The *Caenorhabditis elegans* anchor cell transcriptome: ribosome biogenesis drives cell invasion through basement membrane

Daniel S. Costa, Isabel W. Kenny-Ganzert, Qiuyi Chi, Kieop Park, Laura C. Kelley, Aastha Garde, David Q. Matus, Junhyun Park, Shaul Yogev, Bob Goldstein, Theresa V. Gibney, Ariel M. Pani and David R. Sherwood
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MS TITLE: The C. elegans Anchor Cell Transcriptome: Ribosome Biogenesis Drives Cell Invasion through Basement Membrane

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I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Most importantly, as pointed out by reviewer 1, much of the text needs to be toned down to align with the findings and, where needed, caveats should be included to align the interpretations with the findings. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The C. elegans anchor cell (AC) is a tractable model to dissect genetic and cell biological regulation of cell invasion. Unlike other models, AC invasion occurs in a stereotyped manner; a property the authors used to isolate ACs and define the transcriptome of an invading cell in vivo. Using endogenously tagged constructs, RNAi-mediated knockdown of candidates, and cell-specific protein depletion, led to discovery of several new regulators of AC invasion, including an ortholog of the transcriptionally controlled tumor protein TCTP (TCT-1).

Moreover, the authors provide evidence that the invading AC undergoes an upregulation of translational capacity (defined by an increase in proteins related to ribosome biogenesis, expansion of the endomembranous secretory system and activation of the ER stress machinery) that is required for AC invasion through the basement membrane.

This work makes several important contributions. First, it defines, for the first time, the transcriptome of an invading cell in vivo. Second, it identifies several heretofore unknown players in cell invasion, although of these only TCT-1 is explored in further detail. Finally, this work shows that upregulation of the translational machinery plays a critical role in the invasive potential of cells; a feature that could be exploited in future approaches to target invasive activity in cancer.

Comments for the author

Although the work presented is compelling, my major concern is that overall, it feels like the two stories presented (discovery of new invasion regulators and the proposed function of translational regulation in invasion) do not coalesce into a cohesive finding. Only one of the newly-discovered factors, TCT-1, is analyzed in some detail, but the discovery that this factor is required for F-actin, ZMP-1, and mitochondrial enrichment does not provide significant novel insights into the molecular function of TCT-1/TCTP, and there is no assessment of how TCT-1 may, or may not, be involved in translational regulation, which would help connect the first and second parts of this work.

Another concern is that some of the data is overinterpreted. For example, it is stated throughout that ribosomes and ER are enriched at the same sites prior to AC invasion. However, none of the data shown are from strains where fluorescently tagged ribosomes and ER are co-expressed in the same animal. Unless the authors perform co-localization studies using a different tag for one of the components (for example, they could use a split-wrmScarlet as described in Goudeau, et al 2021), they cannot state that ribosomes are enriched at the ER. If it is not possible to create a new reporter for the ER or ribosomal markers, it would be best if the authors tone down statements of ER enrichment of ribosomes, for example by stating that ribosome accumulation is “consistent” with enrichment at the ER based on similar patterns of localization. Moreover, experiments showing that disruption of the ER (e.g., sec-61 RNAi) disrupts ribosomal localization would also help support the model that ER expansion and ribosomal accumulation in the AC are functionally related.

The interpretation, and/or presentation, of some of the transcriptomic findings are not well supported and should be discussed further. For example, the authors state (pg. 6, line 135) that “To validate our AC transcriptome, we compiled a list of 52 genes previously shown with fluorescent reporters to be expressed in the AC during invasion (Table S3) and found that 51/52 of these genes were present with at least 10 copies in one of the AC libraries”. However, only ~10 of these genes were enriched in the AC, defined as >2-fold change over whole-body in the transcriptome data. This warrants further discussion as to why the >2-fold threshold was chosen and what this threshold means in terms of false negative rates of discovery, or alternatively whether there is something to be learned about the reliability of transcriptional reporters.
Similarly, the authors state (pg. 7, line 170) that they identified 84 putative novel AC invasion regulators which they tested by RNAi (although the number should be 82, as the 84 genes listed in Table S5 include a negative and a positive control). Of these, RNAi-mediated knockdown of only 13 (~16%) caused significant AC invasion defects.
Again, further discussion of this apparent low rate of true positive hits, at least with respect to their function invasion, would be welcome. Finally, the authors noted (pg. 9, line 209) that “26 AC transcriptome enriched genes encoded translational regulators, and 12 encoded ribosomal proteins of the ribosomal large subunit (RPLs, Table S6). These observations suggest that ribosome biogenesis and protein translation may be upregulated to promote BM invasion”. It is not clear to me that 38 genes, out of 1,502 transcripts with significantly elevated expression (pg. 6, line 132), suggests a strong requirement for ribosomes and ribosomal biogenesis in invasion. The authors should provide pathway enrichment analyses to support this statement.

Minor comments:
1) Pg. 7, line 148: dmd-3 “was solely detected in the AC”. Then what are the arrowheads in Fig. S3B pointing at? Other sites of expression or something else (background)?

2) Pg. 14. line 342: “The late loss of RPL-31 …”. Late with respect to what?
The AC-specific RNAi? What is the relative timing of knockdown via AC-specific RNAi vs. ZIF-1-mediated depletion?

3) Pg. 18, line 434: “modest reduction in an RPL (~20%, RPL-31) blocked invasion …”. Authors should specify in the main text how they reached this conclusion, rather than have it in the Methods (or at least point to the methods section where this is explained).

