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TRAIL signalling promotes entosis in colorectal cancer

Emir Bozkurt, Heiko Dussmann, Manuela Salvucci, Brenton Cavanagh, Sandra Van Schaeybroeck, Daniel Longley, Seamus Martin, and Jochen Prehn

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November 23, 2020

Re: JCB manuscript #202010030

Prof. Jochen H M Prehn
Royal College of Surgeons in Ireland
Physiology and Medical Physics
123 St Stephen's Green
Dublin, Dublin D02 YN77
Ireland

Dear Prof. Prehn,

Thank you for submitting your manuscript entitled "TRAIL signalling promotes entosis in colorectal cancer". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We sincerely apologize for the delay in communicating our decision to you. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that the reviewers - and we agree - found the core observations interesting. They asked for controls and suggested experiments to better understand the process observed - entosis or other cell death pathways, the dependence on caspase-8 - that we feel are valid and relevant. Addressing these points would bolster the conclusions and strengthen the description of entosis downstream of TRAIL. We encourage you to address their points in full, with the exception of Rev#1's suggestion to test other apoptosis inducers. This point would not alter the core conclusions, nor would it affect the novelty or interest if this observation was not generalizable beyond TRAIL signaling. Please let us know if you anticipate any issue addressing these points or have any question, we'd be happy to discuss the revisions as needed.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, https://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Pier Paolo Di Fiore, MD, PhD
Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

In the manuscript entitled "TRAIL signaling promotes entosis in colorectal cancer" Bozkurt et al demonstrate a role for TRAIL in promoting entosis. Utilizing high-resolution fluorescence microscopy, time-lapse video, and a novel automated high-throughput screening platform, the authors show entosis frequency was upregulated in response to TRAIL exposure in HCT116 colon cancer cell line. Experiments employing lysotracker and fluorescent reporters of caspase activity and MOMP showed the entosed cells either entered apoptosis and were degraded in the lysosome or did not undergo apoptosis and escaped. Utilizing CLEM, the group was able to visualize entotic structures in high resolution and further classified 3 stages of entotic structure formation. The authors show TRAIL induced entosis still occurred when Caspases and apoptosis were inhibited by z-VAD (caspase inhibitor). Caspase 8 null cells, however, did not increase entosis frequency after TRAIL treatment, though apoptosis was blocked similarly as for z-VAD treated cells. When apoptosis was blocked genetically, this increased the probability of release/escape by the entosed cell. Finally, the authors display a correlation between the detection of CIC structures at the periphery of tumors with frequency of relapse.

Experiments were generally rigorous and conclusions of entosis were well supported. The central finding of the study is novel and interesting, but is somewhat limited in scope and descriptive.

Major Points:

1. It is not clear why Caspase 8 null cells fail to undergo an increase in entosis frequency after Trail (Fig 4D), but z-VAD treated cells do increase entosis frequency after Trail (Fig 4B). z-VAD blocks all caspases, including caspase 8, and is just as potent at blocking Trail induced apoptosis (4A vs 4C). This lack of agreement needs further investigation and explanation. Would an enzyme-dead Caspase 8 rescue the phenotype in Caspase 8 null cells, thus demonstrating a non-enzymatic role for Caspase 8 in entosis, as speculated by the authors?

2. One weakness of the study is its scope is entirely limited to TRAIL. One major question that arises from the findings presented is whether other inducers of apoptosis can induce entosis. Examining apoptosis inducing factors closely related to Trail (TNF super family members) and also unrelated to Trail would be informative. Even if these ligands fail to actually induce apoptosis, perhaps entosis will still be induced, as in the z-VAD treated HCT116 cells. Further, if HCT116 cells lack receptors for various apoptosis inducing ligands, perhaps other cell lines that are sensitive to that stimulus can be used. The key question to examine is how general (or specific to Trail) the induction of entosis is by apoptosis inducing factors, related and unrelated to TRAIL.

3. The authors show that TRAIL (an activator of apoptosis) is initiating apoptotic events and entosis. Because Caspase blockade does not block Trail induced entosis, this raises the question if DR4/DR5 mediate the effect. Knockout/knockdown/blockade of these receptors would help determine the mechanism of Trail induced entosis.

4. One important factor not explored is whether Trail is acting on (and required by) the inner cell, outer cell, or both. The authors should block/inhibit (genetically or otherwise) Trail signaling (and induction of entosis) in a population of marked cells (eg, GFP), and mix with a population of wild type cells (eg, RFP). After Trail addition, determine inner cell and outer cell frequency for each cell type. These or similar experiments would demonstrate if TRAIL stimulation is required by the inner cell, outer cell, or both.

5. Fig. 6E, displaying protein levels of genes in TRAIL pathway in tumor cells, is not adequately explained. The authors should provide images of the staining and technical validation of antibodies used.

6. Entosis was originally observed in cells that were not adhered to a substrate, but in solution or on low adherence plates. Entosis can also be induced after adherence is lost during mitosis. Thus, lack of substrate adherence is an important factor in entosis. The authors should examine their images and data and, using their expertise in apoptosis pathways, provide their judgement on whether loss of substrate adhesion caused as part of the apoptotic process, has a role in TRAIL induced apoptosis.

Minor comments:
1. Fig 6B - Unclear how many cells were counted to give this number of cell-in-cell structures. The data should be normalized, such as to the total number of cells scored. Further, the presence of CIC structures in a tumor cannot be directly attributed to entosis, of course. Other potential mechanisms, such as engulfment by senescent cells, should be considered as a possibility.

2. The effect of Cyclohexamide should be better explained. Why does a translation inhibitor cause increased apoptosis in TRAIL treated cells?

3. Unclear whether the experiments were performed on ultra-low bind plates or adherent conditions. In materials and methods, both are explained well, but unsure whether the adherent conditions, or non-adherent conditions were used in each experiment.

4. In the results section, "Characterisation of entotic ultrastructures and features of entotic cell death induced by TRAIL treatment", the statement "although some inner cells displayed apoptotic nuclear morphology, most inner cells did not show an apoptosis-like nuclear pattern" is not clearly supported by images or quantitative data. Either expound, explain, or remove.

5. In the results section, "Clinical characterization among TRAIL signaling, cell-in-cell structures and colorectal cancer", the statement "(We dropped OS from the plots)" appears to be an author's comment and should be removed.

Reviewer #2 (Comments to the Authors (Required)):

In Bozkurt et al the authors show evidence that the cell engulfment and death mechanism entosis is induced by treatment with TRAIL, in parallel to apoptosis and in a manner that is independent of caspase activity but requires the presence of Caspase-8. They also demonstrate that apoptotic regulators contribute to promoting entotic cell death. The authors further show in colorectal cancers an associated between expression of TRAIL signaling proteins and entotic cell structures, and also correlation with poor prognosis for structures near the invasive front of cancerous lesions.

