "sticky" and the controls you have do a good job demonstrating that the MCC detected is likely legitimate.
3) In the methods section, additional information is needed:
a) The antibody dilutions used for both biochemistry and imaging experiments
b) What buffers were used during IF staining and western blot incubations?
Just PBS? or PBS with some Triton?
c) What objectives were used for imaging?
d) Be specific regarding all post-image processing you performed in Fiji?
e) Please clarify - for the organoid cultures, did you change the media on Days 3, 4, and 5, and add fresh inhibitor to maintain a concentration of 5 micromolar or did you add additional drug each day to the same media?

First revision

Author response to reviewers' comments

Reviewer 1 – Responses to the comments:

1. The authors claim that MCC migrates from the Centrosome to the MTOC. This is not supported by data for several reasons:

a) The dynamics of MCC in cells are not shown.

We interpret the comment “dynamics of MCC in cells” to mean that the Reviewer is requesting live cell imaging of MCC protein (Commnet 5 below) as it relocalizes from the centrosome (MTOC) in proliferating crypt cells to the apical membrane (ncMTOC) in differentiated villus cells. Indeed, this is a fascinating and exciting, yet technically challenging question. We are currently building the requisite experimental tools to pursue live cell imaging of Mcc relocalization in murine intestinal organoids. We have already designed and tested gRNAs for CRISPR/Cas9 gene editing of the *Mcc* locus that will allow the introduction of an N-terminal fluorescent reporter (*e.g.*, GFP or tdTomato) into the 5’ end of the *Mcc* gene via homologous recombination (Schwank and Clevers, 2016). The modified allele will encode a fluorescent Mcc fusion protein that is wild-type in function, and we anticipate that the relocalization of Mcc from the centrosome to the ncMTOC can be captured with time-lapse confocal live imaging. These experiments are underway.

Respectfully, however, we believe that such sophisticated and time-consuming experiments will only generate supplemental information to the main findings of our current submission, and therefore are not critical to underpin the data presented. We advocate that our current findings detailed in this revised manuscript provide exhaustive and incontrovertible evidence that finally repositions MCC as a novel centrosomal protein after more than 30 years since its discovery and lingering controversy surrounding its subcellular localization.

To better convey our findings, we have incorporated insets of both merge and individual channels showing at high magnification the co-localization between Mcc and Pericentrin, which is a well-established component of the centrosome in crypt cells and the apical domain of villus cells (ncMTOC) (**Figure 3** and **Supplementary Figure 3**). Additionally, we have included new images showing the co-localization between Mcc and Ninein, another well- established centrosome component previously described to relocalize to the apical ncMTOC of villus differentiated cells (Goldspink et al., 2017a; Muroyama et al., 2018), **Supplementary Figure 3G**. In the new **Supplementary Figure 4A**, we show the co-localization of Mcc and Ninein at both the centrosome in crypt cells and the apical membrane of differentiated cells in mouse intestinal organoids. More examples of the distinct localization of Mcc in proliferating versus differentiated cells can be observed in **Supplementary Figure 4-B**, **C**, and **E**.

Altogether, these data demonstrate the dynamic relocalization of Mcc from the centrosomal punctum in crypt cells to the apical membrane of differentiated cells *in vivo* mouse and human intestinal epithelium and *ex vivo* intestinal organoids (**Figure 3** and **4** and **Supplementary Figure 3** and **4**).

b) The localization of MCC to the apical membrane does not necessarily mean localization to the MTOC, and no MTOC marker is used in the study.

See response to Comment 01 (a). We now introduce two different centrosomal markers (Pericentrin and Ninein) in our immunolocalization studies of Mcc in intestinal cells.

c) The MTOC and the centrosome share many proteins; hence the localization of MCC to the centrosomes and the MTOC may be via the same protein-protein interactions.

We wish to clarify that the centrosome is a canonical microtubule-organizing center (MTOC) in most dividing cells. However, centrosomes are often inactivated during cell differentiation, and the MTOC function is reassigned to non-centrosomal microtubule-organizing centers (ncMTOC). Several components of the centrosome have also been described to relocalize to non-centrosomal MTOCs during cell differentiation (Muroyama and Lechler, 2017; Muroyama et al., 2016; Sanchez and Feldman, 2017). Specifically, differentiated villus cells, which lack centrosomes, have γ-tubulin and Ninein at the apical ncMTOC (Goldspink et al., 2017b, 2017a; Komarova and Vorob'ev, 1994; Muroyama et al., 2018; Salas, 1999). The localization of Mcc at the apical membrane of intestinal differentiated cells is independent of its interactions at the centrosome, as the centrosome is inactive in these cells. We also demonstrated that CK1ε phosphorylation changes MCC affinity to NDE1 (**Figure 4D** and **Supplementary Figure 2C**), a canonical centrosomal protein not reported at apical membrane of villus cells.

2. The main reason for the authors to suggest centrosomal localization of NCC is based on results obtained in HEK cells, which (as indicated by the authors) do not endogenously express MCC. In figure 3, the authors use IF to substantiate these findings in physiologically relevant cells. However, no centrosomal/ MTOC markers were used, and hence this remained questionable.

We wish to clarify with the reviewer that in our manuscript we specify that HEK293 cells were selected for the mass spectrometry experiments because these cells, in fact, express MCC endogenously (Arnaud et al., 2009). (**Results** section **'Proteomics analyses reveal MCC as a centrosomal protein',** first paragraph).