4) Fig. 5A: the lines occlude the nucleolus. Also, these data would be more convincing if the nucleolar area were normalized to the AC area. Authors could measure IFB-1::eGFP in the background of a red AC marker and compare AC area to nucleolar area. It would also help to compare nucleolar/cell area of another cell (nearby UC or VPC) to determine if the nucleolar size increase is significant and AC-specific.

5) Fig. 5E: It is difficult to see the difference between breached and intact BMs via DIC in these figures. Can a different colored fluorescent BM marker be used?

Reviewer 2

Advance summary and potential significance to field

By first identifying the AC transcriptome and genes enriched in this cell, Costa and colleagues show that TCTP and ribosomal proteins are required for AC invasion. While a requirement for ribosomal proteins in a cell biological event is not surprising per se, the novel insights presented here are that ribosome biogenesis is upregulated right as the AC invades, and ER-localized ribosomes especially become enriched. This coincides with the increased expression of several proteins previously shown to be important for AC invasion. The authors propose that increased ribosome synthesis is needed for a burst in translation that accompanies, and is needed for, AC invasion through the basement membrane.

These findings should be of broad interest given that the AC is a model for cell invasion during tumor metastasis. A strength of the analysis is that the authors were able to profile ACs as they were invading in a living animal, which is not feasible with tumor cell models. In addition to the basic biological insights provided by the study, the authors’ findings suggest new avenues to consider when developing strategies to inhibit tumor metastasis. I have only minor suggestions for improving the manuscript, which was compelling and well-written.

Comments for the author

Minor comments

1. The criteria for identifying genes with >2-fold elevated expression in ACs uses a p value of 0.1 or less, so the 1,502 genes identified in this manner is not much greater than the number expected by chance alone with a p value of $p < 0.1$. Some discussion of this point, or even better a supplemental graph showing expected versus actual numbers of genes binned by p value, would be useful for the reader to interpret the data.

2. The mean values for zmp-1::mNG expression in 2-cell control versus tct-1 is only slightly different, but the difference in the image shown appears dramatic and not representative of the quantification. Are a few outliers in the control making it appear that there is a real difference here? One concern is that the expression levels vary with precise stage (as shown later), and more ACs with higher expression were imaged by chance in the controls. Showing that this small difference is reproducible in independent experiments would make the interpretation of this experiment more convincing.

3. Although the reduction-of-ribosomal-protein experiments are warranted, AC invasion defects could result from general problems in cell physiology due to lower levels of housekeeping proteins that have nothing to do with invasion directly. This caveat should be acknowledged in the text when discussing the interpretation of these experiments.

4. I don't understand the significance of the sma-5 experiment showing that a small AC does not have an invasion defect. These are small AC in a small body, which does not address whether a small AC in a normal body would have an invasion defect. The sma-5 ACs also increase in size like wild-type ACs.

5. It would be useful to compare the dramatic increase in ribosomal protein synthesis in the AC to that of other adjacent cells to understand whether the increase is stage-specific or cell-type specific. In the images in Fig 5B,C it does appear that the AC might have a stronger increase relative to adjacent uterine cells.

First revision

Author response to reviewers' comments

Thank you for reviewing our manuscript **"The C. elegans Anchor Cell Transcriptome: Ribosome Biogenesis Drives Cell Invasion through Basement Membrane".** We are delighted that you and the two reviewers are supportive of our study being published in Development once we address the requested changes. We have now addressed all the requested changes highlighted by the reviewers and are grateful for their thoughtful comments and suggestions, which have strengthened the manuscript. Our work generating the first transcriptome of an invading cell breaching basement membrane in an *in vivo* context has identified many new regulators of invasion and revealed that a burst of ribosome biogenesis and expansion of the endomembrane system occurs prior to invasion. We believe this work significantly advances our understanding of cell invasion and will extensively impact the field of invasion and cell migration.

We have made the following changes to our manuscript based on the advice of you and the two external reviewers.

All changes in the manuscript are in blue. We have provided a manuscript file with all of our changes accepted for easier reading, however, we have saved a version with tracked changes that we can provide upon request.

Note: In making these changes, we have tried to be concise in our response, however, we are now slightly over the word limit for Development. We feel that further shortening of the manuscript would leave out important information, but are open to suggestions on where to shorten if further cutting is necessary.

Comment from handling editor:

"Most importantly, as pointed out by reviewer 1, much of the text needs to be toned down to align with the findings and, where needed, caveats should be included to align the interpretations with the findings."

We thank the reviewers (especially reviewer #1) for the suggestions on better aligning our interpretations. In the response to the reviewers below, we have toned down our comments on co-localization of ribosomes with Sec-61 (reviewer #1 request) and suggested that ribosome biogenesis could possibly have a more general role in cell health that inhibits invasion rather than a specific role in expanding translation capacity to produce pro-invasive proteins that carry out cell invasion (reviewer #2 request).

Reviewer comments:

Reviewer #1 comments to author:

Advance Summary and Potential Significance to Field:

"The C. elegans anchor cell (AC) is a tractable model to dissect genetic and cell biological regulation of cell invasion. Unlike other models, AC invasion occurs in a stereotyped manner; a property the authors used to isolate ACs and define the transcriptome of an invading cell in vivo. Using endogenously tagged constructs, RNAi-mediated knockdown of candidates, and cell-specific protein depletion, led to discovery of several new regulators of AC invasion, including an ortholog of the transcriptionally controlled tumor protein TCTP (TCT-1). Moreover, the authors provide evidence that the invading AC undergoes an upregulation of translational capacity (defined by an increase in proteins related to ribosome biogenesis, expansion of the endomembranous secretory system, and activation of the ER stress machinery) that is required for AC invasion through the basement membrane.