This study is interesting and the experiments are well performed. The imaging studies, which have been critical in this field, are high quality and very convincing. The combined approaches of time-lapse, CLEM, the 96 well imaging platform, and imaging-based quantification of entosis in colorectal cancer, are excellent. While the mechanism underlying caspase-8 function in entosis remains to be uncovered, this reviewer feels that this question is appropriately left for future studies. This current work opens up new interesting questions to be further explored, a hallmark of good science. As such, this reviewer does not have major suggestions or comments. This is an important set of findings for the cell death field where (1) entosis still remains poorly understood and (2) crosstalk talk between different death mechanisms is actively being uncovered and is likely critical to understanding pathophysiology.

Minor comment:

1. In Figure 3G the LAMP1 signal appears to localize to the membrane surrounding the inner cell upon the initiation of the death process - which is also evident in the movie. The interpretation of lysosomal movements during this death process should more clearly indicate this in the text. In its current form, it reads as if all major lysosomal activities are within the inner cell.

Reviewer #3 (Comments to the Authors (Required)):

Using various means of single-cell microscopy analysis, the manuscript by Bozkurt et al. shows that TRAIL stimulation leads to the induction of entosis and subsequent entotic death of the inner cells in colon cancer cells. It remains unclear however if apoptosis and entosis are independent events. Besides this major concern, several additional major points need to be addressed as listed below.

Major points:

1. The authors claim that TRAIL treatment induces both apoptosis and entosis independently of one another. However, the independence of these two processes is not sufficiently demonstrated. According to the paper in which entosis was first described (Overholtzer et al., 2007), cells that are positive for cleaved caspase 3 are not undergoing entotic cell death but apoptosis. Supplementary Figure 2B of the current manuscript shows that the vast majority of inner cells are positive for cleaved caspase 3 suggesting that these cells undergo apoptosis. How do the authors explain this discrepancy?

2. In Figure 2 the definition and identification of early entotic versus late entotic cells is not clear. How can you distinguish between truly early entotic cells that will subsequently die and those that are going to be released from the outer cell? The use of beta-catenin as an additional marker to define cells undergoing entosis would be a nice addition for the imaging analysis to further differentiate early and late entotic events. Furthermore, in panels 2E and 2F the authors should include either a caspase 8 or a pan-caspase inhibitor to determine if the death and the entosis induced by TRAIL and the combination of TRAIL and CHX can be rescued, as utilized in Figure 4.

3. In all panels of Figure 1 controls are missing. There are no images showing the basal conditions in which the cells have not been treated with TRAIL in order to have a direct comparison to determine how much entosis can occur despite TRAIL treatment. Concerning this point, the use of DR4 or DR5 KO cells would serve as a useful control. Authors need to perform certain experiments also with PS-liposomes as a means to inhibit phagocytosis to prove experimentally that the internalization observed is entotic and not a consequence of apoptosis.

4. In Figure 4, the experiments shown are not sufficient to conclude that TRAIL induced entosis and apoptosis are independent processes. The quantification of entosis only allows for a quantification of the cells that are being internalized by another cell. However, the cells could afterwards be released or die and there is no quantification for specific entotic cell death. The fact that caspase inhibition does not affect entosis percentage only means that internalization process is not caspase dependent. Nevertheless, cell death happening afterwards could still be apoptotic. It would be nice to add an inhibitor of lysosomal cell death, such as concanamycin A, and determine if all cell death observed in inner cells can be rescued by this treatment. Additionally, in fig. 4A-D Caspase 8 ko HCT116 cells basally undergo more entosis than WT cells, reaching 1 % after 72h. As percentages of cells undergoing entosis are low overall, how can authors be sure that they do not observe increased entosis upon TRAIL treatment in Caspase 8 ko cells sipoly because maximum amount of entosis is already reached basally?

5. Supplementary Figure 3 would benefit from the addition of treatment with a caspase inhibitor in panel A, to determine whether entosis observed in these additional cell lines is apoptosis-dependent, as done in Figure 4.

6. Western Blot controls confirming that knockout cells used (Caspase 8 ko in Fig. 4 and Supplementary 6 and Bax/Bak ko in Fig. 5 and Suppl.fig. 3) are indeed kos.

7. Panel 5D shows that there are no differences in cathepsin activity upon TRAIL treatment. However, authors claim that cells undergo entotic cell death which is cathepsin-dependent after TRAIL stimulation. How do authors explain this? Is the cell death observed in inner cells apoptotic as suggested by the reduction in cell death and increase in cell release in the presence of caspase inhibitors? It is not clear how the authors determine when a cell is undergoing entotic cell death and it would help if they quantified the co-localization of Lysotracker and Cathepsin B.

8. The authors should explore mechanistically how TRAIL induces entosis which they show to be ROCK-dependent.

Minor points:

• Panels 1J and 1K are not referred to or discussed.

• In Fig. 4, authors should show representative microscopy figures in addition to the entotic percentage quantifications to see morphological changes and differences in staining of the markers used with different treatments.

• In Fig 3, although characterization of structural features is complete for TRAIL-treated cells, the same images should also be shown for control cells for side-by-side comparison.

Dear Editor,

We greatly appreciate your and the reviewers valuable time and effort for assessing our manuscript. We have now addressed the reviewers' points, revised the manuscript based on their suggestions and highlighted the changes in the revised manuscript. Please find our point-by-point response to reviewers' comments below.

Point by point reply by Bozkurt et al.

Reviewer #1:

We appreciate that the reviewer considers our study interesting and well conducted. We thank the reviewer for the constructive suggestions.

Major points:

1-) "*It is not clear why Caspase 8 null cells fail to undergo an increase in entosis frequency after Trail (Fig 4D), but z-VAD treated cells do increase entosis frequency after Trail (Fig 4B). z-VAD blocks all caspases, including caspase 8, and is just as potent at blocking Trail induced apoptosis (4A vs 4C). This lack of agreement needs further investigation and explanation. Would an enzyme-dead Caspase 8 rescue the phenotype in Caspase 8 null cells, thus demonstrating a non-enzymatic role for Caspase 8 in entosis, as speculated by the authors?*"

We thank reviewer 1 for this suggestion. We have re-introduced a catalytically inactive CASP8 mutant (C360A) (1) into CASP8 -/- cells and quantified the entosis rate with or without TRAIL and in the absence or presence of Y-27632. Re-insertion of active site mutant CASP8 restored TRAIL-induced entosis suggesting that Caspase-8 indeed plays a non-enzymatic role in TRAIL-induced entosis. We have updated Fig. 4 (I-K) and the related sections in results (page 9, 10) "**Entosis induced by TRAIL requires DR4 and DR5, and structural presence of Caspase-8**" as well as in discussion (page 13).