In **Figure 3**, we showed the co-localization between Mcc and Pericentrin (Pcnt), which is a well-characterized centrosomal marker. Notably, the Mcc signal observed by immunofluorescence in **Figure 3** is characteristic of centrosome localization in proliferating cells of the intestinal crypt compartment. In these cells, the centrosome is found concentrated in a punctum that is apically situated as seen in **Figure 3A-B** and **H,** and previously described (Goldspink et al., 2017b, Muroyama et al., 2018). For example, the canonical centrosome protein Ninein localizes in apical puncta at the centrosomes of crypt proliferating cells and at the apical membrane in differentiated cells of villus-like domains (Goldspink et al., 2017a, Muroyama et al., 2018). Similarly, we observe Mcc localizing at the centrosomal punctum in crypt cells and the apical membrane of differentiated cells (**Figure 3** and **Supplementary Figure 3 and 4**).

To address the reviewer's concern and conclusively establish the localization of Mcc at the centrosome, we have incorporated insets showing the co-localization between Mcc and Pcnt (merge and individual channels) at high magnification at the centrosome of crypt cells and the apical domain of villus cells (**Figure 3F-H**). Additionally, we have included new images showing the co-localization between Mcc and Pcnt and Mcc and Ninein, another established centrosome marker, at the apical membrane of villus cells in **Supplementary Figure 3F** and **G,** respectively.

3. The conclusion that MCC phosphorylation affects its localization in 3D cyst cells is also problematic. The entire morphology of the cyst is disrupted, and given the many roles of kinases, the documented effect on MCC localization can be indirect.

We have included new supplementary material (**Supplementary Figure 4**) showing immunohistochemistry images to address these concerns.

We agree with the reviewer that the morphology of the PF-670462-treated is affected. However, these organoids present fully polarized enterocytes, as demonstrated by apical alkaline phosphatase staining (**Supplementary Figure 4G-H)**, which is observed along the apical membrane of villus cells. The enzyme alkaline phosphatase is uniquely secreted by the brush border (microvilli) of differentiated cells of intestinal villi (Sato et al., 2009; Sussman et al., 1989). Additionally, we also observed cell proliferation in the crypt domain of PF-670462- treated organoids as demonstrated by Ki67 immunostaining (**Supplementary Figure 4E-F)**. It is likely that the CK1δ/ε inhibitor influences overall organoid development, as we see fewer Ki67 positive cells in the crypt domain and the overall size of PF-670462-treated organoids is relatively smaller compared to non-treated controls (**Figure 4E-H** and **Supplementary Figure 4C-H**).

Importantly, it has been previously demonstrated that genetic depletion of CK1δ/ε significantly affects the intestinal stem cell niche *in vivo* and *in vitro* (Morgenstern et al., 2017). Our organoid experiment presented in **Figure 4** and **Supplementary Figure 4** was based on this study, and the culture conditions were strictly controlled. Specifically, a constant concentration of 5 μM of the CK1δ/ε inhibitor (PF-670462) (Badura et al., 2007) over 3 days was sufficient to prevent the relocalization of Mcc from the centrosome to the apical membrane of differentiated cells without affecting the ability of intestinal *Lgr5*+ stem cells to generate organoids.

Lastly, both Casein Kinases 1 delta and epsilon (CK1δ and CK1ε) were identified as direct interactors of MCC by our mass spectrometry experiments (**Figure 2A**). The physical interaction between MCC and CK1ε was further confirmed by independent immunoprecipitation experiments (**Figure 2B**). We have also demonstrated that CK1ε enzymatic activity in HEK293 and SW480 cells results in the phosphorylation of MCC, altering its molecular weight (93 kDa for the wild type) and its binding affinity for interacting proteins (**Figure 4**) whose identity was revealed by our proteomic analysis. We also present evidence that inhibition of CK1δ/ε activity in intestinal organoids directly affects the Mcc protein, impairing its redistribution from the centrosome to the apical membrane of terminally differentiated cells in intestinal organoids (**Figure 4E-H** and **Supplementary Figure 4C-F**).

4. Last, the role of MCC in centrosomes is not addressed. While a model is proposed in the discussion, no experiments were performed to support the model.

We wish to share with the Reviewer that, although *Mcc* null mutant mice were reported by us to be viable and fertile with no ostensible phenotypes (Young et al., 2011), after introducing the Mcc^{lacZ} null allele onto an inbred, sensitized C57BL/6 genetic background, several prominent phenotypes emerged among the homozygotes. One of which can be divined from the images in **Supplementary Figure 1C**, attention to which is not drawn in the manuscript— that is, there is a prominent villus phenotype in *Mcc* ($Mcc^{-/-}$) null mutant animals. The origins of this phenotype (abnormal villus morphology accompanied by barrier defects) have been extensively investigated by our group, and our unpublished results have provided us with abundant insight into the role of Mcc not only in the maintenance of intestinal homeostasis, but also in the context of disease. Our findings will be described in a follow up, seven-figure manuscript that will be submitted in the next several months. We acknowledge that data regarding the "role of MCC in centrosomes" are of fundamental

interest to the Reviewer, but such data are not essential to support this brief, four-figure initial submission that focuses exclusively on the Mcc protein, demonstrating its subcellular localization, elaborating its interactome for the first time as well as the signaling pathways governing its redeployment from the centrosome to the apical membrane. In the Time of COVID, we encountered numerous challenges with animal experiments and the procurement of reagents, and we have opted for expediency to divide the Mcc story into two parts, with the submission of Tomaz *et al*. to the *Journal of Cell Science* being the first installment.

