Article information: https://dx.doi.org/10.21037/tcr-24-1375

Reviewer #1

Question 1. Line 160: Differential Expression of *CALD1* Between Cancer and Normal Samples The authors should report the classification of the ovarian cancer samples in the tissue microarray, as differences in *CALD1* expression could be present in different types and stages of ovarian cancer.

Response: Thank you very much for the invaluable feedback. We fully agree that the expression of *CALD1* may vary across different types and stages of ovarian cancer. Based on your suggestions, we have re-evaluated our tissue microarray data and have supplemented Table 1 with additional patient information to provide a more detailed classification of the ovarian cancer samples. Our tissue microarray comprises 50 ovarian cancer samples, distributed as follows: Tumor Grade: Grade 1: 4 cases (8%), Grade 1-2: 2 cases (4%), Grade 3: 44 cases (88%). FIGO Staging: Stage I: 24 cases (48%), Stage II: 4 cases (8%), Stage III: 14 cases (28%),Stage IV: 8 cases (16%).

Supplementary Table 1. Clinicopathological characteristics of clinical samples with OV in tissue microarray chips

Features	No. of patients
Patients	50
Healthy controls	11
Age (years)	
< 59	27
≥ 59	34
Sex	
Male	0
Female	61
FIGO Staging	
Stage I	24
Stage II	4
Stage III	14
Stage IV	8
Tumor grade	
1	4
1-2	2
3	44
Lymphatic metastasis (N)	

N0	49
N1	1
T infiltrate	
T1	26
T2	4
T3	20
Cancer Subtype	
Serous carcinoma	46
Squamous cell carcinoma	2
Clear cell carcinoma	2

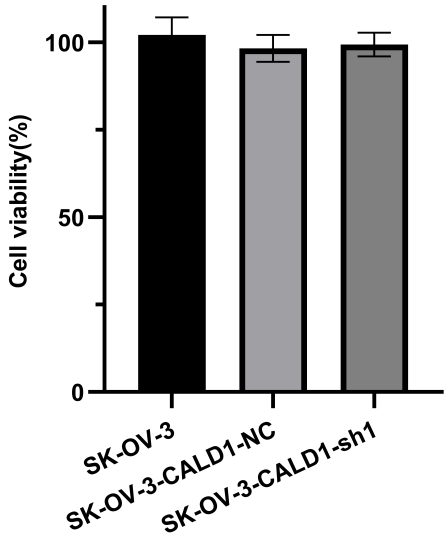
In analyzing the relationship between *CALD1* expression and tumor grading and FIGO staging, we encountered several challenges:

Uneven Sample Distribution: Our sample size, totaling 50 cases, is relatively small and unevenly distributed. For example, grade 3 samples (44 cases) significantly outnumber grade 1 (4 cases) and grade 1-2 (2 cases). Similarly, there are notably fewer stage II samples (4 cases) compared to other stages.

Statistical Analysis Limitations: Due to this uneven distribution among different grades and stages, it is challenging to conduct meaningful statistical comparisons. For subgroups with fewer samples, such as grade 1, grade 1-2, and stage II, the statistical power is limited, making it difficult to draw definitive conclusions about any observed differences. This suggests that in our subsequent experiments, we may need to significantly increase the sample size, particularly focusing on including more low-grade tumors and early-stage FIGO samples to ensure balanced group sizes.

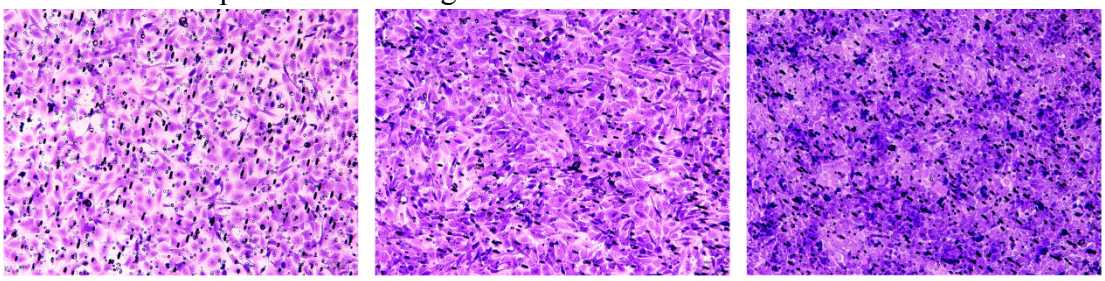
Question 2. Line 192: Impact of *CALD1* Knockout on Cell Viability The authors should verify whether the *CALD1* knockout affects cell viability.

Response: Thank you for your suggestion. The construction of stable cell lines was performed using lentiviral infection, followed by continuous selection with puromycin. We acknowledge that we overlooked the assessment of cell viability during the culturing and passaging processes. To address this, we have used the CCK-8 assay to measure the viability differences between different groups. The results have been included in the supplementary materials as Supplementary Figure 1(page 20, line 424-427).