1- Henry, C.M., and S.J. Martin. 2017. Caspase-8 Acts in a Non-enzymatic Role as a Scaffold for Assembly of a Pro-inflammatory "FADDosome" Complex upon TRAIL Stimulation. Molecular Cell. 65:715-729.e5. doi:10.1016/j.molcel.2017.01.022.

2-) *"One weakness of the study is its scope is entirely limited to TRAIL. One major question that arises from the findings presented is whether other inducers of apoptosis can induce entosis.*

Examining apoptosis inducing factors closely related to Trail (TNF super family members) and also unrelated to Trail would be informative. Even if these ligands fail to actually induce apoptosis, perhaps entosis will still be induced, as in the z-VAD treated HCT116 cells. Further, if HCT116 cells lack receptors for various apoptosis inducing ligands, perhaps other cell lines that are sensitive to that stimulus can be used. The key question to examine is how general (or specific to Trail) the induction of entosis is by apoptosis inducing factors, related and unrelated to TRAIL."

We agree with reviewer #1 that it would be interesting to see whether other members of TNF superfamily can induce entosis. However, we believe that this would be out of scope of this paper. We focused our study on TRAIL signalling as TRAIL ligands are potential anti-cancer agents. We, however, performed a series of experiments with DR4 -/- DR5 -/- cell lines to further understand whether induction of entosis was specific to TRAIL, as these experiments were also suggested by this reviewer in comment 3 (below).

3-) "*The authors show that TRAIL (an activator of apoptosis) is initiating apoptotic events and entosis. Because Caspase blockade does not block Trail induced entosis, this raises the question if DR4/DR5 mediate the effect. Knockout/knockdown/blockade of these receptors would help determine the mechanism of Trail induced entosis."*

We thank reviewer 1 for this suggestion. We have performed HCS-based entosis quantification in HCT116 DR4 -/- DR5 -/- cells and their wild type counterparts treated with or without TRAIL in the absence or presence of Y-27632. We observed that, while TRAIL increased the rate of entosis in wild type cells, there was no increase in entosis rate in DR4 -/- DR5 -/- cells suggesting that death receptors were indeed required for TRAIL induced entosis. We have updated Fig. 4 (D-F) and the related sections in results (page 9) "**Entosis induced by TRAIL requires DR4 and DR5, and structural presence of Caspase-8**" as well as in discussion (page 13).

4-) *"One important factor not explored is whether Trail is acting on (and required by) the inner cell, outer cell, or both. The authors should block/inhibit (genetically or otherwise) Trail signaling (and induction of entosis) in a population of marked cells (eg, GFP), and mix with a population of wild type cells (eg, RFP). After Trail addition, determine inner cell and outer cell frequency for each cell type. These or similar experiments would demonstrate if TRAIL stimulation is required by the inner cell, outer cell, or both."*

To understand whether TRAIL signalling is required for the inner cell, outer cell, or both, we labelled WT cells with CellTracker Green, DR4 -/- DR5 -/- cells with CellTracker Red, and treated

the co-cultured cells with or without TRAIL in the absence or presence of z-VAD-fmk or Y-27632. We then performed HCS imaging and quantified the frequency of inner and outer cells for all possible consequences (green in green, green in red, red in red, red in green). We found that, regardless of treatment, the large majority of cells, which became inner cells were wild type. Moreover, DR4 -/- DR5 -/- cells dominantly became outer cells suggesting that TRAIL signalling (death receptors) may be required for inner cells during cell internalisation. We have updated Fig. 4 (G and H) and the related sections in results (page 9) "**Entosis induced by TRAIL requires DR4 and DR5, and structural presence of Caspase-8**" as well as in discussion (page 13).

5-) "*Fig. 6E, displaying protein levels of genes in TRAIL pathway in tumor cells, is not adequately explained. The authors should provide images of the staining and technical validation of antibodies used.*"

Semi-quantitative immunohistochemistry data for TRAIL, DR4, DR5, Caspase-8 and c-FLIP and clinical data used in this study were historical data obtained from a previous study by McLornan et al (1), where authors Sandra Van Schaeybroeck and Dan Longley are co-authors. Antibodies used, images of staining and technical validation of staining can be found in the related publication, and this is now referred to in this study. We have added the methodology for c-Met immunohistochemistry staining to the methods section (page 20). Images of H&E staining used to detect entosis and c-Met staining are provided in Fig. 6, A.

1- McLornan DP, Barrett HL, Cummins R, McDermott U, McDowell C, Conlon SJ, Coyle VM, Van Schaeybroeck S, Wilson R, Kay EW, Longley DB, Johnston PG. Prognostic significance of TRAIL signaling molecules in stage II and III colorectal cancer. Clin Cancer Res. 2010 Jul 1;16(13):3442-51. doi: 10.1158/1078-0432.CCR-10-0052. Epub 2010 Jun 22. PMID: 20570920; PMCID: PMC2896551.

6-) "*Entosis was originally observed in cells that were not adhered to a substrate, but in solution or on low adherence plates. Entosis can also be induced after adherence is lost during mitosis. Thus, lack of substrate adherence is an important factor in entosis. The authors should examine their images and data and, using their expertise in apoptosis pathways, provide their judgement on whether loss of substrate adhesion caused as part of the apoptotic process, has a role in TRAIL induced apoptosis.*"

As shown in an example of a cell undergoing apoptosis in Fig. 1, A, loss of substrate adhesion during apoptosis occurs after caspase activation. Furthermore, this study as well as previous studies (e.g. Overholtzer, 2007) have shown that Caspase activity does not play a role in cell

internalisation during entosis. Here we show that these findings also apply to TRAIL-induced entosis. For instance, in the presence of z-VAD-fmk, cells do not detach from the surface in response to TRAIL, however, we still observe an increase in entosis rate suggesting that loss of substrate adhesion during apoptosis would not be the main mechanism during in TRAIL-induced entosis.