Regarding the Model in **Figure 4**, we rigorously reinterrogated our original MCC interactome data (see response to Reviewer 2) and have extensively revised the description of these data in the revised manuscript. Consequently, we promulgate that the results underpinning our model have been significantly strengthened even further. We solidly agree that a "Model is just a Model" and experiments are underway to test our model both *in vivo* and *in vitro*. If the Reviewer firmly believes that our Model is unsubstantiated, it can be removed altogether from **Figure 4** and the Discussion.

5. To conclude, live imaging, in-situ co-localization with centrosomal and MTOC markers, and performing experiments that will allow suggesting a feasible model for the role of MCC in centrosomes will considerably improve the manuscript.

These points were thoroughly addressed in our above responses.

Reviewer 2 – Response to the comments:

Essential:

1. **Discuss the mass spec strategy in the text. Why did you use a gel-digest and a gel- free approach? Why are there differences between the results of each method? Does that tell you anything?**

MCC affinity purifications (AP) for mass spectrometry (MS) were analyzed using both gel- based and gel-free approaches with the intent of demonstrating consistency (overlap) in the identification of MCC interactors with both methodologies. We agree with the Reviewer that the reasoning for including both data sets in our first submission was unclear and somewhat confusing, and admittedly not necessary for the story.

The MS samples that underpin this manuscript were originally acquired at a time when the proteomics workflow (carried out in the laboratories of the late Tony Pawson (RIP 2013) and our current co-author Anne-Claude Gingras) was transitioning between gel-based and gel- free approaches. Since we did not produce enough replicates for gel-based AP-MS that would allow us to better evaluate whether the differences with the gel-free approach were reproducible, we have removed the gel-based data from the revised manuscript and re- analyzed the replicates of the gel-free AP-MS samples. We now present (with emphasis on **gel-free**) proteomic results of triplicate analysis of FLAG-MCC purifications in comparison to an empty vector control, also purified and analyzed in triplicates (**Figure 2A, Supplementary Figure 2,** and **Table 02**).

Additionally, we have also formalized the data analysis of the FLAG-MCC purifications by using Significance Analysis of INTeractome (SAINTexpress), which enabled us to stringently define high-confidence interactors, i.e., those that pass a Bayesian False Discovery Rate threshold of 1%. All interactions passing this threshold are presented in a new Table **(Table 02** in the **Supplemental Materials)**, alongside spectral counts, the unique number of peptides associated with each protein across the triplicates, and statistical scores. We have also included a diagram (**Supplementary Figure 2A**) of high-confidence MCC interactions indicating previously reported interactions from the BioGRID interaction database (thebiogrid.org), protein expression levels from Proteomics DB (proteomicsdb.org), and RNA expression levels from Protein Atlas (proteinatlas.org). Lastly, all MS datasets associated with the complete protein evidence and the full SAINTexpress file are now deposited in the public repository for MS results MassIVE (Mass Spectrometry Interactive Virtual Environment), which is a member of the ProteomeXchange consortium.

MassIVE ID: MSV000089258.
ProteomeXchange: PXD03326. Direct link:
https://massive.ucsd.edu/ProteoSAFe/dataset.jsp?task=0a7c6e08c0d14dd8933e887a0575d26e

We have accordingly revised the **Materials and Methods** section (**Immunoprecipitation and Mass Spectrometry**), **Figure 2A**, **Supplementary Figure 2**, the **Results** section (**Proteomics analyses reveal MCC as a centrosomal protein),** and the **Discussion** to comprehensively clarify our MS experimental strategy. New citations have been included in the manuscript to reflect these changes.

2. **Figure 1H - Please explain why no MCC transcript is detected in the Mcc lacZ/lacZ crypts. I must be missing something because I thought this animal expressed LacZ under the promoter of MCC and that the animals would have wild type levels of MCC.**

In this manuscript, we employed the Mcc^{lacZ} reporter allele (officially $Mcc^{Gt(D062B07)GGTC}$) that was generated by a gene-trap insertional mutation previously characterized by our group (Young et al., 2011). This gene-trap functions as an *Mcc* reporter allele and knockout model, wherein ß-galactosidase expression is driven by the endogenous *Mcc* promoter and the presence of the strong polyadenylation sequence within the gene trap cassette downstream of the first coding exon results in premature termination of the *Mcc* transcript. Further analysis by Western blot and IF reveals that no Mcc protein is detectable in assorted tissues (Young *et al.*, 2011 *Developmental Dynamics* and **Figure 3E** and **Supplemental Figure 3C** and **D** of this resubmission). The Mcc^{lacZ} gene-trap reporter allele is therefore a loss-of-function mutation. The Reviewer is invited to review additional experimental evidence supporting this conclusion in Figure 3 of Young et al., 2011.

To clarify this, we have amended the **Results** section '***Mcc* is specifically expressed in crypts of the intestinal epithelium**' [Page 3 ; Line 92-95] as the following:

> "We previously reported that *Mcc* is expressed in the adult mouse intestine using a Mcc^{lacZ} reporter allele generated by a gene-trap insertional mutation that results in premature termination of the *Mcc* transcript and, consequently, produces no Mcc protein (Young et al., 2011)."

We additionally amended **Figure 1H** legend text as:

> *"(…) (H)*: qPCR analysis for *Mcc* and *Lgr5* in purified crypt and villus fractions from WT and homozygous Mcc^{lacZ} (*Mcc* null)."