This work makes several important contributions. First, it defines, for the first time, the transcriptome of an invading cell in vivo. Second, it identifies several heretofore unknown players in cell invasion, although of these only TCT-1 is explored in further detail. Finally, this work shows that upregulation of the translational machinery plays a critical role in the invasive potential of cells; a feature that could be exploited in future approaches to target invasive activity in cancer."

We thank the reviewers thoughtful summary and for appreciating the breadth of the work and the significance of the first transcriptome of a cell invading cell through BM in an in vivo context.

Reviewer 1 Comments for the Author:

1. *Although the work presented is compelling, my major concern is that overall, it feels like the two stories presented (discovery of new invasion regulators and the proposed function of translational regulation in invasion) do not coalesce into a cohesive finding. Only one of the newly-discovered factors, TCT-1, is analyzed in some detail, but the discovery that this factor is required for F- actin, ZMP-1, and mitochondrial enrichment does not provide significant novel insights into the molecular function of TCT-1/TCTP, and there is no assessment of how TCT-1 may, or may not, be involved in translational regulation, which would help connect the first and second parts of this work.*

We greatly appreciate the reviewers point. Our intention was to show how the AC transcriptome could be used to screen classes of upregulated genes to identify individual genes promoting invasion (*tct-1*) as well as pathways/cellular processes (ribosome biogenesis). We have thus now added the following in the Results section, which links the two sections:

Lines: 214-221
"The *C. elegans* TCT-1 homolog TCTP binds several translation regulatory factors, but the functional significance of these interactions are unclear (Bommer and Telerman, 2020). Protein expression from reporter genes in the AC after RNAi-mediated knockdown of TCT-1 was unchanged (Fig. S4), suggesting that TCT-1 does not modulate translation to promote invasion. However, using the Database for Annotation, Visualization, and Integrated Discovery (DAVID), which identifies enriched biological programs in gene expression data sets (Dennis et al., 2003a), we found that translation was one of the top ten pathways most prevalent in AC enriched genes (Table S6). "

We note that although TCTP binds to multiple translation regulatory factors (ribosomes, translation elongation, elongation factor GEF, RACK1) the functional importance of these interactions and a role in translation is unclear. Our new data, as the reviewer requests, **"***there is no assessment of how TCT-1 may, or may not, be involved in translational regulation, which would help connect the first and second parts of this work"* did not find a role for TCT-1 (TCTP) in regulating translation in the AC. Thus, this is also an advance for the field. Once again, we thank the reviewer for this suggestion.

2. Another concern is that some of the data is overinterpreted. For example, it is stated throughout that ribosomes and ER are enriched at the same sites prior to AC invasion. However, none of the data shown are from strains where fluorescently tagged ribosomes and ER are co-expressed in the same animal. Unless the authors perform co-localization studies using a different tag for one of the components (for example, they could use a split-wrmScarlet as described in Goudeau, et al, 2021), they cannot state that ribosomes are enriched at the ER. If it

is not possible to create a new reporter for the ER or ribosomal markers, it would be best if the authors tone down statements of ER enrichment of ribosomes, for example by stating that ribosome accumulation is "consistent" with enrichment at the ER based on similar patterns of localization.
This is an excellent point and we agree that we overinterpreted our data in this case. We have now toned down the way that we interpret ribosome and ER colocalization. Because we are over the Development word limit, for brevity, we often indicate that our localization data indicates that the ribosomes localize to the region near the ER or Sec61.

Specific examples include:

Abstract

Lines: 37-38
Was: "Ribosomes also strongly localize to the AC's endoplasmic reticulum (ER) and the endomembrane system expands prior to invasion."

Changed to: "Ribosomes also enrich near the AC's endoplasmic reticulum (ER) SEC-61 translocon and the endomembrane system expands prior to invasion."

Results Section

Line: 336
Was: "Ribosomes localize to the ER prior to AC invasion and promote invasion at this time"

Changed to: "Ribosomes localize to the ER region and promote AC invasion"

Lines: 362-365
Was: "Taken together these results suggest that AC ribosomes enrich at SEC-61/ER prior to invasion and that their activity is required for BM breaching at this time."

Changed to: "These results indicate that AC ribosomes enrich around the nucleus near the SEC- 61 translocon prior to invasion and that ribosome perinuclear enrichment is dependent on SEC-61. Further, our results show that ribosome activity is required for BM breaching at this time."

Line: 368
Was: "The localization of the ribosomes to the ER

Changed to: "The localization of the ribosomes to the SEC-61/ER region"

We have also changed the graph headings on Figure 5B and 5C to "ER Region Enrichment".

Discussion Section

Lines: 469- 471
Was: "By using a split-GFP approach to label endogenous ribosomes (Noma et al., 2017), we found that AC-ribosomes enriched at the ~~site~~ of Sec61/ER ~2.5h prior to BM invasion."

Changed to: "By using a split-GFP approach to label endogenous ribosomes (Noma et al., 2017), we found that AC-ribosomes enriched at the region of Sec61/ER ~2.5 h prior to BM invasion."

3. Experiments showing that disruption of the ER (e.g., sec-61 RNAi) disrupts ribosomal localization would also help support the model that ER expansion and ribosomal accumulation in the AC are functionally related.