Question 3. Line 204: Transwell Invasion Assay The assay was performed after 24 hours; during this time, cells can both migrate and proliferate, which may impact the number of cells found in the lower part of the transwell. The authors should repeat the test after 16 hours, which is the duration typically used for this kind of assay.

Response: Thank you for your valuable feedback on the Transwell Invasion Assay and your suggestion to perform the assay at 16 hours to minimize the effects of cell proliferation. We have conducted additional experiments with a 16-hour duration, and the results are consistent with those obtained at 24 hours, supporting our original conclusions (results shown in the figure below). However, we observed that the cell density may have been too high, potentially affecting the accuracy of the results. Recognizing the importance of optimizing cell density to ensure reliable data, we plan to repeat the experiments with reduced cell density and conduct multiple replicates to ensure robust and reproducible findings.



Question 4. Regarding the same test, in Figure 5, SKOV3-*CALD1* -NC cells show enhanced invasion compared to SKOV3 cells, and this difference is particularly evident in the corresponding representative images. The graph and these images suggest that the difference might be significant.

Response: Thank you for your insightful comments regarding the enhanced invasion observed in SKOV3-*CALD1*-NC cells compared to SKOV3 cells. We believe that the observed differences may be attributed to the following factors:

Selection Pressure: The use of puromycin for selection after lentiviral infection imposes a selection pressure on the cells. This process may preferentially allow the survival of cells that are more adaptable to the selection conditions, potentially leading to a population with enhanced proliferation and invasion capabilities.

Changes in Gene Expression: The lentiviral infection and subsequent selection process might induce changes in gene expression within the cells. These changes could result in the upregulation of genes associated with increased cell activity and invasion potential.

Question 5. Line 205: Quality of Images and Stress Fibers The quality of the images does not allow for clear observation of stress fibers. Additionally, SKOV3-*CALD1*-NC cells appear to have more stress fibers than SKOV3 cells and also seem larger. Can the authors explain this finding?

Response: Thank you for your insightful comments regarding the image quality and stress fibers. We acknowledge that the use of standard immunofluorescence microscopy, rather than confocal microscopy, may have limited the resolution of our images. Currently, our laboratory lacks a laser confocal microscope. We have contacted Wuhan Servicebio Technology Co., Ltd. for confocal imaging services. However, the shipping time from Xinjiang to Wuhan is approximately 4-5 days, raising concerns about fluorescence quenching. Despite these challenges, we plan to send a batch of samples to test the feasibility of obtaining high-quality confocal images.

Increased Stress Fibers in SKOV3-*CALD1*-NC Cells: Regarding the observation that SKOV3-*CALD1*-NC cells appear to have more stress fibers and seem larger than SKOV3 cells, we believe this may be due to the selection pressure and potential changes in gene expression induced by the lentiviral infection and puromycin selection process. These factors could lead to enhanced stress fiber formation and increased cell size as a cellular response.

Question 6. Line 212 and Discussion: Interpretation of Focal Adhesion and Invasion The authors show that SKOV3-*CALD1*-sh1 cells display a reduction in focal adhesion and conclude that this reduction, together with the decrease in the amount of stress fibers, culminates in increased cell invasion. I do not agree with this conclusion. Stress fibers and focal adhesions are crucial in the migration/ invasion process as they are involved in the epithelial-to-mesenchymal transition (https://doi.org/10.1098/rsob. 130001). To confirm an increase in invasion, the authors should also evaluate canonical markers of EMT (e.g., E-cadherin, N-cadherin, transcription factors such as Zeb1 and Snail).

Response: We fully acknowledge your concerns regarding our conclusions. As

highlighted in the literature you cited (https://doi.org /10.1098/rsob.130001), stress fibers and focal adhesions are widely recognized as critical components in cell migration and invasion, particularly during epithelial-mesenchymal transition (EMT). We concur with this established perspective.

Interestingly, our findings seem to diverge from this general understanding. In SKOV3-*CALD1*-sh1 cells, we observed a concurrent reduction in stress fibers and focal adhesions, coupled with an enhancement in cell invasion capability. This unexpected phenomenon may reflect the inherent complexity of cell migration and invasion mechanisms, which can vary significantly across different cell types and experimental conditions.

To address this apparent discrepancy, we propose the following explanations:

(1) Cell Type Specificity: SKOV3, an ovarian cancer cell line, may exhibit behaviors distinct from other mesenchymal cell types, potentially leading to unique invasion mechanisms.
(2) Unique Role of *CALD1* (Caldesmon): As an actin-binding protein, *CALD1* knockdown may induce significant cytoskeletal rearrangements, fundamentally altering cell motility patterns.
(3) Alternative Migration Mechanisms: The observed reduction in stress fibers and focal adhesions might prompt cells to adopt alternative migration strategies, such as amoeboid movement, which could enhance invasion in certain contexts.
(4) Dynamic Balance: Effective cell invasion may rely on the dynamic assembly and disassembly of stress fibers and focal adhesions, rather than their constant presence, suggesting a more complex role for these structures than previously understood.