3. **Figure 2E - The shape of the MCC signal in the two panels is different. In the cell with Pcnt the MCC appears to be a few internal Puncta, but in the cell with Nenien, MCC appears elongated and more dispersed. Additional angles and or additional examples could resolve this.**

We agree with the Reviewer's observations. To address this issue, we have included a new image showing MCC and PCNT co-localization using 3D structured-illumination microscopy (SIM) (**Figure 2 E**). Additionally, we include a 3D-SIM video (**Video 01**) showing MCC and PCNT co-localization at different angles in the **Supplementary Material**.

4. **Figure 3A and 3B and 3F - The punctate signal in 3A and 3B is consistent with the hypothesis that MCC is localized to the centrosome in tissue. However, demonstrating that MCC is centrosome localized is only accomplished by the co- localization with an established marker, such as pericentrin in 3F. 3F is not conclusive because we cannot see the Pcnt signal clearly in the crypt without single color channels.**

We thank the reviewer for raising this important point. We indeed agree that to conclusively claim that Mcc resides at the centrosome in intestinal crypt cells, it is necessary to demonstrate that Mcc co-localizes with another well-characterized centrosome marker such as Pericentrin (Pcnt). In the revised manuscript, we have therefore incorporated insets showing the co- localization between

Mcc and Pcnt (merge and individual channels) in high magnification at the centrosome of crypt cells and the apical domain of villus cells (**Figure 3F-H**). Additionally, we have included new images showing the co-localization between Mcc and Pcnt and Mcc and Ninein, another well-established centrosome component, at the apical membrane of villus cells in **Supplementary Figure 3F** and **G,** respectively.

5. **Figure 3C and 3D - co-stain with a centrosome marker and determine if any MCC remains associated with the centrosome.**

Figures 3C and D of our original manuscript showed Mcc localization at the apical membrane of mouse intestinal villus cells.

In villus differentiated cells, the centrosomes are inactive (Goldspink et al., 2017b, 2017a; Komarova and Vorob'ev, 1994; Muroyama et al., 2018). Although these cells maintain Centrin puncta on their apical side, microtubules are not enriched around them (Muroyama et al., 2018). Nevertheless, in the revised manuscript, we present data showing Mcc co- immunostaining with two centrosome components that relocalize to the apical ncMTOC of villus differentiated cells, Pericentrin and Ninein (**Figure 3F-H** and **Supplementary Figure 3F-G**).

6. **Figure 3C and 3D - Please include panels (or insets of panels) showing a single channel.**

We have now included insets showing the individual channel for Mcc immunolocalization in mouse small intestine crypt and villus cells (**Figure 3A-D** and **Supplementary Figure 3C-D).**

Additionally, we have included insets showing the individual channel for MCC localization in human small intestine crypt and villus cells (**Supplementary Figure 3A-B)** and for Mcc immunolocalization in mouse colonic cells (**Supplementary Figure 3E).**

7. **Figure 4D - Quantitation of replicate experiments in addition to the displayed panel is necessary to support the conclusions regarding changes in MCC association with RASL2 and NDE1 upon phosphorylation.**

We wish to clarify with the reviewer that the biochemistry experiments in this study were performed using biological replicates (of at least N = 3). Unfortunately, some of the data are inaccessible due to unforeseen circumstances. Nevertheless, we have included quantification analysis for **Figure 4D** to allow a better assessment of the data presented (please, see **Supplementary Figure 2C**). Western Blot quantification analysis was performed using ImageJ following Hossein Davarinejad's method (York University - Canada). (https://www.yorku.ca/yisheng/Internal/Protocols/ImageJ.pdf).

8. **Beta-catenin was not detected in the proteomic analysis. Although the manuscript says that MCC "overlaps with beta-catenin specifically at the apico-lateral junction" there is little evidence of contact or interaction. the junction complexes create boundaries between the apical and basolateral surfaces. It seems likely that at high resolution beta catenin and MCC would be on different sides of the junctions. In addition, the fraction of the total pool of each of these proteins at the interface is very small. These data cannot be used to support the**

Although our proteomics studies did not identify ß-catenin as an interactor of MCC, such interaction has been previously demonstrated *in vitro* using HCT116 colon cancer cells (Benthani et al., 2018). Additionally, another study using *in vitro* overexpression experiments has shown that MCC binds ß-catenin in the nucleus to negatively regulate canonical WNT signaling in cancer cell lines and to inhibit cell proliferation (Benthani et al., 2018; Fukuyama et al., 2008).

However, we agree with the reviewer that the co-localization between MCC and ß-catenin analyzed by conventional confocal microscopy in our manuscript is not sufficient to validate their interaction *in vivo*. Therefore, we have removed the images showing co-staining between MCC and ß-catenin from **Supplementary Figure 4G** and **H** of the original manuscript. We have amended the text to avoid this conclusion and our model diagram for the relocalization of MCC has also been revised (**Figure 4J)**

9. Figure 4 - E, F, G and H - I have several questions related to the organoids. They are cool but I am not sure they are conclusive.

a) Where are the centrosomes? Can you find MCC at the centrosomes?

b) Are the organoids representative of both villa and crypts? if so, which portions are imaged in G and H? The WT signal looks like the crypts in Fig. 3. It would be good to show that there are differences in MCC localization in both crypt and villus like regions of organoids.