We thank the reviewer for this excellent suggestion that could strengthen the connection between ribosomes and SEC-61/the ER. We have thus used RNAi to deplete the SEC-61 gamma subunit (*sec-61.G)* using RNAi and then investigated the localization of RPL-4::GFP11 and RPL-31::GFP11. Notably we found that both large ribosomal subunit proteins no longer enriched in the region around the nucleus, where SEC-61 localized most strongly. This shows that ribosomes are

dependent on SEC-61 for their enrichment near the SEC-61 ER translocon and further supports the idea that ribosomes localized in this region are mediating translation of secretory proteins.
We have now included these data in Figure S6D and its corresponding figure legend as well as the following text to the result section

Lines: 347-351
"Consistent with an important role of SEC-61 and ER translocated proteins, RNAi targeting *sec-61.G* in the AC-specific RNAi strain, lead to a strong invasion defect (Table 1). Furthermore, RNAi-mediated depletion of SEC-61.G disrupted the enrichment of both RPL-4::GFP11 and RPL-31::GFP11 around the nucleus where the SEC-61 translocon localizes (Figure S6D)."

Lines: 362-365
Was: "Taken together these results suggest that AC ribosomes enrich at SEC-61/ER prior to invasion and that their activity is required for BM breaching at this time."

Changed to: "These results indicate that AC ribosomes enrich around the nucleus near the SEC-61 translocon prior to invasion and that ribosome perinuclear enrichment is dependent on SEC-61. Further, our results show that ribosome activity is required for BM breaching at this time."

4. The interpretation, and/or presentation, of some of the transcriptomic findings are not well supported and should be discussed further. For example, the authors state (pg. 6, line 135) that "To validate our AC transcriptome, we compiled a list of 52 genes previously shown with fluorescent reporters to be expressed in the AC during invasion (Table S3) and found that 51/52 of these genes were present with at least 10 copies in one of the AC libraries". However, only ~10 of these genes were enriched in the AC, defined as >2-fold change over whole-body in the transcriptome data. This warrants further discussion as to why the >2-fold threshold was chosen and what this threshold means in terms of false negative rates of discovery, or alternatively whether there is something to be learned about the reliability of transcriptional reporters.

These are excellent points that the reviewer makes. We did not do an adequate job of writing this section and clearly explaining our rationale for selecting the > 2-fold change of AC gene enrichment over whole-body expression. Actually of the 51 genes previously shown with fluorescent reporters that we found were present in AC transcriptome, 26 of the 51 had > 2-fold increase in enrichment versus WB transcripts (log2 fold change greater than 1). To test whether the 2-fold or greater cut off was robust for identify additional genes expressed at high levels in the AC, we endogenously tagged 5 additional genes that were present at 2-fold or greater expression. We also examined 9 genes that had been previously endogenously tagged and were present at 2-fold or greater levels in the AC versus WB transcriptome. The proteins from all 14 of these genes were present at high levels in the AC, and 12 were higher than neighboring non-invasive uterine cells. We also looked at 5 additional genes that showed 1-2 fold enrichment. All were present in the AC, but were generally present at the same levels as neighboring non-invasive uterine cells, and only ZMP-1 expressed more strongly than neighoring non-invasive cells. The clear high expression of 12/14genes in the AC showing 2- fold or greater enrichment was thus our justification for using this as a threshold for further analysis. We have now re-written this section as follows to make our justification more clear (rewritten parts underlined):

Lines: 136 – 166
"To begin to assess our AC transcriptome, we compiled a list of 52 genes previously shown with fluorescent reporters to be expressed in the AC during invasion (Table S3) and found that 51/52 of these genes were present with at least 10 copies in one of the AC libraries (Tables S1,S3). Notably, 26 of these genes had elevated expression (> 2-fold, log2 fold change great than 1.0, Table S3), suggesting > 2-fold enrichment identifies many key genes expressed in the AC. To further validate our transcriptome and a > 2-fold enrichment threshold as identifying genes with elevated expression in the AC, we also created genome edited *C. elegans* strains where mNeonGreen (mNG) was inserted in frame with the protein coding region of five genes enriched at 2-fold or greater —*tct-1* (translationally controlled tumor protein), s*nb-1* (synaptobrevin), *eif-1.A* (translation initiation factor EIF1A), *lin-3* (EGF ligand) and *rab-11.1* (Rab11 small GTPase). We also examined 9 other previously tagged genes enriched at > 2-fold and 7 that were either slightly enriched or underenriched (Fig. S2, Table S4). Examination of ACs at the time of BM breaching (P6.p 2-cell stage, n ≥ 5 animals each) revealed that the 14 proteins encoding genes that were

annotated as enriched 2-fold or greater (Fig. S2) were all present at high levels in the AC, and 12 of the 14 were expressed at levels higher than neighboring uterine cells (Fig. 1E, Fig. S2). TCT-1, which was highly enriched in the AC, was also at high levels in neighboring uterine cells (Fig. S3A). Notably, the DMD-3 protein (doublesex), whose encoding mRNA was extremely enriched (~45-fold enrichment), was solely detected in the AC at the mid-L3 stage (Fig. S3B). The proteins encoded by genes whose transcripts were slightly enriched or at equivalent levels in the AC (~1-to-2-fold enrichment versus WB) were generally present at equivalent levels to uterine cells (Fig. S2). An exception was the ZMP-1 protein (GPI anchored MMP), which was present at high levels in the AC, but was not highly enriched in the transcriptome. Lack of *zmp-1* mRNA enrichment in the AC versus WB was likely a result of high levels in the WB from *zmp-1* expression in non-uterine and non-vulval tissues (Kaletsky et al., 2018). AIR-2 (Aurora B) and PAT-3 (b integrin) proteins were also examined and both were underenriched in the AC transcriptome (< 0.5 fold). Consistent with this, AIR-2 was at low-to- undetectable levels in the AC, but present in neighboring uterine cells (Fig. 1E; Fig. S3C). However, PAT-3 protein was present at high levels in the AC (Fig. S2). The low enrichment of *pat-3* was likely because of strong *pat-3* expression in body wall muscles (Gettner et al., 1995). We conclude that the AC transcriptome has a high fidelity and that a threshold of a > 2-fold enrichment identifies genes expressed at high levels in the AC."