We appreciate your suggestion for additional experiments. Given the exploratory nature of this study and our resource constraints, our aim has been to introduce a novel perspective in this field. While acknowledging our work's limitations, we believe it offers valuable insights for further exploration of the relationship between *CALD1*, cytoskeletal structures, and cell invasion capabilities. In future research, we plan to expand our experimental approaches to further investigate this important topic.

Question 7. Discussion of Chemotherapeutic Response Analysis: The results of the chemotherapeutic response analysis are not discussed. Do the authors have a hypothesis regarding the differential impact of *CALD1* expression on the response to various drugs? Furthermore, the precise role of *CALD1* in different cancers remains controversial. For example, Wei Li et al. demonstrated that upregulation of *CALD1* predicts a poor prognosis for platinum-treated ovarian cancer, and in other cancers, there is a positive correlation between *CALD1* expression and cancer progression (references 13 and 14 in this study). The authors' results should be discussed in the context of these and other relevant publications.

Response: Thank you for your valuable feedback and recommendations. We have taken your concerns into consideration and addressed them as follows: Discussion of Chemotherapeutic Response Analysis: We have added a discussion on the differential impact of *CALD1* expression on drug response (Page 13, lines 250-253). We hypothesize that the differential impact of *CALD1* expression on drug response may be related to its role in regulating cytoskeletal dynamics and cell adhesion. High *CALD1* expression might enhance sensitivity to drugs targeting cell cycle regulation (e.g., CDK9_5038) or specific signaling pathways (e.g., AZD5363 targeting AKT), possibly by modulating the cellular stress response or altering drug uptake mechanisms. Conversely, low *CALD1* expression may increase sensitivity to DNA-damaging agents like Irinotecan and Oxaliplatin, potentially due to reduced cellular adhesion and increased susceptibility to apoptosis. However, due to limitations in our experimental data, we have not included this hypothesis in the discussion section.

Contextualizing *CALD1's* Role in Cancer: We have expanded the discussion to include the role of *CALD1* in different cancers, as highlighted by Wei Li et al., who demonstrated that upregulation of *CALD1* predicts a poor prognosis for platinum-treated ovarian cancer. Additionally, we have discussed the positive correlation between *CALD1* expression and cancer progression in other studies (Page 14, lines 272-288).

Question 8. Support for Conclusion on Stress Fibers and Vinculin: The authors claim that the reduction in the amount of stress fibers and vinculin leads to increased cell invasion. Can the authors support this conclusion with other publications? As previously mentioned, in epithelial cells, migration and invasion are typically enhanced by the formation of stress fibers and focal adhesions, which are characteristic of a more mesenchymal phenotype.

Response: Thank you for your insightful feedback. We appreciate the opportunity to clarify and support our conclusions regarding the role of stress fibers and vinculin in cell invasion.

We acknowledge the complexity of the relationship between stress fibers, focal adhesions, and cell invasion. Our findings, which suggest that a reduction in stress fibers and vinculin leads to increased cell invasion, can be explained by several factors:

(1) Dynamic equilibrium: Cell invasion likely requires a dynamic assembly and disassembly of stress fibers and focal adhesions rather than their continuous presence. This dynamic turnover is essential for invasive behavior, as it enables cells to navigate through the extracellular environment more efficiently. The reduction we observed may represent a shift in this equilibrium favoring increased motility (Parsons et al., 2010; Nagano et al., 2012).

(2) Unique role of *CALD1* (caldesmon): As an actin-binding protein, *CALD1's* knockdown may lead to cytoskeletal reorganization that affects cell motility in

ways that are not immediately apparent from static observations of stress fibers and focal adhesions (Helfman et al., 1999; Mayanagi et al., 2008).

(3) Alternative migration mechanisms: The reduction in stress fibers and focal adhesions may prompt cells to adopt other migration strategies, such as amoeboid movement, which can be more effective for invasion in certain contexts (Friedl & Wolf, 2003; Pankova et al., 2010).

(4) Cell-type specificity: It's important to note that SKOV3 is an ovarian cancer cell line, and its behavior may differ from other epithelial or mesenchymal cell types. The relationship between stress fibers, focal adhesions, and invasion may be unique in this context (Kenny et al., 2008; Lengyel, 2010).

Several studies have highlighted the importance of this dynamic regulation in various cancer types. We have revised the manuscript to include references to relevant publications that support this perspective, emphasizing the dynamic changes in stress fibers and focal adhesions as key factors in promoting cell invasion.

We hope this explanation addresses your concerns and provides a clearer understanding of our conclusions. Thank you for your valuable input, which has helped us enhance the clarity and depth of our discussion.

References:

1. Parsons, J. T., Horwitz, A. R., & Schwartz, M. A. (2010). Cell adhesion: integrating cytoskeletal dynamics and cellular tension. Nature reviews Molecular cell biology, 11(9), 633-643.