Minor points:

1-) "*Fig 6B - Unclear how many cells were counted to give this number of cell-in-cell structures. The data should be normalized, such as to the total number of cells scored. Further, the presence of CIC structures in a tumor cannot be directly attributed to entosis, of course. Other potential mechanisms, such as engulfment by senescent cells, should be considered as a possibility."*

We identified and counted cell-in-cell structured in high resolution images of tissue microarray (TMA) core sections (Fig. 6A) stained with either H&E or c-MET. We believe the counts estimates can be used "as is" without further normalization (for example, by total number of scored cells), as we identified and investigated cell-in-cell structures in a consistent manner for all patients from standardized core sections of ~1.5 mm. We observed a strong correlation when comparing cell-in-cell events identified from HE- and c-MET-stained TMA cores (Fig. S5, B). Thus, for downstream analyses, we pooled counts from cell-in-cell structures estimated from HE and c-MET staining and considered them as biological replicates, totalling up to 6 cores per patient from tumour tissue. For each patient we tallied the number of cell-in-cell structures detected across the corresponding core sections and we then computed the aggregated statistics (median, minimum and maximum values) shown in Fig. 6B. To aid visualization and comparison of intra- and inter-patient heterogeneity, we graphed our findings as a waterfall plot where patients (x-axis) are sorted by median number of cell-in-cell structures. For each patient, we indicated the median number of cell-in-cell structures with a marker symbol and its range (minimum and maximum) with a gray shaded area.

We revised the legend of Fig. 6, B, as "Inter- and intra-patient heterogeneity in cell-in-cell (CIC) events detected in tumour tissue stained with either HE or c-MET and computed for each patient of the NI240 cohort (n=223). Multiple TMA section cores (minimum 2, median 6) were examined for each patient and summary statistics for number of cell-in-cell events (median, minimum and maximum) were computed across all examined cores for each patient. Patients (x-axis) are sorted in decreasing order of median CIC events (y-axis). The median number of detected cell-in-cell structures is indicated by the line symbol while the shaded area indicates the range (minimum and maximum).

2-) "*The effect of Cyclohexamide should be better explained. Why does a translation inhibitor cause increased apoptosis in TRAIL treated cells?"*

CHX has been shown to inhibit parallel activation of survival pathways during TRAIL-induced apoptosis. It downregulates endogenous cFLIP and inhibits downstream NF-kappaB activation that provides survival during apoptosis (1). CHX has been widely used to sensitize many types of cells to TRAIL induced apoptosis by our group (2) as well as by other groups (e.g. 3). This information has now been added to the manuscript (page 13).

1- Wajant H, Haas E, Schwenzer R, Muhlenbeck F, Kreuz S, Schubert G, Grell M, Smith C, Scheurich P. Inhibition of death receptor-mediated gene induction by a cycloheximide-sensitive factor occurs at the level of or upstream of Fas-associated death domain protein (FADD). J Biol Chem. 2000 Aug 11;275(32):24357-66. doi: 10.1074/jbc.M000811200. PMID: 10823821.

2- Hellwig CT, Kohler BF, Lehtivarjo AK, Dussmann H, Courtney MJ, Prehn JH, Rehm M. Real time analysis of tumor necrosis factor-related apoptosis-inducing ligand/cycloheximide-induced caspase activities during apoptosis initiation. J Biol Chem. 2008 Aug 1;283(31):21676-85. doi: 10.1074/jbc.M802889200. Epub 2008 Jun 3. PMID: 18522940.

3- Guseva, N. V., Rokhlin, O. W., Taghiyev, A. F., & Cohen, M. B. (2007). Unique resistance of breast carcinoma cell line T47D to TRAIL but not anti-Fas is linked to p43cFLIPL. Breast Cancer Research and Treatment, 107(3), 349–357. doi:10.1007/s10549-007-9563-2

3-) *"Unclear whether the experiments were performed on ultra-low bind plates or adherent conditions. In materials and methods, both are explained well, but unsure whether the adherent conditions, or non-adherent conditions were used in each experiment."*

All experiments except preparation of spheroids were performed in adherent conditions (96-Well optical-bottom plates #165305, Thermo Fisher Scientific, or 12-mm glass-bottom WillCo-dishes, WillCo Wells B.V.) as stated in the manuscript. The ultra-low attachment plates were only used to prepare spheroids (Corning Spheroid Microplates #4515).

4-) "*In the results section, "Characterisation of entotic ultrastructures and features of entotic cell death induced by TRAIL treatment", the statement "although some inner cells displayed apoptotic nuclear morphology, most inner cells did not show an apoptosis-like nuclear pattern" is not clearly supported by images or quantitative data. Either expound, explain, or remove."*

We thank Reviewer#1 for this comment, we have removed the statement "although some inner cells displayed apoptotic nuclear morphology, most inner cells did not show an apoptosis-like nuclear pattern" from the manuscript.

5-) *"In the results section, "Clinical characterization among TRAIL signaling, cell-in-cell structures and colorectal cancer", the statement "(We dropped OS from the plots)" appears to be an author's comment and should be removed."*

We have removed the statement "(We dropped OS from the plots)" from the manuscript.

Reviewer #2:

"In Bozkurt et al the authors show evidence that the cell engulfment and death mechanism entosis is induced by treatment with TRAIL, in parallel to apoptosis and in a manner that is independent of caspase activity but requires the presence of Caspase-8. They also demonstrate that apoptotic regulators contribute to promoting entotic cell death. The authors further show in colorectal cancers an associated between expression of TRAIL signaling proteins and entotic cell structures, and also correlation with poor prognosis for structures near the invasive front of cancerous lesions.

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<u>Minor points:</u>

1-) *"In Figure 3G the LAMP1 signal appears to localize to the membrane surrounding the inner cell upon the initiation of the death process - which is also evident in the movie. The interpretation of lysosomal movements during this death process should more clearly indicate*

this in the text. In its current form, it reads as if all major lysosomal activities are within the inner cell."

We thank reviewer #2 for this comment, we have edited the related paragraph according to reviewer #2's suggestions (page 8, "As shown in Fig. 3, F, during internalisation....").

Reviewer #3:

"Using various means of single-cell microscopy analysis, the manuscript by Bozkurt et al. shows that TRAIL stimulation leads to the induction of entosis and subsequent entotic death of the inner cells in colon cancer cells. It remains unclear however if apoptosis and entosis are independent events. Besides this major concern, several additional major points need to be addressed as listed below."

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We thank Reviewer #3 for this valuable comment. According to Reviewer #3's points, we have edited the entire manuscript to better clarify the induction of apoptosis and entosis by TRAIL. We categorised the interaction between these two mechanisms into two main steps: (1) cell internalisation during entosis and (2) downstream entotic cell death. Our findings suggest that the cell internalisation process during entosis is independent of caspase activity (Fig. 4, A-C;

results section "**Entosis induced by TRAIL requires DR4 and DR5, and structural presence of Caspase-8**" (page 8); and page 13 in discussion), however, inhibition of caspase activity or knockout of Bax and Bak changes inner cell fate during downstream entotic cell death. Moreover, an increase in cleaved caspase-3 does not necessarily mean that these cells will complete (and die by) apoptosis (1, 2). Therefore, although TRAIL increases cleaved caspase-3 levels due to activation of apoptosis, this activation does not play a direct role in cell internalisation during entosis.