5. Similarly, the authors state (pg. 7, line 170) that they identified 84 putative novel AC invasion regulators, which they tested by RNAi (although the number should be 82, as the 84 genes listed in Table S5 include a negative and a positive control). Of these, RNAi- mediated knockdown of only 13 (~16%) caused significant AC invasion defects. Again, further discussion of this apparent low rate of positive hits, at least with respect to their function invasion, would be welcome.

We thank the Reviewer for pointing out our error of Table S5. We have now corrected this in the manuscript. We also thank the reviewer for pointing out the low percentage of genes that gave AC invasion defects after RNAi knockdown. We did not do an adequate job of explaining why this is likely the case. While this percentage of invasion defects might seem low, biological processes are robust/redundant. For example, only ~20% of yeast genes are necessary for viability:

https://www.nature.com/articles/nrg2085

As this review points out, there are *"~200,000 synthetic-lethal/sick double-mutant combinations, indicating that there are ~200-fold more ways of creating the same mutant phenotype through a digenic interaction."*

We have found this robustness/redundancy extensively in the AC, where a number of genes expressed in the AC don't have phenotypes on their own, but only in combination with other genes—e.g. ZMP-1 matrix metalloproteinase, MIG-2 Rho GTPase, CED-10 Rho GTPase, MIG- 10 lamellipodin, FGT-2 glucose transporter (PMIDs: 35316617, 30686527, 24553288). One of the strengths of the AC-transcriptome is providing a list of genes to prioritize for future synthetic genetic interaction screens. To address this important point, we have rewritten the ending of the paper as follows:

Lines: 500-506
Was: "As cell invasion has feedback and adaptive mechanisms that ensure robustness (Parlani et al., 2022), focused screens in mutant backgrounds might be an especially powerful approach to reveal mechanisms underlying invasion and potential new strategies for targeting invasive activity in cancer."

Changed to: "Importantly, the AC and other invasive cells have feedback and adaptive mechanisms that ensure robustness (Garde et al., 2022; Kelley et al., 2019; Parlani et al., 2022; Wang et al., 2014a), which is likely the reason that few individual genes that had increased expression in the AC had invasion defects (only 16%) in our RNAi screen. Thus, synthetic screens in mutant backgrounds (Boone et al., 2007) might be an especially powerful approach to reveal mechanisms underlying invasion and new strategies for targeting invasive activity in cancer."

6. Finally, the authors noted (pg. 9, line 209) that "26 AC transcriptome enriched genes encoded translational regulators, and 12 encoded ribosomal proteins of the ribosomal large subunit (RPLs, Table S6). These observations suggest that ribosome biogenesis and protein translation may be

upregulated to promote BM invasion". It is not clear to me that 38 genes, out of 1,502 transcripts with significantly elevated expression (pg. 6, line 132), suggests a strong requirement for ribosomes and ribosomal biogenesis in invasion. The authors should provide pathway enrichment analyses to support this statement.

We thank the reviewer for pointing out that our statements regarding ribosomal biogenesis and protein translation genes that are upregulated based on the transcriptome needs support from pathway transcriptome analysis. We have now provided a table (Table S6) using the Database for Annotation, Visualization, and Integrated Discovery (DAVID), which identifies enriched biological programs in gene expression data sets. While 25 translation associated genes (it was 26 in our original submission, however, one was counted twice as it was associated with two human isoforms), might not sound like many, DAVID considers the total number of genes in the pathway when determining enrichment. See:

https://david.ncifcrf.gov/helps/functional_annotation.html
The total genes within the translation pathway is 181 of which 25 are enriched in the AC transcriptome (> 2 fold enrichment versus whole body expression). DAVID examines whether 25/1,502 transcripts is significant versus 181/total *C. elegans* genome background. We note in the Table S6 that by far the most upregulated pathway is cuticular collagens. We suspect that these are deposited by the AC into the apical extracellular matrix after the AC fuses with the uterine cells shortly after AC invasion. We have noted this in Table S6 figure legend.
We have now added the following to the results section:

Lines: 214-223
"The *C. elegans* TCT-1 homolog TCTP binds several translation regulatory factors, but the functional significance of these interactions are unclear (Bommer and Telerman, 2020). Protein expression from reporter genes in the AC after RNAi-mediated knockdown of TCT-1 was unchanged (Fig. S4), suggesting that TCT-1 does not modulate translation to promote invasion. However, Using the Database for Annotation, Visualization, and Integrated Discovery (DAVID), which identifies enriched biological programs in gene expression data sets (Dennis et al., 2003a; Sherman et al., 2022), we found that translation was one of the top ten pathways most prevalent in AC enriched genes from the AC transcriptome (Table S6). Of the 25 AC enriched genes encoding translational regulators, 13 were ribosomal proteins of the ribosomal large subunit (RPLs, Table S7)."

We have also added Table S6 and the Table S6 figure legend:

"Table S6. Pathway analysis of AC enriched genes.
Table of AC-enriched annotated terms and protein domains (referred to as pathways in text for simplicity) sorted by p-values identified using Database for Annotation, Visualization and Integrated Discovery (DAVID). Terms and domains are arranged in order of their significance. Total genes annotated with the term or domain is indicated in the Count column, while the total genes annotated with term or domain lists the total number of genes in the *C. elegans* genome in with that term or domain. Fisher Exact p-value was used for gene-enrichment analysis.
Resource database used for each term or domain is shown. Notably, cuticular collagens were the most enriched genes in the AC. This is likely for a post-invasion role of the AC in contributing to the apical extracellular matrix of the vulva."