Response to comment 9 (a) and (b):

In intestinal crypt cells, the centrosomes are found concentrated in a punctum at the apical domain as seen in **Figure 3A-B** and **H,** and previously described (Goldspink et al., 2017b, 2017a; Muroyama et al., 2018). Notably, *ex vivo* intestinal organoids are reported to faithfully mimic the cellular composition, stem-cell hierarchy, and epithelial cell architecture of the *in vivo* intestinal epithelium (Sato et al., 2009), including centrosomal and microtubule reorganization during differentiation (Goldspink et al., 2017b, 2017a).

Morphologically, mouse small intestine organoids consist of a central lumen lined by villus-like differentiated cells and numerous surrounding budding crypt-like domains (Sato et al., 2009). Fully differentiated epithelial cells of the villus exhibit distinct apical ncMTOCs that line the central lumen. For example, the centrosome protein Ninein localizes in puncta at the centrosomes of crypt proliferating cells and along the apical membrane in differentiated cells of villus-like domains (Goldspink et al., 2017b, 2017a; Muroyama et al., 2018). Similarly, we observe Mcc localizing at the centrosomal punctum in crypt cells and the apical membrane of differentiated cells in intestinal organoids (**Figure 4G**).

To better allow the assessment of Mcc centrosomal and apical localization, we have included a new figure (**Supplementary Figure 4**) to show the distinct subcellular localization of Mcc at the centrosome and apical ncMTOC in villus cells of mouse intestinal organoids. Accordingly, we have revised the text to reference the new data (**Discussion** - third paragraph and **Results** section '**Phosphorylation by CK1δ/ε triggers MCC redeployment to the ncMTOC at the apical membrane of villus cells',** last paragraph).

In **Figure 4-G and H** (same in the revised manuscript), the high magnification insets show differentiated cells of the villus-like domains. Note Mcc localization in **Figure 4G** (WT organoid) distributed along the apical membrane of differentiated cells, facing the lumen.

Additionally, in the new **Supplementary Figure 4A**, we show the co-localization of Mcc and Ninein in both the centrosome in crypt cells and the apical membrane of differentiated cells (villus-like domain). More examples of the distinct localization of Mcc in proliferating versus differentiated cells can be observed in **Supplementary Figure 4-B**, **C**, and **E**.

c) The P-670462 influences overall organoid development (likely through cell division defects). Are the drug treated organoids polarized? Is there an apical membrane that MCC could go to?

We have included new immunohistochemistry images in **Supplementary Figure 4** to address these questions.

Indeed, it is likely that the CK1δ/ε inhibitor influences overall organoid development, as we see fewer Ki67 positive cells in the crypt domain (**Supplementary Figure 4E-F)** and the size of PF670462-treated organoids is relatively smaller compared to non-treated controls (**Figure 4E-H** and **Supplementary Figure 4C-H**). However, the treatment with the CK1δ/ε inhibitor in our assay does not affect intestinal cell differentiation in organoids. PF670462-treated organoids exhibit fully polarized enterocytes, as demonstrated by apical alkaline phosphatase staining (**Supplementary Figure 4G-H)**, which is observed along the apical membrane of villus cells, facing the central

lumen. The enzyme alkaline phosphatase is uniquely secreted by the brush border (microvilli) of differentiated cells of intestinal villi (Sato et al., 2011; Sussman et al., 1989).

A previous study demonstrated that genetic depletion of CK1δ/ε dramatically affects the intestinal stem cell niche *in vivo* and *in vitro* (Morgenstern et al., 2017). Our organoid experiment presented in **Figure 4** and **Supplementary Figure 4** was based on this study, and the culture conditions were strictly controlled. Specifically, a constant concentration of 5 μM of the CK1δ/ε inhibitor (PF670462) (Badura et al., 2007) over 3 days was sufficient to prevent the relocalization of Mcc from the centrosome to the apical membrane of differentiated cells without affecting the ability of intestinal *Lgr5*+ stem cells to differentiate.

Recommended:

1. **Provide full mass spec dataset as a supplemental file. Include information that can provide information about the confidence or frequency of individual hit discovery.**

This recommendation has been addressed. Please, refer to our response for the **Essential - Comment 1**.

2. **Revise images using a different coloring scheme that is accessible for more readers (see the journal instructions regarding manuscript preparation - last paragraph of section 4.1**

We thank the reviewer for this important observation. In the revised manuscript, we have kept the same color scheme as the original manuscript in order to maintain the quality of the data and to allow assessment of the conclusions made in the text. However, we have included insets displaying single channels for most fluorescent microscopy images to make them comprehensible to more readers. In some cases, the insets displaying single channels are also labeled to indicate the marker used, allowing better interpretation of the data presented.

3. **Provide higher magnification inset of centrosomes in each channel for figure 2C, 2D**

We thank the reviewer for this suggestion. We have included additional high magnification insets displaying single channels for MCC and other centrosomal proteins in **Figure 2C and D**.

4. **Wherever possible include the single channels images in addition to the merged image (not needed for Hoechst). In some images like Sup Fig. 3B, it would be sufficient to show the single channel of only the zoomed images.**

We thank the reviewer for this suggestion. We have included additional high magnification insets displaying single channels for MCC and other centrosomal proteins in **Figure 3A – D**, **Supplementary Figure 2D** and **E**, **Supplementary Figure 3A – G**, and **Supplementary Figure 4A – F**.

5. **Strengthen the discussion of the difference in location of MCC transcription (Crypt only) and MCC protein (crypts and villi). This is interesting and it is only highlighted in the last sentence of the 1st paragraph of the discussion.**

We agree with the reviewer and have now redrafted the first and second paragraphs of the **Discussion** to further explore this topic. The new citations have been updated in the **References**.