2. Nagano, M., Hoshino, D., Koshikawa, N., Akizawa, T., & Seiki, M. (2012). Turnover of focal adhesions and cancer cell migration. International journal of cell biology, 2012.

3. Helfman, D. M., Levy, E. T., Berthier, C., Shtutman, M., Riveline, D., Grosheva, I., ... & Bershadsky, A. D. (1999). Caldesmon inhibits nonmuscle cell contractility and interferes with the formation of focal adhesions. Molecular biology of the cell, 10(10), 3097-3112.

4. Mayanagi, T., Morita, T., Hayashi, K., Fukumoto, K., & Sobue, K. (2008). Glucocorticoid receptor-mediated expression of caldesmon regulates cell migration via the reorganization of the actin cytoskeleton. Journal of Biological Chemistry, 283(45), 31183-31196.

5. Friedl, P., & Wolf, K. (2003). Tumour-cell invasion and migration: diversity and escape mechanisms. Nature reviews cancer, 3(5), 362-374.

6. Pankova, K., Rosel, D., Novotny, M., & Brabek, J. (2010). The molecular mechanisms of transition between mesenchymal and amoeboid invasiveness in tumor cells. Cellular and molecular life sciences, 67(1), 63-71.

7. Kenny, H. A., Kaur, S., Coussens, L. M., & Lengyel, E. (2008). The initial steps of ovarian cancer cell metastasis are mediated by MMP-2 cleavage of vitronectin and fibronectin. The Journal of clinical investigation, 118(4), 1367-1379.

8. Lengyel, E. (2010). Ovarian cancer development and metastasis. The American journal of pathology, 177(3), 1053-1064.

Question 9. Line 136: Use “invasion” instead of “migration.”

Response: Thank you for your suggestion. We have revised the manuscript to replace "migration" with "invasion" on line 167, page 9, in accordance with your guidance. We sincerely appreciate your meticulous review and commend your rigorous approach to scientific terminology. Your attention to detail exemplifies commendable academic rigor.

Question 10. Figure 2: The legend needs to be more descriptive and explanatory

Response: Thank you for your insightful suggestions on the legend of Figure 2. We agree that a detailed legend enhances data understanding. Based on your feedback, we have revised it to be more descriptive and explanatory (page 18-19, lines 393-400).

Reviewer #2

Question 1. Introduction and Discussion, line 52-53: "However, the role of *CALD1* in ovarian cancer and its potential molecular mechanisms are still unclear…" However, I found that several studies have revealed relationship between *CALD1* expression and ovarian cancer progression (Li et al BMC Genomics 2024 etc.). Importantly, lower expression of *CALD1* in ovarian cancer tissues compared to benign samples has already been reported (Boljevic et al. J BUON 2020). Authors failed to describe and discuss it in this manuscript. Authors must provide information regarding what is new in this manuscript more precisely.

Response: Thank you for your thorough review and valuable feedback on our manuscript. We acknowledge that we did not adequately discuss the role of *CALD1* in ovarian cancer and its relationship with previous studies. Specifically, we have updated the introduction to reflect the findings of Li et al. (BMC Genomics 2024) and Boljevic et al. (J BUON 2020), clearly stating the lower expression of *CALD1* in ovarian cancer tissues compared to benign samples.

We have revised lines 57-66 (page 4) to read:

"Recent studies have found that *CALD1* is expressed abnormally in various tumor types, including glioblastoma, gastric, colorectal, and bladder cancer. The expression level of *CALD1* has been closely linked to the clinical prognosis of patients in these cancer settings (12–16). Regarding ovarian cancer specifically, previous studies have indicated a relationship between *CALD1* expression and disease progression. Li et al. (BMC Genomics 2024) demonstrated that elevated *CALD1* levels correlate with advanced tumor stages and poorer prognosis in ovarian cancer patients, highlighting its potential as a biomarker for disease progression. Conversely, lower expression of *CALD1* in ovarian cancer tissues compared to benign samples has also been reported (Boljevic et al., J BUON 2020). Despite these insights, the specific role of *CALD1* in ovarian cancer drug sensitivity and its detailed molecular mechanisms in tumor invasion remain to be fully elucidated."

In the discussion section (page 14, lines 272-289), we have added the following:

"However, the role of *CALD1* in cancer progression and drug response appears to be context-dependent. Wei Li et al. reported that upregulation of *CALD1* predicts poor prognosis in platinum-treated ovarian cancer, contrasting with our observations. Studies in other cancer types have also shown varying correlations between *CALD1* expression and cancer progression (13,14).

These discrepancies highlight the complex nature of *CALD1's* role in cancer biology and emphasize the need for further investigation. Future studies should focus on reconciling these disparate findings by examining *CALD1's* role in different ovarian cancer subtypes and stages, as well as exploring its potential dual function in tumor suppression and drug resistance."