Minor comments:

7. Pg. 7, line 148: dmd-3 "was solely detected in the AC". Then what are the arrowheads in Fig. S3B pointing at? Other sites of expression or something else (background)?

We thank the reviewer for this comment and their careful reviewing of our manuscript. We had forgotten to mention that the arrowheads were pointing to autofluorescent gut granules in the image. We have now updated the figure legends as follows.
Figure S3 legend:
"Arrowheads indicate autofluorescent gut granules"

8. Pg. 14. line 342: "The late loss of RPL-31 …". Late with respect to what? The AC-specific

RNAi? What is the relative timing of knockdown via AC-specific RNAi vs. ZIF-1-mediated depletion?
We thank the reviewer for this comment. AC-specific RNAi knockdown occurs right at the time of AC specification, ~6 h before invasion), while loss of RPL-31 via ZIF-1 occurs ~2 h prior to invasion. We agree with the reviewer that this statement is not clear enough. In the full context, we had meant to convey that the loss of RPL-31 occurs just prior to invasion when ribosomes concentrate around the region of SEC-61, thus implicating translation at this time as being required for invasion. We have thus changed the wording to the following (underlined).

Lines: 359-365
"At this time, loss of RPL-31::ZF1::GFP11 was also first observed (n=6/10 no signal). By the P6.p 2-cell stage (~2 h prior to invasion), RPL-31::ZF1::GFP11 was not detected in the AC (Fig. 5E, n=10/10). This ZIF-1 mediated loss of RPL-31 ~2 h prior to invasion resulted in a highly penetrant invasion defect (Table S7). These results indicate that AC ribosomes enrich around the nucleus near the SEC-61 translocon prior to invasion and that ribosome perinuclear enrichment is dependent on SEC-61. Further, our results show that ribosome activity is required for BM breaching at this time."
Note, we also state when AC-specific RNAi knockdown occurs in the results section as follows:

Lines: 230-233
"Using an AC-specific RNAi strain, where the AC becomes sensitive to RNAi shortly after the time of its specification (~6h prior to invasion, Methods), we targeted 10 AC-enriched RPLs, as well as *rpl-4* and *rpl-6* which encode core RPL proteins in mouse embryonic stem cells (Shi et al., 2017)."

9. Pg. 18, line 434: "modest reduction in an RPL (~20%, RPL-31) blocked invasion …". Authors should specify in the main text how they reached this conclusion, rather than have it in the Methods (or at least point to the method section where this is explained).

We thank the reviewer for this point and we have added the following text.

Lines: 448-449
"modest reduction in an RPL (~20%, RPL-31(Methods, RNAi Knockdown Efficiency))"

10. Fig. 5A: the Lines: occlude the nucleolus.

We agree with the reviewer that the Lines: are too large. However, they are important as they highlight the nucleolus size. Thus, we have decreased the opacity of the Lines: around the nucleolus in Fig. 5A and decreased the line width in half.

11. Also, these data would be more convincing if the nucleolar area were normalized to the AC area. Authors could measure FIB-1::eGFP in the background of a red AC marker and compare AC area to nucleolar area. It would also help to compare nucleolar/cell area of another cell (nearby UC or VPC) to determine if the nucleolar size increase is significant and AC-specific.

We appreciate the reviewers suggestion. This analysis assumes that nucleous size scales with cells size and this is not the case. In fact sometimes it is the reverse

See: https://www.cell.com/current-biology/pdfExtended/S0960-9822(15)00014-7

We therefore think this is not appropriate to present this data in the paper, as it is confusing and not pertinent to our findings.

We use nucleolus size and FIB-1 as independent markers for ribosome biogenesis—the nucleolus size of a cell over time is a well-established marker for ribosome biogenesis and we further support these findings by using FIB-1, an independent marker marker for ribosome biogenesis.

See: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3345250/

To address the reviewers request for examination of the nucleolus/ribosome biogenesis in another cell, we have now added Figure S6A (and updated the figure legend), which shows that

neighboring non-invasive uterine cells (these are cells that are lineagely most closely related to the AC) do not show an increase in nucleolus size/ribosome biogenesis.
Results section

Lines: 304-307
"Strikingly, the AC nucleolus showed an ~50% increase in cross-sectional area from the early 1- cell stage (~6 h prior to invasion) to late 1-cell stage (~2.5 h prior to invasion) and then decreased in size during invasion (Fig. 5A). Notably, neighboring non-invasive uterine cells did not show a change in nucleolus size during this time (Fig. S6A)."

Finally, to further test whether ribosomes and ribosome biogenesis promote the increase in AC size, which is important to our study and we thought needed stronger testing, we have added new RNAi data targeting the ribosome biogenesis factor *nifk-1*. We found that similar to targeting *rpl-31* and *rpl-4* RNAi treatment did not reduce AC size. This data is now added to Figure 3B (and text of the figure legend).

Results Section

Lines: 260-262

"RNAi-mediated loss of RPL-4 and RPL-31, and the ribosome biogenesis factor NIFK-1, however, did not reduce AC size expansion (Fig. 3B)."

12. Fig. 5E: It is difficult to see the difference between breached and intact BMs via DIC in these figures. Can a different colored fluorescent BM marker be used?

We thank the reviewer for bring up this point. Due to the difficulty in crossing additional markers into these strains we have replaced the DIC images in Fig. 5E with better DIC images that more clearly show the intact and breached BM.