1- Lamkanfi, M., Festjens, N., Declercq, W. et al. Caspases in cell survival, proliferation and differentiation. Cell Death Differ 14, 44–55 (2007). https://doi.org/10.1038/sj.cdd.4402047
2- Khalil H, Peltzer N, Walicki J, et al. Caspase-3 protects stressed organs against cell death. Mol Cell Biol. 2012;32(22):4523-4533. doi:10.1128/MCB.00774-12

2-) *"In Figure 2 the definition and identification of early entotic versus late entotic cells is not clear. How can you distinguish between truly early entotic cells that will subsequently die and those that are going to be released from the outer cell? The use of beta-catenin as an additional marker to define cells undergoing entosis would be a nice addition for the imaging analysis to further differentiate early and late entotic events. Furthermore, in panels 2E and 2F the authors should include either a caspase 8 or a pan-caspase inhibitor to determine if the death and the entosis induced by TRAIL and the combination of TRAIL and CHX can be rescued, as utilized in Figure 4."*

We thank Reviewer #3 for this comment. Morphological stages of entosis have been described by Garanina et al. (1), based on morphological changes of inner cell shape, structure of nucleus, and state of cytoplasm during entosis. According to this study, events that occur prior to lysotracker accumulation can be inferred as "early stages", and after lysotracker accumulation as "late stages". These observations have been corroborated by time-lapse images in the above cited study, as well as many studies in the field including ours. Because our findings as well as the literature strongly suggest that lysotracker accumulation in inner cells is a late-stage event for entosis (and occurs during entotic cell death), we used the term "late stage" when cells showed lysotracker accumulation. For events that occur before lysotracker accumulation in inner cells, we used the term "early stage". We, however, agree with the reviewer and are aware of the fact that it would not be possible to determine inner cell fate by using an end-point HCS assay, which was used to obtain data in Fig. 2. Moreover, definition of "early and late stage entosis" does not affect our findings as we quantify both as entotic in our analysis. Therefore, we removed the terms "early stage" and "late stage" from Fig. 2, all related sections and figure legends, but kept those terms from Fig. 3 onwards as we here have additional beta-catenin, TEM and time-lapse analysis to support our observations.

By preparing Fig. 2, we aimed to introduce our large-scale, unbiased HCS-based entosis and apoptosis quantification workflow that we used throughout our study. Our second aim was to give a general overview of the increase in apoptosis and entosis by TRAIL after showing several representative time-lapse images in Fig. 1. However, we did not include the addition of CHX in these investigations as CHX exposure is biologically or clinically not a very relevant paradigm (and its effects was already shown in Fig. 1, C). As we aimed the assess downstream TRAIL signalling in a great detail in Fig. 4 but not Fig. 2, we did not include results from caspase inhibitors in Fig. 2. We, however, prepared a supplementary figure (Fig. S2, A) from a manual entosis analysis that includes beta-catenin staining, as well as the addition of caspase inhibitor z-VAD-fmk or Y-27632.

1- Garanina, A.S., Kisurina-Evgenieva, O.P., Erokhina, M.V. et al. Consecutive entosis stages in human substrate-dependent cultured cells. Sci Rep 7, 12555 (2017). https://doi.org/10.1038/s41598-017-12867-6.

3-) *"In all panels of Figure 1 controls are missing. There are no images showing the basal conditions in which the cells have not been treated with TRAIL in order to have a direct comparison to determine how much entosis can occur despite TRAIL treatment. Concerning this point, the use of DR4 or DR5 KO cells would serve as a useful control. Authors need to perform certain experiments also with PS-liposomes as a means to inhibit phagocytosis to prove experimentally that the internalization observed is entotic and not a consequence of apoptosis."*

We have added representative images from control experiments to Fig. 1 (Fig. 1, E and F), and Fig. 3 (Fig. 3, A) for side-by-side comparison of the morphology of entotic structures. We have added a statement to page 7 that we do not observe morphological differences in plasma membranes, nuclei, and lysosomal accumulation when we compared entotic structures in controls with the ones in TRAIL-treated cells. As it was also suggested by reviewer #1, we have performed a series of experiments with HCT116 DR4 -/- DR5 -/- cells and their wild type counterparts to further understand the role of TRAIL signalling in entosis; and presented our findings in Fig. 4, D-H. Our findings suggest that DR4 and DR5 are required for TRAIL-induced entosis, and are required for inner cells during cell internalisation (results section: **Entosis induced by TRAIL requires DR4 and DR5, and structural presence of Caspase-8,** discussion page 13).

A recent paper from Tonnessen-Murray et al., (1) provided a great example for distinguishing entosis and phagocytosis. The study showed that senescent-related engulfment by phagocytosis-like activity is distinct from entosis. Importantly, ROCK inhibitor Y-27632, the same inhibitor we used in our study, did not affect phagocytotic activity while it effectively blocked entosis. Because Y-27632 consistently inhibited the formation of TRAIL-induced cell-in-

cell structures throughout our study, we conclude that entosis was induced. We, therefore, did not perform additional experiments with PS-liposomes to inhibit phagocytosis. In addition, as we show in Fig. S1, B; and Videos 1 and 2, we observed a distinctive movement of inner cells towards outer cells, which is another hallmark for entosis (Overholtzer, 2007) but not phagocytosis.

1- Tonnessen-Murray CA, Frey WD, Rao SG, Shahbandi A, Ungerleider NA, Olayiwola JO, Murray LB, Vinson BT, Chrisey DB, Lord CJ, Jackson JG. Chemotherapy-induced senescent cancer cells engulf other cells to enhance their survival. J Cell Biol. 2019 Nov 4;218(11):3827-3844. doi: 10.1083/jcb.201904051. Epub 2019 Sep 17. PMID: 31530580; PMCID: PMC6829672.