Opportunities:

1. **It would be interesting to know if you could detect Ezrin association with MCC using a pulldown assay. Could you do the pulldown on organoid lysates or villa tissue lysate?**

Interestingly, a previous study of protein-protein interactions in human cells using a MS-based approach identified MCC as an interactor of Ezrin (Ewing et al., 2007). It remains to be determined whether the interaction of MCC to Ezrin is direct or indirect, especially given that MCC directly interacts with NHERF1/2, which are scaffold proteins that form apical protein complexes with Ezrin

(Weinman et al., 2006). We agree with the Reviewer that this is an interesting question, and we are performing a series of co-immunoprecipitation assays to compare the interactions of endogenous Mcc at the centrosome versus apical membrane in lysates of purified villus and crypt fractions from mouse small intestine. Ezrin is one of the candidates being assessed. However, such experiments reside downstream of the main findings of our current submission and are aimed at addressing future questions regarding Mcc function *in vivo*.

2. **There is additional MCC signal in some of the images that suggest that MCC might also localize to centriolar satellites. It would be interesting to stain with a centriolar satellite marker and look and see if MCC is in both.**

We agree with the reviewer that this is an interesting point to be addressed. We have been screening for antibodies against centriolar satellites proteins that could work for immunolocalization with Mcc in tissues. We have not found a successful candidate yet.

3. **If you generate a movie from the data presented in Fig. 2E, others could more fully appreciate the 3D information in the SIM data.**

We agree with the reviewer's suggestion, and we will include a 3D-SIM video showing MCC and PCNT colocalization in SW480 cells as supplementary material (**Video 01**).

4. **I don't see MCC listed as identified in a proteomic study that identified binding partners of CK1 delta and epsilon (Guillen et al Sci Reports 2020). There are several proteomic studies of centrosomes. Can you find MCC included in any of those?**

We thank the reviewer for this observation. Indeed, endogenous MCC was not recovered as prey in this study by the Gould lab (Guillen et al., 2020). Additionally, we wish to mention that the lab of our co-author Anne-Claude Gingras has been conducting proteomic studies using FLAG AP-MS and the proximity-dependent biotinylation approach BioID across thousands of baits in HEK293 cells (data are available at humancellmap.org and prohits-web.lunenfeld.ca) and detecting MCC endogenously is very infrequent. One probable explanation is the relatively low expression level of MCC in HEK293 cells (*). Nevertheless, in agreement with our findings, a previous large-scale comparative analysis of the interactomes of 32 human kinases has identified MCC as an interactor of CSNK1E (Varjosalo et al., 2013). MCC was also recovered as prey in another independent AP-MS proteomics study in HeLa cells having CSNK1E as a bait (Hein et al., 2015).

(*)https://www.proteomicsdb.org/proteomicsdb/#protein/proteinDetails/54121/expression (in *Biological Source*, tick *Cell Line* box and note MCC expression levels across various human tissues and cell lines).

5. **The closing paragraph of the discussion is interesting and could be strengthened by incorporating more ideas related to the data presented. For example, can centrosome localization or movement of MCC be related to the concepts discussed?**

We thank the Reviewer for this suggestion. We have redrafted our closing paragraph to elaborate better concluding ideas.

Important Details:

1. **In the pdf, but not the Word file, the text in many figures is unclear. There may have been a conversion error.**

We thank the reviewer for this observation. We will alert the editors about this issue.

2. **Good you have negative controls for anti-MCC antibody. The centrosome can be "sticky" and the controls you have do a good job demonstrating that the MCC detected is likely legitimate.**

This is an important point, and we thank the reviewer for this interesting observation. Given the confusion within the literature surrounding MCC subcellular localization, we carefully considered

whether our fixation techniques impacted our interpretation for MCC centrosomal localization. In a previous study, (Hua and Ferland, 2017) thoroughly investigated the effect of buffers and fixatives and their impact on the localization of centrosomal and ciliary proteins after conflicting reports emerged regarding the localization of Centrosome and Spindle Pole Associated Protein 1 (CSPP1). For example, these authors reported that methanol (MeOH) fixation produced less cytoplasmic background while enhancing centrosome and microtubules staining when compared to paraformaldehyde (PFA) fixation in assorted cell lines. In a different study by (Goldspink et al., 2017b), it was reported that PFA fixation commonly results in the loss of antigenicity of centrosomal proteins. These findings were carefully considered when developing the experimental immunostaining protocols described in the Materials and Methods and when interpreting our results. Most significantly, the absence of Mcc staining at the centrosome in intestinal tissue harvested from *Mcc* homozygous null mice validates the specificity of the anti-MCC antibody and allows us to christen Mcc for the first time as a novel centrosomal protein in our study.

3. In the methods section, additional information is needed:

a) The antibody dilutions used for both biochemistry and imaging experiment?

We thank the reviewer for this question and have now clarified that in the **Materials and Methods (Histology** and **Western Blot)** section. Briefly, in both immunohistochemistry and biochemistry experiments, each antibody used was diluted at the optimal concentration following the manufacturer's instructions.

b) What buffers were used during IF staining and western blot incubations? Just PBS? or PBS with some Triton?

We have now included this information in the **Materials and Methods (Histology** and **Western Blot)** section.

c) What objectives were used for imaging?

This information has now been specified in the **Materials and Methods (Imaging)** section.

d) Be specific regarding all post-image processing you performed in Fiji?