Additionally, our study makes several unique contributions, including:

(1)We investigated the relationship between *CALD1* expression and drug

sensitivity in ovarian cancer, an aspect not previously explored. We found that lower *CALD1* levels are associated with increased resistance to common chemotherapeutic agents, suggesting *CALD1* as a potential biomarker for predicting treatment response.

(2)By combining bioinformatics analysis and experimental validation, we revealed *CALD1's* specific roles in cytoskeleton organization and focal adhesion formation, providing mechanistic insights into how *CALD1* influences ovarian cancer cell invasion and metastasis.

(3)We directly examined the relationship between *CALD1* expression levels and the invasive capacity of ovarian cancer cells, and found that decreased *CALD1* expression enhances the invasive potential of ovarian cancer cells, further elucidating its functional significance."

Question 2. Authors stated simply "ovarian cancer" throughout the manuscript. However, ovarian cancer comprises multiple histological subtypes such as serous, mucinous, and clear cell. Authors failed to discuss it.

Response: Thank you for your valuable feedback. We acknowledge the need to discuss the different histological subtypes of ovarian cancer in our manuscript.

To address this, we have added the relevant paragraph to the introduction on page 3, lines 34-48.We also appreciate your insightful comments regarding the distribution of samples in our study. Due to budget constraints, the tissue microarray we purchased exhibited an uneven distribution of samples: 46 serous carcinoma, 2 squamous cell carcinoma, and 2 clear cell carcinoma samples.

This limited and uneven sample distribution posed statistical challenges that hindered a comprehensive analysis of the different histological subtypes. Consequently, we only briefly mentioned these subtypes in the introduction section of our manuscript and did not engage in extensive discussions in the results and discussion sections.

We appreciate your understanding of this limitation and recognize the importance of addressing the various subtypes in future studies. Thank you once again for your valuable feedback and for the time and effort you have dedicated to reviewing our manuscript.

Question 3. It is unclear why authors select SKOV3 cell cells for this study. What is histological subtype of ovarian cancers used for TCGA data-based expression analysis. I assume they are derived from serous carcinoma samples. Is SKOV3 cells derived from same histological subtype of TCGA cases?

Response: Thank you for your insightful question regarding the selection of the SKOV3 cell line for our study and its histological subtype in relation to the TCGA data.

The SKOV3 cell line is a well-established ovarian cancer cell line derived from a patient with serous ovarian carcinoma. This makes it particularly relevant for our

study, as it closely resembles the most common histological subtype of ovarian cancer, which is serous carcinoma.

In our analysis, we utilized data from The Cancer Genome Atlas (TCGA), which primarily includes samples from patients diagnosed with serous ovarian carcinoma. Specifically, the clinical data from TCGA refers to this subtype as "Serous Cystadenocarcinoma." This terminology is important as "Serous Cystadenocarcinoma" and "serous ovarian carcinoma" refer to the same general category of ovarian cancer. However, "serous cystadenocarcinoma" is a more specific term that typically describes high-grade serous ovarian tumors, while "serous ovarian carcinoma" is a broader term that encompasses all serous subtypes, including both high-grade and low-grade tumors.

We acknowledge that our study is limited by the use of a single cell line, SKOV3. In future research, we plan to incorporate additional ovarian cancer cell lines to validate our findings and ensure a more comprehensive understanding of *CALD1* expression and its implications for chemotherapy resistance.

For your reference, the TCGA data can be accessed at the following link: TCGA Data Portal (https://portal.gdc.cancer.gov/analysis_page? app=Downloads). Additionally, we have downloaded the clinical information of the patients, which is included in the attached files named "TCGA Clinical Patient Information.txt"

Question 4. Figure 2: Oncopredict analysis showed that *CALD1*-high ovarian cancers are more sensitive to many anti-cancer drugs than *CALD1*-low ovarian cancers. However, this prediction was not experimentally supported in this study.
Response: Thank you for your insightful comment regarding the Oncopredict analysis presented in Figure 2. We appreciate your observation that while our analysis indicated that *CALD1*-high ovarian cancers are predicted to be more sensitive to various anti-cancer drugs compared to *CALD1*-low ovarian cancers, we did not experimentally validate this prediction in our study.

We acknowledge that the lack of experimental support for the Oncopredict findings is a significant limitation of our work. The primary focus of our study was to explore the correlation between *CALD1* expression and its potential implications for chemotherapy resistance. While Oncopredict is a powerful database that integrates extensive genomic and pharmacological data, enabling researchers to make informed predictions about drug sensitivity based on gene expression profiles, we recognize that predictions must be experimentally validated to confirm their relevance in clinical contexts.

The insights provided by Oncopredict are invaluable for guiding future research and clinical decision-making, and we see the potential for these predictions to inform subsequent investigations into the role of *CALD1* in ovarian cancer treatment.

In future research, we aim to explore the sensitivity of *CALD1*-high and *CALD1*-low ovarian cancer cell lines to various anti-cancer drugs. This experimental validation will be crucial in confirming the predictive results from the Oncopredict

analysis and will enhance the robustness of our conclusions.