4-) *"In Figure 4, the experiments shown are not sufficient to conclude that TRAIL induced entosis and apoptosis are independent processes. The quantification of entosis only allows for a quantification of the cells that are being internalized by another cell. However, the cells could afterwards be released or die and there is no quantification for specific entotic cell death. The fact that caspase inhibition does not affect entosis percentage only means that internalization process is not caspase dependent. Nevertheless, cell death happening afterwards could still be apoptotic. It would be nice to add an inhibitor of lysosomal cell death, such as concanamycin A, and determine if all cell death observed in inner cells can be rescued by this treatment. Additionally, in fig. 4A-D Caspase 8 ko HCT116 cells basally undergo more entosis than WT cells, reaching 1 % after 72h. As percentages of cells undergoing entosis are low overall, how can authors be sure that they do not observe increased entosis upon TRAIL treatment in Caspase 8 ko cells simply because maximum amount of entosis is already reached basally?"*

We are grateful for this suggestion by reviewer #3. We now refer to cell internalization during entosis and downstream entotic cell death as two separate processes throughout the manuscript. Our findings suggest that the cell internalisation process during entosis is independent of caspase activity (Fig. 4, A-C; results section "**Entosis induced by TRAIL requires DR4 and DR5, and structural presence of Caspase-8**" (page 8); and page 13 in discussion), however, inhibition of caspase activity or knockout of Bax and Bak changes inner cell fate during downstream entotic cell death.

We do not have any experimental evidence to explain why basal levels of entosis is moderately higher in caspase-8 -/- cells compared to wild type cells. A recent study from Liccardi et al., (1) found that Caspase-8 (and also RIPK1) is required for the maintenance of chromosome stability and consequent aneuploidy during mitosis. In addition, a direct link between increase in entosis and

aneuploidy has been shown by Krajcovic et al., (2). This might be a possible explanation for higher basal levels of entosis in caspase-8 -/- cells. We, however, are not concerned that caspase-8 -/- cells may have already reached maximum levels of entosis. As demonstrated in glucose starvation experiments (Fig. S4, C), when we put caspase-8 -/- cells under glucose starvation, we still observe further increase in entosis rate.

1- Liccardi G, Ramos Garcia L, Tenev T, Annibaldi A, Legrand AJ, Robertson D, Feltham R, Anderton H, Darding M, Peltzer N, Dannappel M, Schünke H, Fava LL, Haschka MD, Glatter T, Nesvizhskii A, Schmidt A, Harris PA, Bertin J, Gough PJ, Villunger A, Silke J, Pasparakis M, Bianchi K, Meier P. RIPK1 and Caspase-8 Ensure Chromosome Stability Independently of Their Role in Cell Death and Inflammation. Mol Cell. 2019 Feb 7;73(3):413-428.e7. doi: 10.1016/j.molcel.2018.11.010. Epub 2018 Dec 28. PMID: 30598363; PMCID: PMC6375735.

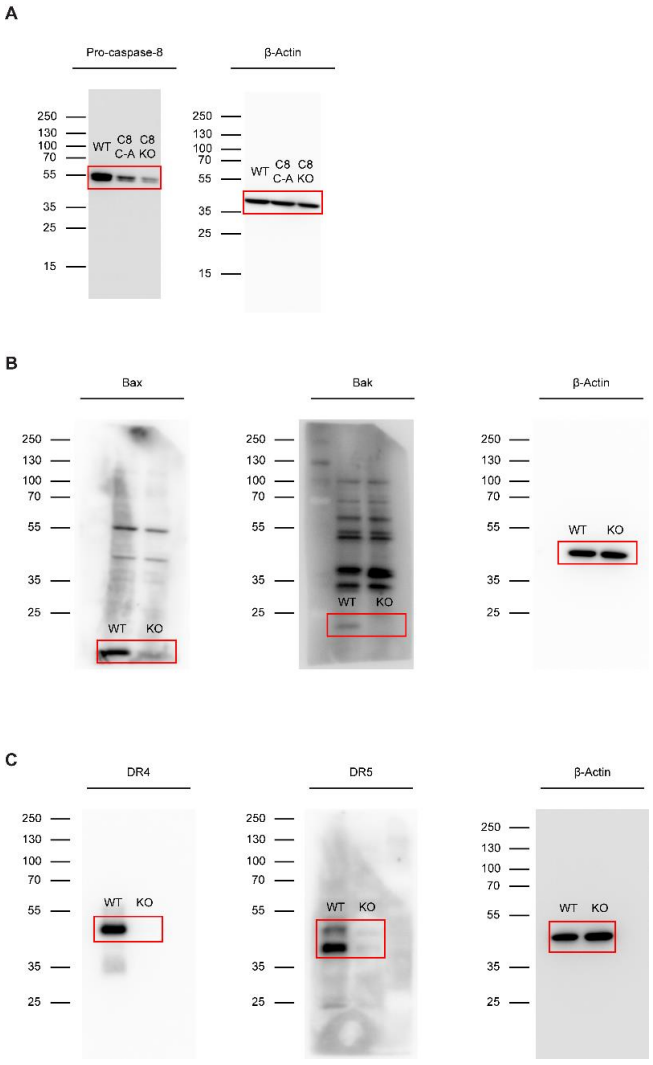
2- Krajcovic M, Johnson NB, Sun Q, Normand G, Hoover N, Yao E, Richardson AL, King RW, Cibas ES, Schnitt SJ, Brugge JS, Overholtzer M. A non-genetic route to aneuploidy in human cancers. Nat Cell Biol. 2011 Mar;13(3):324-30. doi: 10.1038/ncb2174. Epub 2011 Feb 20. PMID: 21336303; PMCID: PMC3576821.

5-) *"Supplementary Figure 3 would benefit from the addition of treatment with a caspase inhibitor in panel A, to determine whether entosis observed in these additional cell lines is apoptosis-dependent, as done in Figure 4."*

We prepared Fig. S3 (old) Fig. S2 (new) in order to show that the increase in entosis by TRAIL can be expanded to different models. Because we do not discuss downstream signalling in Fig. 2, we did not include the results from experiments with caspase inhibitors or ROCK inhibitors. We, however, prepared a supplementary figure (Fig. S2, A) that includes beta-catenin staining, caspase inhibitor and ROCK inhibitor addition in HCT116 cells as suggested by the reviewer.

6-) *"Western Blot controls confirming that knockout cells used (Caspase 8 ko in Fig. 4 and Supplementary 6 and Bax/Bak ko in Fig. 5 and Suppl.fig. 3) are indeed kos."*

We have confirmed all knock out cell lines used in the study by western blot as well as by a functional assay (e.g. quantification of PI-positive cells and/or caspase-activation in response to TRAIL and inhibitors). We have added representative western blots to Fig. 4 and Fig. 5. Because we are allowed to have maximum 5 supplementary figures, we have attached the full-length western blots of cropped images shown in Fig. 4 and Fig. 5 below and removed Fig. S6 (old).