Reviewer #2 comments to author:
Advance Summary and Potential Significance to Field:
By first identifying the AC transcriptome and genes enriched in this cell, Costa and colleagues show that TCTP and ribosomal proteins are required for AC invasion. While a requirement for ribosomal proteins in a cell biological event is not surprising per se, the novel insights presented here are that ribosome biogenesis is upregulated right as the AC invades, and ER-localized ribosomes especially become enriched. This coincides with the increased expression of several proteins previously shown to be important for AC invasion. The authors propose that increased ribosome synthesis is needed for a burst in translation that accompanies, and is needed for, AC invasion through the basement membrane.

These findings should be of broad interest given that the AC is a model for cell invasion during tumor metastasis. A strength of the analysis is that the authors were able to profile ACs as they were invading in a living animal, which is not feasible with tumor cell models. In addition to the basic biological insights provided by the study, the authors' findings suggest new avenues to consider when developing strategies to inhibit tumor metastasis. I have only minor suggestions for improving the manuscript, which was compelling and well-written.

We thank the reviewer for their comments on the importance of our work. We also thank the reviewer for highlighting the long term impact of our study in its use in developing strategies to halt tumor metastasis.

Minor comments:

1. The criteria for identifying genes with >2-fold elevated expression in ACs uses a p value of 0.1 or less, so the 1,502 genes identified in this manner is not much greater than the number expected by chance alone with a p value of $p < 0.1$. Some discussion of this point, or even better a supplemental graph showing expected versus actual numbers of genes binned by p value, would be useful for the reader to interpret the data.

This is an excellent point brought up by the reviewer. We would like to note that we are using the False Discovery Rate (FDR) adjusted p-value not just the p-value. The reviewer is correct in that if we were to have used the p-value (without adjustments) our genes expected by chance would be almost equivalent to our number of hits. However, with this large dataset we thought it appropriate to use an adjusted p-value to reduce the proportion of false positives by using the Benjamini-Hochberg correction to control the FDR. Our adjusted p-value < 0.1 means that 10% of the ~1500 differentially expressed genes will be false positives. Thus ~150 genes would be expected to be false positives, not close to 10% of the total genes in the AC transcriptome from the non-adjusted p-value. Neverthelss, we agree with the reviewer that it is critical to rigorously address our threshold of >2-fold elevated expression. To do this we examined endogenous proteins levels of 21 genes whose transcripts were enriched at various levels in the AC transcriptome. This analysis supported our use of the > 2-fold level of enrichment threshold as identifying genes whose expression is upregulated in the AC, as the protein products of all 14 genes assessed with a > 2-fold level of enrichment the AC were present at high levels in the AC, and 12/14 were present at higher levels than neighboring non-invasive uterine cells. We have now better outlined this reasoning in detail in the results section as follows (new portions underlined):

Results Section

Lines: 136-166
"To begin to assess our AC transcriptome, we compiled a list of 52 genes previously shown with fluorescent reporters to be expressed in the AC during invasion (Table S3) and found that 51/52 of these genes were present with at least 10 copies in one of the AC libraries (Tables S1,S3). Notably, 26 of these genes had elevated expression (> 2-fold, log2 fold change great than 1.0, Table S3), suggesting > 2-fold enrichment identifies many key genes expressed in the AC. To further validate our transcriptome and a > 2-fold enrichment threshold as identifying genes with elevated expression in the AC, we also created genome edited *C. elegans* strains where mNeonGreen (mNG) was inserted in frame with the protein coding region of five genes enriched at 2-fold or greater —*tct-1* (translationally controlled tumor protein), s*nb-1* (synaptobrevin), *eif-1.A* (translation initiation factor EIF1A), *lin-3* (EGF ligand) and *rab-11.1* (Rab11 small GTPase). We also examined 9 other previously tagged genes enriched at > 2-fold and 7 that were either slightly enriched or underenriched (Fig. S2, Table S4). Examination of ACs at the time of BM breaching (P6.p 2-cell stage, n ≥ 5 animals each) revealed that the 14 proteins encoding genes that were annotated as enriched 2-fold or greater (Fig. S2) were all present at high levels in the AC, and 12 of the 14 were expressed at levels higher than neighboring uterine cells (Fig. 1E, Fig. S2). TCT-1, which was highly enriched in the AC, was also at high levels in neighboring uterine cells (Fig. S3A). Notably, the DMD-3 protein (doublesex), whose encoding mRNA was extremely enriched (~45-fold enrichment), was solely detected in the AC at the mid-L3 stage (Fig. S3B). The proteins encoded by genes whose transcripts were slightly enriched or at equivalent levels in the AC (~1-to-2-fold enrichment versus WB) were generally present at equivalent levels to uterine cells (Fig. S2). An exception was the ZMP-1 protein (GPI anchored MMP), which was present at high levels in the AC, but was not highly enriched in the transcriptome. Lack of *zmp-1* mRNA enrichment in the AC versus WB was likely a result of high levels in the WB from *zmp-1* expression in non-uterine and non-vulval tissues (Kaletsky et al., 2018). AIR-2 (Aurora B) and PAT-3 (b integrin) proteins were also examined and both were underenriched in the AC transcriptome (< 0.5 fold). Consistent with this, AIR-2 was at low-to- undetectable levels in the AC, but present in neighboring uterine cells (Fig. 1E; Fig. S3C). However, PAT-3 protein was present at high levels in the AC (Fig. S2). The low enrichment of *pat-3* was likely because of strong *pat-3* expression in body wall muscles (Gettner et al., 1995). We conclude that the AC transcriptome has a high fidelity and that a threshold of a > 2-fold enrichment identifies genes expressed at high levels in the AC."