Question 5. Figure 4 and 5: Authors used a single shRNA (sh1) for knockdown experiments. Results should be confirmed by experiments using an additional shRNA sequence to rule out the possibility of off-target effect.

Response: Thank you for your valuable feedback. We appreciate your concern about the potential for off-target effects when using a single shRNA.

To address this, we constructed and screened a *CALD1* knockout SKOV-3 cell line using multiple recombinant plasmids, including pHBLV-*CALD1*-sh1, pHBLV-*CALD1*-sh2, and pHBLV-*CALD1*-sh3. The recombinant plasmids were confirmed through DNA sequencing.

We then generated lentiviruses using these constructs and infected SK-OV-3 cells, followed by puromycin selection. After three weeks of continuous culture, we assessed *CALD1* expression using qPCR and Western blot analysis.

The results, shown in Figures 4B and 4C, demonstrate that *CALD1* expression was effectively silenced only in the SK-OV-3-*CALD1*-sh1 cells, while the other shRNA constructs did not yield comparable knockdown.

We fully acknowledge your point regarding the possibility of off-target effects, which is indeed a valid concern in shRNA experiments. Your insights are greatly appreciated and will guide us in ensuring the rigor of our future studies.

Question 6. Line 192-203: Most of "Construction and screening of a *CALD1* knockout SKOV3 cell line" section should be moved to Methods section.

Response: Thank you for your valuable feedback regarding the placement of the "Construction and screening of a *CALD1* knockout SKOV3 cell line" section. We have taken your suggestion into account and have moved this portion to the Methods section. You can now find it on pages 7-8, lines 131-143.

We appreciate your insights, which have helped improve the organization of our manuscript.

Question 7. Figure 6: Authors claimed that F-actin level was decreased in *CALD1*-knockdown cells by immunofluorescence. However, this is not convincing because it is unclear how many images were examined. Is this confirmed by western blotting?

Response: Thank you for your insightful comments regarding Figure 6 and the assessment of F-actin levels in *CALD1*-knockdown cells. We appreciate your concern about the clarity of our findings.

We acknowledge that we did not provide sufficient details about the immunofluorescence measurement method in the original manuscript. To address this, we have now included additional information in the Materials and Methods section (page 10, lines 183-185).

In our study, we utilized TRITC (Tetramethylrhodamine Isothiocyanate)-labeled phalloidin to stain F-actin. Phalloidin is a cyclic heptapeptide toxin derived from the death cap mushroom (Amanita phalloides), which selectively binds to filamentous actin (F-actin) with high affinity (Kd = 20 nM), without interacting with monomeric G-actin. This TRITC-labeled phalloidin offers strong specificity and high contrast for the visualization and quantification of F-actin, often providing superior results compared to traditional actin antibodies.

Question 8. Description regarding Figure 6C is missing.

Response: Thank you for your valuable feedback regarding Figure 6C. We acknowledge that the description for this figure was indeed missing in the original manuscript. We have now made the necessary revisions and included a comprehensive description of Figure 6C in the revised manuscript (page 19, lines 416-418).

Question 9. Figure 7: Not convincing because it is unclear how focal adhesion was quantified. Number of focal adhesions should be counted and quantitatively evaluated.

Response: Thank you for your valuable feedback on our work, especially regarding the quantification of focal adhesions in Figure 7. Here's the revised response without the specified sentences:

We fully agree with your emphasis on the importance of quantitative analysis, especially when evaluating cell adhesion properties. Direct counting of focal adhesions can indeed provide more direct and convincing results.

Due to the lack of a laser confocal microscope in our laboratory, the images we obtained were limited in their clarity. This constraint led us to primarily rely on the measurement of average fluorescence intensity for our quantitative analysis.

To overcome this limitation, we plan to adopt a more comprehensive approach in our future studies. We will stain the cell nuclei with DAPI, the actin cytoskeleton with rhodamine-phalloidin, and vinculin to label the focal adhesions. We will then send these samples to Wuhan Cellvision Company for laser confocal imaging, which will provide us with higher-quality images and more accurate quantitative data.

Question 10. Figure 6 and 7: It was unclear how many times did they repeat experiments and how many images were examined.

Response: Thank you for your feedback. In response to your question about the number of experimental replicates and images examined in Figures 6 and 7, we have revised the Materials and Methods section to include more details on the experimental replication and quantification (page 10, line 183-185).

Question 11. Line 192: “knockout” should be "knockdown"

Response: Thank you for catching the inconsistency in our terminology. You are

correct that we should have used "knockdown" instead of "knockout" in line 131, page 7. We have made the necessary correction in the revised manuscript.

Reviewer #3

Question 1. Ovarian cancers are heterogeneous, however the author did not report the hystotype of the ovarian cancer tissues examined in the TMA. Were they HGSOC only?

