nature portfolio

Peer Review File

VSTM2L protects prostate cancer cells against ferroptosis via inhibiting VDAC1 oligomerization and maintaining mitochondria homeostasis in men

Corresponding Author: Professor Ping Gao

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The current manuscript evaluates the mechanisms of mitochondrial VDAC1 oligomerization in regulating ferroptosis during prostate cancer.
The authors have employed immunoprecipitation coupled to mass spectrometry (IP/MS), identifying VSTM2L as a VDAC1 binding partner in LNCaP androgen sensitive, metastatic prostate cancer cells, supporting previous work in non-prostate cells. The authors use an shRNA mediated knockdown approach to reveal that VSTM2L loss, sensitizes human androgen insensitive metastatic prostate cancer cells to RSL3-induced ferroptosis in, both in vitro and in vivo xenograft assays.
Major observations for this study include that VSTM2L is a VDAC1 binding protein in human prostate cancer cells that can suppress ferroptosis by forming a complex with VDAC1 and HK2, preventing VDAC1 oligomerisation. These observations are important to the field, providing further insight into the mechanisms regulating ferroptosis in prostate cancer, and rationale for targeting VSTM2L in prostate cancer to promote ferroptosis and enhance the efficacy of current treatments.

Specific points:

1. The authors should provide context to the other top candidates identified in Figure 1A (PHB2, Cystatin-A and Albumin) and further justify the rationale for only exploring VSTM2L. It would be helpful if the authors extended the table in Figure 1A to show the top 10 candidates identified. The authors should also comment on the 10th candidate identified RPS3, known to mediate apoptosis and DNA repair.

2. The authors should clarify in the manuscript that the VDAC1:VSTM2L interaction is observed in both androgen sensitive (LNCaP) and androgen insensitive (PC3/DU145) mCRPC cells lines (Figure 1D/E).

3. Please ensure all co-IP blots include total protein/input controls.

4. Figure 1F – please expand on key proteins identified in the Venn diagram that bind both VDAC1 and VSTM2L, was HK2 present in datasets for VSTM2L and VDAC1? What proportion of the proteins identified have been shown to bind VDAC1 and/or VSTM2L previously?

5. What statistical test and n-values are included in Figure 2B?

6. Figure 2D-E – what patient population is represented? Please consider providing a table to provide an overview of key clinicopathological features of the 78 matched (tumour and adjacent normal) samples analysed and more information regarding the samples (including patient age, gleason score, Stage, PSA level etc). The sample type (primary/metastatic? Radical prostatectomy?), patient consent and ethical approval information also needs to be included in the methodology.

7. In addition to mRNA expression (Fig. 2F/G), does VSTM2L copy number variation (e.g., Gain or Amp) also correlate with disease outcome in primary or metastatic prostate cancer patient datasets? Does VSTM2L over-expression predict for poor

outcome in metastatic prostate cancer as well as primary prostate cancer?
8. Figure S2G – please include a Western blot to confirm the VSTM2L KD constructs reduced protein levels as well as mRNA expression in PC3 cells. It is not clear if the presence of the scramble shRNA control vector influences VSTM2L protein expression. The authors should add a parental cell line control to the Western blots shown to determine if the control vector impacts VSTM2L expression.

9. Figure 2K, please normalize the colony formation data and plot the % colony forming efficiency to account for any differences in the seeding densities between groups. How many biological repeats were included in each experiment? The impact on clonogenicity should be discussed in the text, and whether VSTM2L is known to impact stem cell function?

10. Figure 2L – the 22Rv1 wound healing assays appear to show no change in sheet migration in the context of VSTM2L shRNA mediated knockdown, yet the quantitation of this experiment in Fig 2N shows a significant difference. Why?

11. If possible, the RSL3 treatment studies shown in Figure 4 should include analysis of additional ferroptosis markers other than GPX4/GSH (e.g., coenzyme Q10 or BH4), as well as other apoptosis markers (e.g., p53 mediated apoptosis by Cleaved-caspase 3) to further confirm RSL3 triggers ferroptosis in this setting and not other forms of apoptosis.