2. The mean values for zmp-1::mNG expression in 2-cell control versus tct-1 is only slightly different, but the difference in the image shown appears dramatic and not representative of the quantification. Are a few outliers in the control making it appear that there is a real difference here? One concern is that the expression levels vary with precise stage (as shown later), and more ACs with higher expression were imaged by chance in the controls. Showing that this small difference is reproducible in independent experiments would make the interpretation of this experiment more convincing.

We thank the reviewer for these comments and their noticing the ZMP-1::mNG levels. Staging the AC is something we do routinely and we do precisely because of the highly stereotyped nature of invasion. For this experiment, we examined ~15-20 animals at the P6.p 2-cell stage and P6.p 4-cell stage. Both stages showed either a significant (P6.p 2-cell stage) or trending toward significant (P6.p 4-cell stage) decrease in ZMP-1::mNG protein levels after TCT-1 knockdown, supporting the validity of a decrease in ZMP-1 expression after reduction of TCT-1. The reviewer is correct about the variance at the P6.p 2-cell stage in control animals. We suspect this is when ZMP-1 expression normally ramps up and that this ramp up is delayed after TCT-1 reduction and instead occurs later between the 2-cell and 4-cell stages. The reviewer is correct in that we also selected a poor example of a representative image of ZMP-1 expression in the figure.

To address the reviewers concerns we have now replaced the ZMP-1 image after tct-1 RNAi knockdown with a representative image a decrease in ZMP-1 levels (mean fluorescence intensity) at the P6.p 2-cell stage.

control value= 21350 a.u.
tct-1 RNAi value= 20040 a.u.

We have also updated the text in the results section as follows (underlined) to highlight better the complexity of ZMP-1 expression levels:

Lines: 199-202
"RNAi-mediated reduction of *tct-1* led to a decrease in the levels of the ZMP-1::mNG (MMP) at the P6.p 2-cell stage, when the AC initiates breaching the BM, however, ZMP-1 levels recovered to near normal by the P6.p 4-cell stage when the AC completes invasion (Fig. 2B)."

3. Although the reduction-of-ribosomal-protein experiments are warranted, AC invasion defects could result from general problems in cell physiology due to lower levels of housekeeping proteins that have nothing to do with invasion directly. This caveat should be acknowledged in the text when discussing the interpretation of these experiments.

We thank the reviewer for this suggestion and we agree that this caveat should be discussed in the text. We think that the most clear way to convey this point is in the discussion section. We have thus modified the text as follows (underlined):

Lines: 455-457
"While we cannot rule out a role for translation in supporting general housekeeping proteins whose reduction might impinge on the ACs ability to invade, our observations are consistent with the idea that early ribosome biogenesis expands the AC's translation capacity to facilitate the subsequent translation of proteins that execute invasion."

We also soften our interpretation of the role of ribosome biogenesis in the Results Section by stating:

Lines: 333-334
Was: "Together, these results offer compelling evidence that ribosome biogenesis occurs shortly after AC specification to facilitate robust translation of pro-invasive proteins that promote BM invasion."

Changed to: "Together, these results offer compelling evidence that ribosome biogenesis occurs shortly after AC specification, which facilitates robust translation of pro-invasive proteins."

4. I don't understand the significance of the sma-5 experiment showing that a small AC does not have an invasion defect. These are small AC in a small body, which does not address whether a small AC in a normal body would have an invasion defect. The sma-5 ACs also increase in size like wild-type ACs.

The reviewer is correct in that *sma-5* animals also have a small body size. Our point in this experiment was to show that the AC does not need to reach its large size found in wild type animals to have invasive ability. As invasion occurs through a matrix and not cells, we considered the smaller cell size of *sma-5* mutant animals to be a reasonable test of whether small ACs can invade through extracellular matrix/basement membrane (even if the animal is smaller in size). We do not know of a way to make small ACs in a normally sized body. We have left this experiment as is, but would be happy to remove the experiment if the reviewer thinks this is still confusing.

5. It would be useful to compare the dramatic increase in ribosomal protein synthesis in the AC to that of other adjacent cells to understand whether the increase is stage-specific or cell-type specific. In the images in Fig 5B,C it does appear that the AC might have a stronger increase relative to adjacent uterine cells.

We thank the reviewer for this suggestion. We have now quantified the level of RPL-4 and RPL- 31 in neighboring uterine cells and assessed nucleolus size as a measure of ribosome biogenesis.

We present the neighboring uterine levels for RPL-4 and RPL-31 in the graphs now in Figure 5B and 5C, respectively. We found that RPL-4 levels to not change in neighboring non-invasive uterine cells prior to and during AC invasion, while RPL-31 levels increase, but not as significantly in the AC when ribosome levels increase dramatically between the early 1-cell and late 1-cell stages.

Accompanying this new data, we have now added the following text to of the Results section in the manuscript:

Lines: 329-333
"Consistent with a burst of ribosome production occurring shortly after AC specification, levels of RPL-4::GFP11 and RPL-31::GFP11 increased in the AC between the early P6.p 1-cell stage and late P6.p 1-cell stage (Fig. 5B,C). RPL-4 did not increase in levels in neighboring uterine cells at this time, while RPL-31 showed a more modest increase in levels (Fig. 5B,C)."

We have also now added Figure S6A (and updated the figure legend) showing that neighboring non-invasive ventral uterine cells do not show an increase in nucleolus size/ribosome biogenesis.

Results section

Lines: 306-307

"Notably, neighboring non-invasive uterine cells did not show a change in nucleolus size during this time (Fig. S6A)."

Second decision letter

MS ID#: DEVELOP/2022/201570

MS TITLE: The C. elegans Anchor Cell Transcriptome: Ribosome Biogenesis Drives Cell Invasion through Basement Membrane

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ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.