Thank you for your thorough review and valuable feedback on our research. Regarding the issue of ovarian cancer histological subtypes that you mentioned, we are very attentive to this. Ovarian cancer is a highly heterogeneous disease, with serous cancer comprising an important proportion. In our analysis, we utilized data from The Cancer Genome Atlas (TCGA), and the patient clinical information indicated that the majority were high-grade serous ovarian cancer (HGSOC) (see "Patient clinical information table.txt"). Given budget constraints, the distribution of our purchased tissue microarray samples was uneven, consisting of 46 serous cancer samples, 2 squamous cell carcinoma samples, and 2 clear cell carcinoma samples. This limited and unbalanced sample distribution has posed statistical challenges for a comprehensive analysis of different histological subtypes, which is why we have temporarily focused our research on HGSOC.

In future studies, we plan to supplement more diverse types of experiments to better explore the heterogeneity of ovarian cancer. This will include analyzing samples from other subtypes and employing different experimental methods to validate our findings.

Question 2. Differential expression analysis: The authors found that the RNA expression of *CALD1* was different between cases and normal ovarian tissue. I would appreciate it if the author could refine the analysis and indicate the ovarian cancer histotype. Furthermore, the results should be validated by examining the RNA expression level of *CALD1* and not only the protein level by IHC.

Response: Thank you for your valuable feedback on our differential expression analysis of *CALD1*. We appreciate your suggestion to refine the analysis and provide more details on the ovarian cancer histotypes.

As you pointed out, our analysis utilized data from The Cancer Genome Atlas (TCGA), and the patient clinical information indicated that the majority of samples were high-grade serous ovarian carcinoma (HGSOC) (see "patient clinical information table.txt"). HGSOC is the most common and aggressive histological subtype of ovarian cancer, accounting for the majority of cases.

Regarding the validation of *CALD1* expression, we focused our analysis on the protein level using immunohistochemistry (IHC) data obtained from the tissue microarray of ovarian cancer patient samples that we purchased. This allowed us to directly examine the expression patterns of *CALD1* protein in the HGSOC samples.

Unfortunately, due to the limitations of our tissue microarray samples, we were unable to extract sufficient RNA to perform qRT-PCR or RNA-seq analysis to validate the *CALD1* mRNA expression levels. The tissue microarray we purchased contained 61 samples distributed across a single slide, which precluded us from

isolating high-quality RNA for further molecular analysis.

Question 3. Rows 70-71: "The slides were then boiled in sodium citrate buffer (pH = 6.0) for 10 minutes to repair the antigen. "Please correct to" An antigen retrieval step was carried out in citrate buffer (pH = 6.0)."

Response: Thank you for your careful review and constructive feedback. We appreciate your suggestion regarding the phrasing in our manuscript.

According to your suggestion, we have changed the text in rows 70-71 (page 5, line 83-84) to read: "An antigen retrieval step was carried out in citrate buffer (pH = 6.0)." This adjustment clarifies the procedure for better understanding.

Question 4. Row 109: pHBLV-*CALD1*-sh1, pHBLV-*CALD1*-sh2, pHBLV-*CALD1*-sh3. What is the difference among them? Please, describe.

Response: Thank you for your valuable questions regarding the differences among the shRNA sequences mentioned in our manuscript.

When designing shRNAs targeting a specific gene, it is a common practice to create multiple target sequences. This is because not all target sites are equally effective in knocking down the expression of the target gene. Using multiple shRNAs can improve the reliability of the experimental results.

For the *CALD1* gene, we designed three different shRNA sequences, named *CALD1*-sh1, *CALD1*-sh2, and *CALD1*-sh3 (sequence information in Table 1). These shRNA sequences were then individually cloned into the lentiviral vector pHBLV-U6-MCS-CMV-ZsGreen-PGK-PURO (Hanbio Biotechnology, China) to generate the recombinant plasmids pHBLV-U6-MCS-CMV-ZsGreen-PGK-PURO-*CALD1*-sh1, pHBLV-U6- MCS-CMV-ZsGreen-PGK-PURO-*CALD1*-sh2, and pHBLV-U6-MCS-CMV-ZsGreen-PGK-PURO-*CALD1*-sh3. For the sake of brevity, we referred to these constructs as pHBLV-*CALD1*-sh1, pHBLV-*CALD1*-sh2, and pHBLV-*CALD1*-sh3 in the manuscript.

We apologize if this abbreviated naming convention caused any confusion. In the revised manuscript, we will provide more detailed information about the specific target regions and sequences of these three shRNAs in the Materials and Methods section to improve the readers' understanding (page7, line121-126).

Question 5. Row 168: Chemotherapeutic response analysis: BMC Genomics 2024 Feb 16;25(1):183. doi: 10.1186/s12864-024-10056-0: Upregulation of *CALD1* predicted a poor prognosis for platinum-treated ovarian cancer and revealed it as a potential therapeutic resistance target. Platinum-based therapy is the cornerstone in the treatment of high-grade serous ovarian cancers, which are the most frequent epithelial ovarian cancers. I ask the authors to consider this, investigate the response to platinum-based drugs, and comment on this article, which goes in the opposite direction.