We have revised the text in the **Materials and Methods** section **(Imaging)** to clarify our microscopy post-processing image analysis. Briefly, our confocal images were processed using Fiji and 3D-SIM video and images were processed with Imaris.

e) Please clarify - for the organoid cultures, did you change the media on Days 3, 4, and 5, and add fresh inhibitor to maintain a concentration of 5 micromolar or did you add additional drug each day to

We agree that this was not clear in our original manuscript. We have revised the text in the **Materials and Methods** (**Intestinal organoid culture**) section to clarify our methodology for the kinase inhibitor treatment. Briefly, for inhibition of phosphorylation, fresh culture medium (300 µL) containing 5 µM of the Casein Kinase 1 δ/ε inhibitor PF670462 (Sigma #SML0795) was changed on days 3, 4 and 5 of culture to consistently maintain a 5 µM concentration of the inhibitor.

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Second decision letter

MS ID#: JOCES/2021/259272

MS TITLE: MCC is a novel centrosomal protein that relocalizes to the apical ncMTOC during intestinal cell differentiation

AUTHORS: Lucian B. Tomaz, Bernard A Liu, Meroshini M, Sheena L. M. Ong, Ee Kim Tan, Nicholas Stanislaw Tolwinski, Christopher S. Williams, Anne-Claude Gingras, Marc Leushacke, and Norris Ray Dunn
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.organd click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers gave favourable reports but raised some critical points that will require amendments to your manuscript. In particular, reviewer #2 makes some specific suggestions that you will need to carry out in order for us to accept your paper. I hope that you will be able to carry these out because I would like to be able to accept your paper, depending on further comments from 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 revised version includes new experiments that strongly substantiate their findings that MCC is a centrosomal protein and that it localizes to the ncMTOC.
This conclusion was obtained based on several experimental approaches including mass spec, IF of several TC cells, 3D cyst models, and tissues. While these findings are solid, further experiments to demonstrate the relocalization of MCC in live cells and to support a proposed mechanistic model were not performed.
Although I understand the technical difficulties and other considerations raised by the authors in the point-by-point letter I find that the manuscript, in its current state, does not provide conceptual advances or a mechanistic understanding of the function of MCC. Therefore, the manuscript currently does not meet the criteria for publication in JCS.

Comments for the author

non.

Reviewer 2

Advance summary and potential significance to field

The authors demonstrate that MCC - a gene implicated in colon cancer - is transcribed and translated in crypt cells. Mass Spec identified several centrosome proteins that Co-IP with MCC. The nascent protein localizes to centrosomes. Upon differentiation and migration to villi, the MCC protein distributes on the apical surface along with other former centrosome proteins to establish the ncMTOC. The authors investigate MCC localization in both cultured cell, organoids and tissue. In addition, they use biochemical strategies and drug treatment to initiate mechanistic understanding of the change in MCC localization.

The changes made by the authors largely address the essential and recommended comments in my initial review. Due to both the revisions in the experiments and the model, the model is no longer a stretch beyond what the data in the paper support.

If the concerns below were to be addressed, I would have no reservations recommending this study for publication in JCS.

Comments for the author

1 - I am concerned that replicated of the data presented in Figure 4D can not be recovered in order to be quantified. I recommend repeating the experiment.
2 - The revised title includes the term "ncMTOC". This is not an abbreviation that will be obvious to many readers of JCS.
3 - In light of the discussion regarding "dynamics" in the response to reviewers I recommend revising the running title.

Second revision

Author response to reviewers' comments

Reviewer 1 - Advance Summary and Potential Significance to Field:

The revised version includes new experiments that strongly substantiate their findings that MCC is a centrosomal protein and that it localizes to the ncMTOC. This conclusion was obtained based on several experimental approaches including mass spec, IF of several TC cells, 3D cyst models, and tissues. While these findings are solid, further experiments to demonstrate the relocalization of MCC in live cells and to support a proposed mechanistic model were not performed. Although I understand the technical difficulties and other considerations raised by the authors in the point-by-point letter I find that the manuscript, in its current state, does not provide conceptual advances or a mechanistic understanding of the function of MCC. Therefore, the manuscript currently does not meet the criteria for publication in JCS.

We acknowledge and fully appreciate that data regarding the "role of MCC in centrosomes" are of fundamental interest to the Reviewer. Such data, respectfully, are however not essential to support this brief, four-figure submission to *JCS*, an argument which we lengthily articulated in our previous Response to the Reviewers. Tomaz et al. focuses exclusively on the Mcc protein, demonstrating its subcellular localization and elaborating its interactome for the first time (after more than 30 years since its discovery) as well as the signaling pathways governing its redeployment from the centrosome to the apical membrane.

We wish to share with and to remind the Reviewer that, although *Mcc* null mutant mice were reported by us to be viable and fertile with no ostensible phenotypes (Young et al., 2011), after introducing the Mcc^{lacZ} null allele onto an inbred, sensitized C57BL/6 genetic background, several phenotypes emerged among the homozygotes. One of which can be easily divined from the images in Supplementary Figure 1C, attention to which is not drawn in the manuscript—that is, there is a prominent villus phenotype in

Mcc null mutant animals. The origins of this phenotype (abnormal villus morphology accompanied by barrier defects) have been extensively investigated by our group, and our unpublished results have provided us with abundant insight into the role of Mcc not only in the maintenance of intestinal homeostasis, but also in the context of disease. Our findings, addressing the mechanistic function of MCC, will be described in a follow-up, seven-figure manuscript that will be submitted in the next several months. A significant proportion of the data that will be incorporated into this forthcoming submission can be found in the first author's, Dr. Lucian Tomaz's, Ph.D. thesis: https://hdl.handle.net/10356/140724.