12. The authors should further clarify how they scored the IHC staining shown in Fig 4F-I. Were areas of necrosis/stroma avoided? Was a similar number of cells scored per tumour/cohort?

13. To clarify the morphological changes observed, the authors should describe the morphological changes observed in Fig. 5C.

14. The authors should comment on whether a conformational change/folded structure of VDAC1 is likely for the HK2 and VSTM2L interaction to occur given their differences in binding the N- and C-terminal of VDAC1. Does the VDAC1, HK2 and VSTM2L complex occur in normal prostate epithelial cells, or is it unique to prostate cancer cells?

15. The authors should acknowledge alternative mechanisms whereby VBIT4 could reverse the impact of VSTM2L depletion in addition to VDAC1 oligomerisation in vivo, given that VBIT4 can also influence type I interferon signalling and neutrophils.

16. This reviewer is not convinced that VBIT4 is colony forming assays, scratch assays and transwell assay representative images are quantitated accurately in Figure 8, as the quantitation does not appear to correlate with the images. For instance, the colony forming assays shown in figure 8C do not appear to be representative of the colony quantitation shown in Fig 8D for the DMSO control. There appears to be a higher seeding density in the DMSO shRNA control setting. The authors should confirm if the assay was performed at a similar seeding density for each treatment arm and plot the % colony forming efficiency, by normalizing the data to the number of cells plated. The VBIT4 control plate also appears to have less colony formation relative to the DMSO control in Fig 8C, yet this is not observed in the quantitation in Fig 8D. Do the VBIT4 treated arms correlate with increased stem cell activity in vivo?

17. Were there any histopathological differences between the different experimental arms shown in Fig 8M?

18. The authors claim “the IHC and H&E staining results further indicated that the administration of VBIT4 to experimental animals notably reversed the ferroptotic cells death within the tumor tissues caused by VSTM2L suppression (Fig. 8M-N).” however cell death has not been assessed. Does VBIT4 treatment effect the number of cells undergoing apoptosis/ferroptosis in addition to altering proliferation?

Minor points:

- Intro line 2 – please amend the wording: prostate cancer is one of the most “common” (not “popular”) cancer types
- Please add citation for “VSTM2L is the newly identified VDAC1-interacting protein” in the introduction and comment on where VSTM2L is reported to bind to VDAC1.
- Page 6 – last paragraph refers to VSTM2L inhibited 22Rv1 or Du145 cells – this is scientifically incorrect terminology, please amend “inhibited” to “depleted”.
- Page 8, line 17-18 – the authors should indicate the 22Rv1 cell line was used to perform the xenograft study.
- Page 12 -line 2-4 – please amend wording to improve clarity. “is involved in” should be amended to “may involve the”.
- Figure 1H Venn diagram is referred to as proteins in the text and genes in the figure legend – please correct. Same issue with Figure 1I
- Methodology: Please indicate if cell lines were cell authenticated. How was the RNA for qRT-PCR quality checked? What parameters were used to generate the Kaplan-Meier plots in c-BioPortal (i.e. what is high and low referring to, was a z-score threshold used?), and what software was used to plot the data from cBioPortal?

Reviewer #2

(Remarks to the Author)
In this manuscript, Yang et al identified VSTM2L as a previously unrecognized interaction partner of VDAC1. The authors provide evidence that VSTM2L expression correlates with the aggressiveness of prostate cancer, including various in vitro assays and clinical prognosis. Mechanistically, the authors show that VSTM2L can facilitate interaction between VDAC1

and HK2, thereby inhibiting oligomerization of VDAC1 that is required for mitochondrial ROS generation and ferroptosis induction.

The study is overall well designed and executed, and will be of interest to the readership of Nature Communications. This reviewer only has a few minor comments that may improve the work.

(1) It is a bit confusing that cell survival data shown in Figure 3I (22Rv1 cells displayed drastic cell death upon VSTM2L KD) and in Figure 5B (DMSO group, minimal cell death detected upon VSTM2L KD also in the same cell line). The authors should explain why.