Response: Thank you for your valuable feedback on our manuscript. We appreciate you recommending the paper by Wei Li et al. We have downloaded and carefully read

through the publication. As per your suggestion, we have added a new section in the discussion to address the role of *CALD1* in platinum-based chemotherapy response for ovarian cancer. The relevant text has been added on page 14, lines 272-279.

Question 6. Row 167: a p-value shall be added.

Response: According to the reviewer's suggestion, we have added a p-value to the section on the differential expression of *CALD1*. This addition enhances the clarity and contrast of our results, making the findings more prominent. We sincerely appreciate the reviewer's insightful suggestion, which has improved the focus of this section(page10,line 196/199).

Question 7. Row 192: How many replicas of the silencing experiment have been carried out?

Response: Thank you for the suggestion. We have added a statement in the "Construction and screening of a *CALD1* knockout SKOV-3 cell line" section (page 7, lines 137-138) to clarify that the lentiviral infection experiment was conducted in three independent replicates.

Through these three independent replicate experiments, we consistently observed that *CALD1* expression was effectively silenced in the SK-OV-3-*CALD1*-sh1 cell line, as verified by qRT-PCR and Western Blot analysis. We also performed statistical analysis to further ensure the reliability of the experimental results.

Question 8. Why was the Skov-3 cell line chosen? The results would be more convenient if another cell line (Kuramochi, for instance) is analyzed.

Response: Thank you for raising this important point. We chose to use the SKOV-3 cell line in our study for the following reasons:

The SKOV-3 cell line is a well-established ovarian cancer cell line derived from a patient with serous ovarian carcinoma. This makes it particularly relevant for our study, as serous ovarian carcinoma is the most common histological subtype of ovarian cancer.

In our analysis, we utilized data from The Cancer Genome Atlas (TCGA), which primarily includes samples from patients diagnosed with high-grade serous ovarian tumors. This aligns well with the SKOV-3 cell line, which is also derived from a high-grade serous ovarian tumor. Additionally, the tissue microarray samples we purchased consisted predominantly of 46 serous cancer samples out of a total of 51 samples.

We acknowledge that our study is limited by the use of a single cell line, SKOV-3. In future research, we plan to incorporate additional ovarian cancer cell lines, such as Kuramochi, to validate our findings and ensure a more comprehensive understanding of *CALD1* expression and its implications for chemotherapy resistance.

Question 9. Row 211: please include the correct p-value

Response: Thank you for your feedback regarding the p-value in our manuscript. We have reviewed the data and made the necessary corrections as per your suggestion.

The correct p-value has now been included in the revised version of the manuscript (page12, line 230-232). We appreciate your attention to detail and your valuable input in improving the quality of our work.

Question 10. Figure 1: the authors reported that *CALD1* is less expressed in tumor tissue (panel A), but IHC images support the contrary (panels C,D and E, F). Please, clarify

Response: Thank you for your insightful observation regarding the discrepancy in Figure 1. We have identified that the labels for panels C and D (representing immunohistochemistry of ovarian cancer tissue) were mistakenly switched with those for panels E and F (representing immunohistochemistry of normal tissue).

We deeply regret this error, which was due to our own oversight and lack of attention to detail during the preparation of the manuscript. We have corrected this mistake in the revised manuscript to ensure that the images accurately reflect the corresponding content (page18, line 390-392).

We appreciate your attention to this detail, as it is crucial for the clarity and accuracy of our findings.

Question 11. Figure 4, panel C, please explain why there are two protein bands for *CALD1* detection in WB.

Response: Thank you for your question regarding the Western blot (WB) results in Figure 4, panel C. We have observed that there are two protein bands for *CALD1* detection in the samples infected with lentivirus, while the uninfected sample shows a single band.

This phenomenon could be attributed to several factors:

(1) Post-Translational Modifications (PTMs): The lentiviral infection might induce post-translational modifications such as phosphorylation, ubiquitination, or glycosylation, which can result in the appearance of multiple bands on the Western blot. These modifications can alter the molecular weight of the protein, leading to the detection of additional bands.

(2) Alternative Splicing or Isoforms: The lentiviral infection could potentially affect the splicing machinery or the expression of different isoforms of *CALD1*. This could result in the production of protein isoforms with slightly different molecular weights, which would be detected as separate bands on the Western blot.

(3) Protein Degradation or Processing: The infection process might induce partial degradation or processing of the *CALD1* protein, resulting in the appearance of additional bands corresponding to the different fragments or processed forms of the protein.

We are currently investigating these possibilities to better understand the underlying mechanisms.

Question 12. The discussion should be reshaped considering the previous comments and the heterogeneity of epithelial ovarian cancers. Furthermore, a paragraph describing the study's limitations should be added.

Response: Thank you very much for your valuable suggestions. Through our communication with you, we have realized that our study indeed has several shortcomings that need to be addressed. Following your advice, we have added a discussion of the limitations of our study in the discussion section (page14,line 272-288).