7-) *"Panel 5D shows that there are no differences in cathepsin activity upon TRAIL treatment. However, authors claim that cells undergo entotic cell death which is cathepsin-dependent after TRAIL stimulation. How do authors explain this? Is the cell death observed in inner cells apoptotic as suggested by the reduction in cell death and increase in cell release in the presence of caspase inhibitors? It is not clear how the authors determine when a cell is undergoing entotic cell death and it would help if they quantified the co-localization of Lysotracker and Cathepsin B."*

In line with the literature (1-3), we show that, regardless of treatment, all inner cells during entotic cell death exhibit cathepsin-dependent lysosomal cell death. However, we show that inner cells can occasionally show increase in apoptosis markers before lysosomal degradation eventually results in degradation of these markers. Moreover, we (Fig. 5) as well as other studies showed that inhibition of apoptosis either genetically by overexpressing BCL-2 (2) or pharmacologically by using caspase inhibitors (3) results in an increase in the frequency of inner cells that are released during entosis. In Fig. 5, D and E, we aimed to examine whether caspase inhibition or knock out of Bax and Bak affects the decision of inner cell fate during late-stages of entotic cell death. We determine that a cell is undergoing entotic cell death by lysotracker accumulation in inner cells as shown in Fig. 3. In Fig. 5, D, all entotic events shown exhibit lysotracker accumulation in inner cells suggesting that they are undergoing entotic cell death. Fig. 5, D and E show that there are no differences in cathepsin activity in late-stage entotic inner cells upon TRAIL treatment with or without z-VAD-fmk in both wild type and Bax -/- Bak -/- cells. These findings suggest that caspase inhibition or knockout of Bax and Bak does not affect the decision of inner cell fate during late stages of entotic cell death. We, therefore, believe that the decision of why inhibition of apoptosis causes increase in the frequency of released-inner cells might possibly be made during earlier stages of entosis. We have quantified overlap coefficient values for colocalisation of lysotracker and cathepsin B, and added these to Fig. 4, D.

1- Overholtzer, M., A.A. Mailleux, G. Mouneimne, G. Normand, S.J. Schnitt, R.W. King, E.S. Cibas, and J.S. Brugge. 2007. A Nonapoptotic Cell Death Process, Entosis, that Occurs by Cell-in-Cell Invasion. Cell. 131:966–979. doi:10.1016/j.cell.2007.10.040.

2- Florey, O., S.E. Kim, C.P. Sandoval, C.M. Haynes, and M. Overholtzer. 2011. Autophagy machinery mediates macroendocytic processing and entotic cell death by targeting single membranes. Nature Cell Biology. 13:1335–1343. doi:10.1038/ncb2363.

3- Martins, I., S.Q. Raza, L. Voisin, H. Dakhli, A. Allouch, F. Law, D. Sabino, D.D. Jong, M. Thoreau, E. Mintet, D. Dugué, M. Piacentini, M.-L. Gougeon, F. Jaulin, P. Bertrand, C. Brenner, D.M. Ojcius, G. Kroemer, N. Modjtahedi, E. Deutsch, and J.-L. Perfettini. 2018. Anticancer chemotherapy and radiotherapy trigger both non-cell-autonomous and cell-autonomous death. Cell Death & Disease. 9:716. doi:10.1038/s41419-018-0747-y.

8-) *"The authors should explore mechanistically how TRAIL induces entosis which they show to be ROCK-dependent."*

Rho/ROCK signalling regulates several cellular functions such as cell shape, actin cytoskeleton reorganisation, cell contractility, and motility (1, 2). In the context of entosis, Overholtzer et al., showed that Rho/ROCK signalling pathway is specifically required for inner cells during entosis (Overholtzer, 2007). Here we show that, TRAIL death receptors DR4 and DR5 are required for

inner cells during TRAIL-induced entosis suggesting a possible link between TRAIL signalling, apoptosis and Rho/ROCK signalling. Other studies show that during apoptosis activation, proteolytic activity of caspase-3 leads to constitutive activation of ROCK1 resulting in membrane blebbing during apoptosis (3). However, we (Fig. 4, B) and others (4, 5) demonstrated that caspase activity is not required for cell internalisation during entosis suggesting that caspase activity is not the main mechanism of ROCK activation in our study. Interestingly, inhibition of ROCK1 does not seem to affect apoptosis, as ROCK1 inhibited cells still undergo normal apoptosis without showing membrane blebbing upon Fas- (or TNFa-) stimulation (2). ROCK 1 and ROCK 2 share ~65 % structural homology, and both can be cleaved and activated by caspases or granzyme B in addition to their natural activators Rho GTPases (6). Similar to ROCK 1, ROCK 2 seems to have a similar effect on membrane blebbing during apoptosis (7). Although the interaction among ROCK signalling, TRAIL signalling, apoptosis and entosis seem very interesting and worthwhile to investigate further, we believe that exploring those interactions require a detailed experimental investigation and would go beyond the scope of this study.

1- Hajdú, I., Szilágyi, A., Végh, B.M. et al. Ligand-induced conformational rearrangements regulate the switch between membrane-proximal and distal functions of Rho kinase 2. Commun Biol 3, 721 (2020). https://doi.org/10.1038/s42003-020-01450-x.

2- Kurokawa M, Kornbluth S. Caspases and kinases in a death grip. Cell. 2009 Sep 4;138(5):838-54. doi: 10.1016/j.cell.2009.08.021. PMID: 19737514; PMCID: PMC3390419.

3- Sebbagh M, Renvoizé C, Hamelin J, Riché N, Bertoglio J, Bréard J. Caspase-3-mediated cleavage of ROCK I induces MLC phosphorylation and apoptotic membrane blebbing. Nat Cell Biol. 2001 Apr;3(4):346-52. doi: 10.1038/35070019. PMID: 11283607.

4- Overholtzer, M., A.A. Mailleux, G. Mouneimne, G. Normand, S.J. Schnitt, R.W. King, E.S. Cibas, and J.S. Brugge. 2007. A Nonapoptotic Cell Death Process, Entosis, that Occurs by Cell-in-Cell Invasion. Cell. 131:966–979. doi:10.1016/j.cell.2007.10.040.

5- Martins, I., S.Q. Raza, L. Voisin, H. Dakhli, A. Allouch, F. Law, D. Sabino, D.D. Jong, M. Thoreau, E. Mintet, D. Dugué, M. Piacentini, M.-L. Gougeon, F. Jaulin, P. Bertrand, C. Brenner, D.M. Ojcius, G. Kroemer, N. Modjtahedi, E. Deutsch, and J.-L. Perfettini. 2018. Anticancer chemotherapy and radiotherapy trigger both non-cell-autonomous and cell-autonomous death. Cell Death & Disease. 9:716. doi:10.1038/s41419-018-0747-y.

6- Hartmann S, Ridley AJ, Lutz S. The Function of Rho-Associated Kinases ROCK1 and ROCK2 in the Pathogenesis of Cardiovascular Disease. Front Pharmacol. 2015;6:276. Published 2015 Nov 20. doi:10.3389/fphar.2015.00276.