(2) VDAC1 has long been connected with apoptosis regulation. The authors should examine apoptotic activity in cells received short hairpins against VSTM2L.

(3) Related to (2), the authors should examine if the cell death phenotype observed in Figure 3I can be reversed by Fer-1 or Lip-1. (The following figures show Fer-1 rescue in the context of RSL3 treatment, but this is different.

(4) In Figure 1B-1E, the authors should include IgG controls in the immunoprecipitation assays.

(5) Page 12, Line 8-10; Figure 6J, K. It will be highly informative and necessary to provide data on the tripartite complex comprising VSTM2L, VDAC1, and HK2 in a semi-endogenous assay using PCa cells besides the exogenous data in 293T cells in Figure 6J. Or, is VSTM2L co-immunoprecipitated following anti-HK2 immunoprecipitation in the shCtrl DU145 cell lysates showed in Figure 6K?

(6) Page 13, Line 6-8; Figure 7A. For the left representative image, the mitochondrial ROS ratios for shVSTM2L-1 and shVSTM2L-2 seem mislabeled and inconsistent between the actual areas (~50%) and indicated numbers (13.3%, 17.3%).

(7) Page 14, Line 3-4; Figure 7I. The description “The results illustrated an elevation in MMP levels following the suppression of VSTM2L” is not correct and illogical for the data showed in Figure 7I if using JC-1 aggregates/JC-1 monomers ratio to indicate MMP levels.

Reviewer #3

(Remarks to the Author)
In this paper by Yang et al. , the authors found that VSTM2L, a novel VDAC1 binding protein, is positively associated with prostate cancer progression and a key regulator of ferroptosis. VSTM2L knockdown in PCa cells enhanced the sensibility of RSL3-induced ferroptosis. VSTM2L forms a complex with VDAC1 and HK2, preventing VDAC1 oligomerization, thereby inhibiting ferroptosis and maintaining mitochondrial homeostasis.

Here are some concerns regarding this paper.
1. Recently, Lee et al. (2024) published that VSTM2L protects against cancer cell death in cholangiocarcinoma, reducing the novelty of this paper which reports that VSTM2L protects against prostate ca cell death. Therefore, one questions the novelty of this paper.
2. The most novel part of this paper is the claim that VSTM2L interacts with and stabilizes the VDAC1-HK2 interaction, which prevents VDAC1 oligomerization on mitochondrial outer membrane. This is cited as the mechanism by which VSTM2L prevents cancer cell death. However, previous reports indicate that VSTM2L is not localized to mitochondria but is rather a secreted protein. Moreover, according to Mitocarta, which exhaustively identified mitochondria-localized proteins, VSTM2L is not localized to mitochondria. Therefore, it is possible that the effect of VSTM2L on the VDAC1-HK2 interaction is not direct but is indirect, which makes this paper less interesting. The authors need to prove that endogenous VSTM2L is a mitochondrial protein.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I would like to congratulate the authors for addressing all of the reviewer comments in full, and believe that their article would be a valuable addition for the readership of Nature Communications.

Reviewer #2

(Remarks to the Author)
The authors have done a good job in addressing the concerns raised by this reviewer. The manuscript is now ready for the next stage of the publication process.

Reviewer #3

(Remarks to the Author)
I am satisfied with the revision.

NCOMMS-24-34401

VSTM2L protects prostate cancer cells against ferroptosis via inhibiting VDAC1 oligomerization and maintaining mitochondria homeostasis

We thank the Editor and Referees for their valuable time and suggestions to further improve our manuscript. We have now strengthened the data analyses and added extensive experiments in response to the points raised by the referees. In the following pages is our point-by-point response (in blue) to each of specific comments raised by the Reviewers. The changes in the main manuscript and supplementary materials are colored in red.

Reviewer #1 (Remarks to the Author):
Specific points:
1. The authors should provide context to the other top candidates identified in Figure 1A (PHB2, Cystatin-A and Albumin) and further justify the rationale for only exploring VSTM2L. It would be helpful if the authors extended the table in Figure 1A to show the top 10 candidates identified. The authors should also comment on the 10th candidate identified RPS3, known to mediate apoptosis and DNA repair.