Reviewer 1 asserts that "the manuscript currently does not meet the criteria for publication in JCS". As Senior Author, I stridently disagree with this intransigent opinion, one which is wholly unreasonable. As a longtime member of the Advisory Board of *Development*, a sister journal to *JCS*, our work without question crosses the threshold for publication amongst all Company of Biologists journals. Our work is experimentally rigorous, thorough and novel and will be appreciated by hard core cell biologists, developmental biologists and stem cell biologists. My co-authors and I very much appreciate the contrasting, enthusiastic support of Reviewer 2.

Reviewer 2 - Advance Summary and Potential Significance to Field:

The authors demonstrate that MCC - a gene implicated in colon cancer – is transcribed and translated in crypt cells. Mass Spec identified several centrosome proteins that Co-IP with MCC. The nascent protein localizes to centrosomes. Upon differentiation and migration to villi, the MCC protein distributes on the apical surface along with other former centrosome proteins to establish the ncMTOC. The authors investigate MCC localization in both cultured cell, organoids and tissue. In addition, they use biochemical strategies and drug treatment to initiate mechanistic understanding of the change in MCC localization.

The changes made by the authors largely address the essential and recommended comments in my initial review. Due to both the revisions in the experiments and the model, the model is no longer a stretch beyond what the data in the paper support.

If the concerns below were to be addressed, I would have no reservations recommending this study for publication in JCS.

Reviewer 2 - Comments for the Author:

1. **- I am concerned that replicated of the data presented in Figure 4D cannot be recovered in order to be quantified. I recommend repeating the experiment.**

We very much appreciate the concern raised by the Reviewer and wish to emphasize that all biochemistry experiments in this study, including Figure 4D, were performed using biological replicates of at least N = 3, and the data presented in Figure 4D were selected from such biological replicates. Regrettably, repeating this experiment is currently not feasible, and foremost such effort we strongly believe is not necessary to support the conclusions presented in our manuscript.

Why is repeating this experiment currently not feasible? We wish to share with the Editor and the Reviewer the extenuating circumstances we have faced. Bernard Liu, second author of this manuscript, was a postdoctoral fellow in the laboratory of Tony Pawson, the discoverer of the SH2 domain, who died suddenly in 2013. The biochemical experiments presented throughout our manuscript were carried out solely by Bernard in Tony's lab, with mentorship and input from and in close collaboration with our other co-author Anne-Claude Gingras, who is a world-renowned, accomplished protein biologist. Many years later, after Tony's lab was shuddered and Bernard moved on, Bernard reached out generously to my group about his postdoctoral results demonstrating that MCC is a centrosomal protein. My group had struggled for years trying to pin down the subcellular localization of MCC, and Bernard revealing his extensive, unpublished data was a Eureka moment. With Tony's death, the subsequent closure of the Pawson lab and Bernard's rapid departure, it has been a near Herculean task, while assembling this manuscript almost a decade later, to retrieve files from Bernard's numerous replicate experiments. Over the last 90 days or so, all avenues have been exhausted. The files are irretrievable. My lab in Singapore is not a protein biochemistry lab. Attempting to repeat the experiments Bernard initiated is simply not possible. Most importantly and emphatically, with an appeal to the Editor's discretion and compassion, we assert that addressing this Reviewer's request is not critical to ascertain the conclusions made in Figure 4D, as our study provides additional, strong evidence supporting the redeployment of MCC being dependent upon phosphorylation by Casein Kinases 1 delta and epsilon (CK1δ and CK1ε). We wish for our work to see the light of day after a long submission journey. We feel strongly that it belongs in *JCS*. If absolutely necessary, Figure 4D can be removed.

We wish to emphasize that we have included quantification analysis to allow a better assessment of the data presented in Figure 4D (please, see Supplementary Figure 2C). Western Blot quantification analysis was performed using ImageJ following Hossein Davarinejad's method (York University - Canada). (https://www.yorku.ca/yisheng/Internal/Protocols/ImageJ.pdf).

2. **- The revised title includes the term "ncMTOC". This is not an abbreviation that will be obvious to many readers of JCS.**

While "ncMTOC" is a standardized and widely used acronym in the field of non- centrosomal microtubule biology, we agree with the Reviewer that this term would not be fully comprehended by readers outside the field. To reach a broader readership, we have amended the main title to **MCC is a centrosomal protein that relocalizes to apical non-centrosomal sites during intestinal cell differentiation**

3. **- In light of the discussion regarding "dynamics" in the response to reviewers, I recommend revising the running title.**

The running title has been amended to **Redeployment of MCC during intestinal cell differentiation.**

Third decision letter

MS ID#: JOCES/2021/259272

MS TITLE: MCC is a centrosomal protein that relocalizes to non-centrosomal apical sites during intestinal cell differentiation

AUTHORS: Lucian B. Tomaz, Bernard A Liu, Meroshini M, Sheena L. M. Ong, Ee Kim Tan, Nicholas Stanislaw Tolwinski, Christopher S. Williams, Anne-Claude Gingras, Marc Leushacke, and Norris Ray Dunn
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I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks. The paper is now suitable for publication without the more exhaustive work asked by one of the reviewers.