7- Sebbagh M, Hamelin J, Bertoglio J, Solary E, Bréard J. Direct cleavage of ROCK II by granzyme B induces target cell membrane blebbing in a caspase-independent manner. J Exp Med. 2005 Feb 7;201(3):465-71. doi: 10.1084/jem.20031877. PMID: 15699075; PMCID: PMC2213043.

Minor points:

1-) *"Panels 1J and 1K are not referred to or discussed."*

Based on the reviewers' suggestions, we moved these figures to Fig. S1, A and B. We have cited Fig. S1, A and B in page 5 "In separate time-lapse experiments....".

2-) *"In Fig. 4, authors should show representative microscopy figures in addition to the entotic percentage quantifications to see morphological changes and differences in staining of the markers used with different treatments."*

We have added representative field of view as Fig. 4, C. Moreover, we prepared Fig. S4, B to visualize the morphological changes with the different treatments.

3-) *"In Fig 3, although characterization of structural features is complete for TRAIL-treated cells, the same images should also be shown for control cells for side-by-side comparison."*

As Fig. 3, A, we have added representative images from a 3D confocal microscopy analysis of beta-catenin and Hoechst staining showing entotic structures in control cells, as suggested by the reviewer. We have added the following statement to the results section (page 7): "We did not observe morphological differences in plasma membranes, nuclei, and lysosomal accumulation when we compared entotic structures in controls with the ones in TRAIL-treated cells. Thus, we focused on TRAIL-treated cells in further analysis.".

August 6, 2021

RE: JCB Manuscript #202010030R

Prof. Jochen H M Prehn
Royal College of Surgeons in Ireland
Physiology and Medical Physics
123 St Stephen's Green
Dublin, Dublin D02 YN77
Ireland

Dear Prof. Prehn:

Thank you for submitting your revised manuscript entitled "TRAIL signalling promotes entosis in colorectal cancer". The paper has now been seen again by one of the original reviewers (unfortunately, reviewer #3 was not available to re-review the manuscript and since reviewer #2 had supported publication in the first round, we did not return the paper to that referee). As you will see, reviewer#1 has raised a few relatively minor concerns but now supports publication. Therefore, we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

Please be sure to address each of reviewer#1's final comments and provide a point-by-point rebuttal to these issues along with your final revised manuscript.

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Articles is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends. You are currently below this limit but please bear it in mind when revising. If you find that you need to exceed 40,000 characters in order to address rev#1's final comments, we can give you some extra space but please try to be as concise as possible.

2) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

3) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and

methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, since you used parametric tests in your study (e.g. t-tests, ANOVA, etc.), you should have first determined whether the data was normally distributed before selecting that test. In the stats section of the methods, please indicate how you tested for normality. If you did not test for normality, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

4) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described."
Please note that we do not allow "supplementary methods* so you will need to remove this text and move all non-duplicated methods into the main materials and methods section.

5) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies.

6) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

7) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.
Please note that we do not allow "supplementary references" - please remove that section an include the one reference in the main reference list.

8) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles/Tools may have up to 5 supplemental figures. At the moment, you meet this limit but please bear it in mind when revising.
Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

9) eTOC summary: A ~40-50 word summary that describes the context and significance of the findings for a general readership should be included on the title page.
We realize that you have provided one already but the statement should be written in the present tense and refer to the work in the third person. It should also include "First author name(s) et al..." to

match our preferred style.

10) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

11) A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their first and middle initials and full surnames. We encourage use of the CRediT nomenclature (https://casrai.org/credit/).

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Please contact the journal office with any questions, cellbio@rockefeller.edu.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of

Cell Biology.

Sincerely,

Pier Paolo Di Fiore, MD, PhD
Senior Editor
Journal of Cell Biology

Tim Spencer, PhD
Executive Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

The revised MS has been noticeably improved, specifically the addition of data using DR4/5 knockout cells and Caspase 8 mutants have provided mechanistic insight and additional novelty to an already very nice study. Publication is recommended following a few minor text changes, essentially just inclusion of comments and citations used in the rebuttal that will improve readability and reach.
In the rebuttal, authors nicely explain the purpose of adding CHX to TRAIL and cite studies (Wajant, Guseva). Including a short version of this statement and the citation in the results section where CHX is added would make the MS more easily understood by a broad audience.
In the rebuttal, the authors provide a convincing explanation for how they have ruled out phagocytosis as a mechanism by using Y27632 and cite a study (Tonnessen-Murray et al). A version of this explanation and citation should also be in the main text, in the discussion or results section.
In the rebuttal, the authors cite a study supporting classification of entosis stages. This study (Garanina) should be cited.

Dear Editor,

We would like to greatly thank you and the reviewers for this very constructive review process. We are delighted to hear that our paper is now eligible for publication in Journal of Cell Biology. We have now addressed the reviewers' points, revised the manuscript based on the suggestions and highlighted the changes in the revised manuscript. Please find our point-by-point response to reviewers' comments below.

Point by point reply by Bozkurt et al.

Reviewer #1:

<u>Minor point:</u>

1-) "*The revised MS has been noticeably improved, specifically the addition of data using DR4/5 knockout cells and Caspase 8 mutants have provided mechanistic insight and additional novelty to an already very nice study. Publication is recommended following a few minor text changes, essentially just inclusion of comments and citations used in the rebuttal that will improve readability and reach.*

a-) *In the rebuttal, authors nicely explain the purpose of adding CHX to TRAIL and cite studies (Wajant, Guseva). Including a short version of this statement and the citation in the results section where CHX is added would make the MS more easily understood by a broad audience.*

b-) *In the rebuttal, the authors provide a convincing explanation for how they have ruled out phagocytosis as a mechanism by using Y27632 and cite a study (Tonnessen-Murray et al). A version of this explanation and citation should also be in the main text, in the discussion or results section.*

c-) *In the rebuttal, the authors cite a study supporting classification of entosis stages. This study (Garanina) should be cited."*

a-) We thank reviewer 1 for this suggestion. We have added the statement "Translation inhibition by CHX downregulates key survival components during death receptor activation such as cFLIP, MCL-1, and BCL-XL, and is routinely used to sensitize cells to TRAIL-induced apoptosis (Dijk et al., 2013; Wajant et al., 2000; Guseva et al., 2008)" to the results section where CHX is added (page 4).

b-) We have added the statement "ROCK inhibitor Y-27632, which effectively blocks entosis without affecting phagocytosis-like activity (Tonnessen-Murray et al., 2019)" to the discussion section (page 13).

c-) We have cited the paper from Garanina et al. in page 7 “In line with previous findings…”.