Response:

We thank and agree with the reviewer. We have presented the top 10 candidates in ***Revised Figure 1A*** and provide context of the other top candidates as the reviewer kindly suggested (**Page 4, Line 12-13**). Among the top 10 candidates, VSTM2L as top one scored protein, was reported related to few types of cancer, but the biological functions of VSTM2L are not clear. Moreover, the functional role of VSTM2L in prostate cancer cells has never been reported. So, we focus on the investigation for the function of VSTM2L in prostate cancer cells (**Page 4, Line 22 to Page 5, Line 1-4**).
In addition, we also added our comments of RPS3 and other top candidates at **Page 4, Line 12-21** in our manuscript.

2. The authors should clarify in the manuscript that the VDAC1:VSTM2L interaction is observed in both androgen sensitive (LNCaP) and androgen insensitive (PC3/DU145) mCRPC cells lines (Figure 1D/E).

Response:

We thank the reviewer for this suggestion. The observation for the VDAC1:VSTM2L interaction was clarified in the manuscript in **Page 5, Line 9-11**.

3. Please ensure all co-IP blots include total protein/input controls.

Response:

We thank the reviewer for pointing out this issue. We have checked all co-IP blots and uniformly changed all the total protein controls to “Input”. (***Revised Figure 1 B-E and 6 D, G, H, J-M***)

4. Figure 1F – please expand on key proteins identified in the Venn diagram that bind both VDAC1 and VSTM2L, was HK2 present in datasets for VSTM2L and VDAC1? What proportion of the proteins identified have been shown to bind VDAC1 and/or VSTM2L previously?

Response:

We thank the reviewer for pointing out this issue. We have expanded the key proteins identified in the Venn diagram (***Supplementary File 2***). Moreover, HK2 was present in datasets for VDAC1 and VSTM2L (***Figure 1 G, Figure 6 I, Supplementary File 1 and Supplementary File 2***). In addition, we presented the proportion of overlapped VDAC1 or VSTM2L interacted proteins between the published data from NCBI and our IP/MS data in the venn diagram. The data showed that our VDAC1 or VSTM2L IP/MS data identified 24.4% or 16.67% of the published proteins from NCBI (Figure 1).

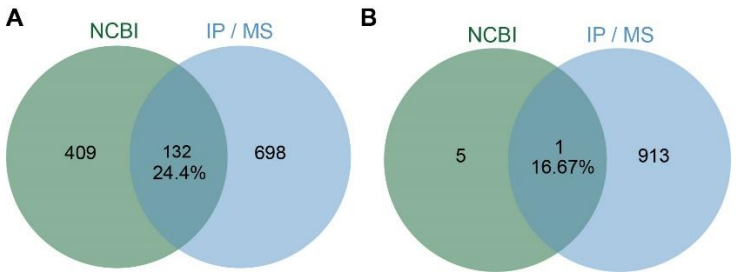

Figure 1 Venn diagram of NCBI reported proteins and IP / MS identified proteins. A Venn diagram showing the 132 (24.4%) proteins obtained by intersecting the IP-MS data of VDAC1 and published data. **B** Venn diagram showing the 1 (16.67%) protein obtained by intersecting the IP-MS data of VSTM2L and published data.

5. What statistical test and n-values are included in Figure 2B?

Response:

We thank the reviewer for pointing out this issue. *P* value was determined by unpaired two-tailed Student's *t*-test and n-values are 492 for tumors (T), 152 for normal (N). We have clarified the items in ***Figure 2 B*** and ***Figure legend for Figure 2*** at **Page 44 Line 17** in our manuscript.

6. Figure 2D-E – what patient population is represented? Please consider providing a table to provide an overview of key clinicopathological features of the 78 matched (tumour and adjacent normal) samples analysed and more information regarding the samples (including patient age, gleason score, Stage, PSA level etc). The sample type (primary/metastatic? Radical prostatectomy?), patient consent and ethical approval information also needs to be included in the methodology.

Response:

We thank the reviewer for pointing out this missing information that need to be provided. We had provided a table of key clinicopathological features (including patient age, gleason score, Stage, PSA level etc) of the 78 paired samples

(***Supplementary file 3***) (**Page 6, Line 21-22**). The sample type was also provided in ***Supplementary file 3***. In addition, the patient consent and ethical approval information had been added in the manuscript as the Reviewer kindly suggested in **Page 34, Line 13-16.**

7. In addition to mRNA expression (Fig. 2F/G), does VSTM2L copy number variation (e.g., Gain or Amp) also correlate with disease outcome in primary or metastatic prostate cancer patient datasets? Does VSTM2L over-expression predict for poor outcome in metastatic prostate cancer as well as primary prostate cancer?

Response:

We thank the reviewer for bringing up this question. We have drawn the relationship between VSTM2L copy number variation (Gain *VS* Other (neutral and Loss)) with disease outcome in primary prostate cancer, suggesting that there is no significant correlation between the copy number variation and poor disease outcome (Figure 2 A, B).

The source data for drawing ***Figure 2 F/G*** is from the TCGA Pan Cancer Atlas Cell 2018 dataset of prostate cancer in Database cBioportal. We cannot predict the VSTM2L copy number variation or overexpression for poor outcome in metastatic prostate cancer using this dataset since it only contains the information of primary prostate cancer tissue samples. To solve this question, we further analyzed the data from SU2CPCF Dream Team PNAS 2019 or MCTP Nature 2012 datasets of metastatic prostate cancer in Database cBioportal. The results showed that there is no correlation between VSTM2L copy number variation with disease outcome in metastatic prostate cancer (Figure 2 C). This might due to the lower gain samples.

Then we analyzed the relationship between VSTM2L over-expression with the poor outcome of metastatic prostate cancer in MCTP Nature 2012 and SU2CPCF Dream Team PNAS 2019 dataset. There is a positive correlation between VSTM2L over-expression with the poor survival rate of metastatic prostate cancer in MCTP Nature 2012 dataset (Figure 2 D). However, there is no positive correlation between VSTM2L over-expression and poor outcome of metastatic prostate cancer in SU2CPCF Dream Team PNAS 2019 dataset (Figure 2 E). This inconsistent result might be caused by the less sample numbers.

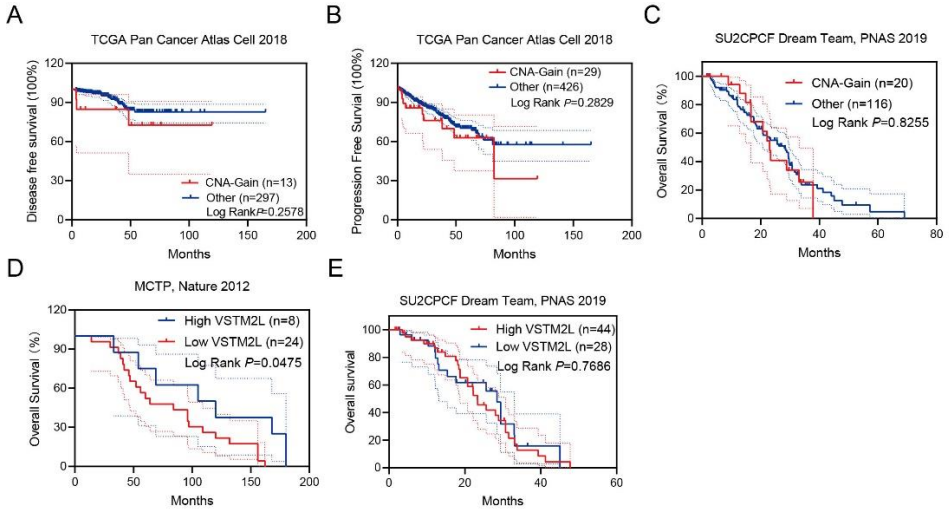

Figure 2 Association between VSTM2L copy number variation / expression and disease outcome. A Association between Disease-free survival of prostate cancer patients and VSTM2L copy number variation from the TCGA database. **B** Association between Progression-free-survival and VSTM2L copy number variation from the TCGA database. **C** Association between Overall survival of prostate cancer patients and VSTM2L copy number variation from SU2CPCF Dream Team database. **D, E** Association between Overall survival of prostate cancer patients and VSTM2L expression level from MCTP (**D**) and SU2CPCF Dream Team (**E**) databases. *P* value was determined by log-rank test.

8. Figure S2G – please include a Western blot to confirm the VSTM2L KD constructs reduced protein levels as well as mRNA expression in PC3 cells. It is not clear if the presence of the scramble shRNA control vector influences VSTM2L protein expression. The authors should add a parental cell line control to the Western blots shown to determine if the control vector impacts VSTM2L expression.

Response:

We thank the reviewer for pointing out this issue. We have added the parental cell lines and tested VSTM2L knockdown efficiency at protein level using western blot in 22Rv1, Du145 and PC3 cells, respectively. The results showed that the scramble shRNA control group unaltered VSTM2L protein level compared with the parental group, and shVSTM2L group markedly reduced VSTM2L protein level compared with the scramble shRNA control group, indicating that the scramble shRNA control vector did not impact VSTM2L protein levels (***Revised Figure S2G***).

9. Figure 2K, please normalize the colony formation data and plot the % colony forming efficiency to account for any differences in the seeding densities between groups. How many biological repeats were included in each experiment? The impact on clonogenicity should be discussed in the text, and whether VSTM2L is known to impact stem cell function?

Response:

We thank the reviewer for pointing out this issue. We have normalized the colony formation data and plot the % colony forming efficiency between different groups, as the reviewer's kindly suggested (***Revised figure 2K and figure 8D***). In the present study, 3 biological repeats were included in each experiment. Moreover, we have discussed the impact of VSTM2L deletion in PCa cells on clonogenicity in the manuscript **Page 7, Line 21-22.**

To date, the roles of VSTM2L in stem cell function have never been reported in the literature. We further detected the expression levels of the genes related with stem cells as described previously[1], the results showed that VSTM2L depletion did not affect the mRNA levels of enhancer of zeste homolog 2 (*EZH2*) and ATP-binding cassette sub-family G member 2 (*ABCG2*) (Figure 3).

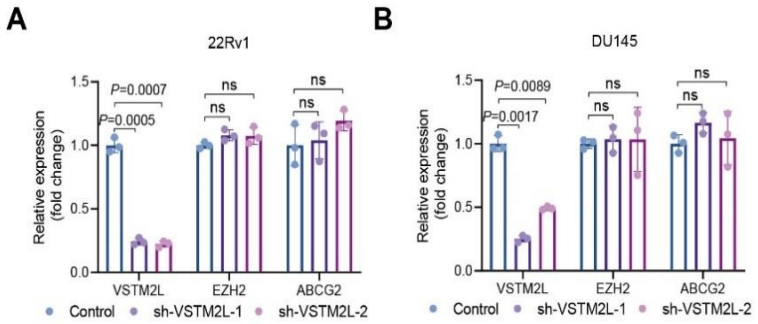

Figure 3 The effect of VSTM2L depletion on the expression of genes related with stem cell. A Relative expression of *EZH2* and *ABCG2* of 22Rv1 cells with VSTM2L depletion. **B** Relative expression of *EZH2* and *ABCG2* of DU145 cells with VSTM2L depletion. Data shown as mean ± SD, n=3. *P* value was determined by two-way ANOVA.

10. Figure 2L – the 22Rv1 wound healing assays appear to show no change in sheet migration in the context of VSTM2L shRNA mediated knockdown, yet the quantitation of this experiment in Fig 2N shows a significant difference. Why?

Response:

We thank the reviewer for pointing out this mistake. We have misused the images at the time point of 24 h. To confirm these results, we repeated this experiment again, and extended the time point to 72 h and corrected this result (***Revised figure 2L, N***).

11. If possible, the RSL3 treatment studies shown in Figure 4 should include analysis of additional ferroptosis markers other than GPX4/GSH (e.g., coenzyme Q10 or BH4), as well as other apoptosis markers (e.g., p53 mediated apoptosis by Cleaved-caspase 3) to further confirm RSL3 triggers ferroptosis in this setting and not other forms of apoptosis.

Response:

We thank the Reviewer for this insightful suggestion. We have evaluated the ferroptosis marker BH4 in the xenograft tumors, the result indicated that VSTM2L knockdown decreased the level of BH4, RSL3 administration further reduced the level of BH4 in VSTM2L depletion tumors (***Revised figure 4F***) (**Page 11, Line 3-6**).

To further confirm RSL3 triggers ferroptosis in this setting and not other forms of apoptosis, we detected the apoptosis marker of Cleaved-caspase 3 in the tumors, the results showed that there is no obvious difference between these four groups. Thereby, the results have confirmed that RSL3 triggers ferroptosis in this setting not other forms of apoptosis (***Revised figure 4G-K***) (**Page 11, Line 17-21**). Moreover, DU145 cells transfected with VSTM2L shRNA (shVSTM2L-1, shVSTM2L-2) or control shRNA (Control) were treated with or without RSL3, Fer-1, 3MA, CQ, Z-VAD-FMK, Z-IETD-FMK and Z-DEVD-FMK for 24h, and CCK-8 assay was performed to detect cell viability. The results showed that RSL3-induced cell death in DU145 shVSTM2L cells was abolished by the ferroptosis inhibitor ferrostatin-1(Fer-1), rather than a panel of apoptosis inhibitors (Z-VAD-FMK, Z-IETD-FMK and Z-DEVD-FMK), as well as autophagy inhibitors 3-Methyladenine (3MA) and Chloroquine (CQ) (***Figure 5A***)

(**Page 12, Line 4-9**).

12. The authors should further clarify how they scored the IHC staining shown in Fig 4F-I. Were areas of necrosis/stroma avoided? Was a similar number of cells scored per tumour/cohort?

Response:

We thank the reviewer for pointing out this issue. We had clarified the method of calculating the staining index of VSTM2L/Ki67/GPX4/Cleaved-Caspase3 in the manuscript as the Reviewer kindly suggested in **Page 35, Line 7-11**.

13. To clarify the morphological changes observed, the authors should describe the morphological changes observed in Fig. 5C.

Response:

We thank the Reviewer for this valuable suggestion. We had described the morphological changes observed in ***Figure 5C*** in the manuscript in **Page 12, Line 11-15**.

14. The authors should comment on whether a conformational change/folded structure of VDAC1 is likely for the HK2 and VSTM2L interaction to occur given their differences in binding the N- and C-terminal of VDAC1. Does the VDAC1, HK2 and VSTM2L complex occur in normal prostate epithelial cells, or is it unique to prostate cancer cells?

Response:

The three-dimensional structure of VDAC1 shows a channel composed of 19 β-strands with an N-terminal α-helix located near the midpoint of the pore, and the majority of models involve significant conformational shifts of this N-terminal region[2]. The mobility and disorder of the N-terminal domain were confirmed in a crowd of biophysical studies, suggesting that it could exist in a dynamic equilibrium between random coil and α-helix[3, 4]. HK2 binds to the N-terminal of VDAC1, thereby restricting it from freely diffusing out of the pore[5]. In our study, we found that mitochondria-localized VSTM2L plays vital role in maintaining mitochondria homeostasis by stabilize mitochondrial ROS and maintain mitochondrial membrane potential (MMP) in prostate cancer cells. MMP is of crucial significance to the conformation of VDAC1[6, 7]. After the MMP rises, the pore of VDAC1 closes and its conformation changes. Our finding indicated that reduced VSTM2L expression in prostate cancer cells led to the decreased MMP, and the pore of VDAC1 became larger. Further, based on the three-dimensional conformation of VDAC1 (Figure 4A)[4], we can discover that the β-helix structure of VDAC1 did not make its C-terminal and N-terminal keep in exactly opposite directions. Considering that HK2 binds to the N-terminal of VDAC1(Figure 4B)[8], VSTM2L binds to the C-terminal of VDAC1 and VSTM2L depletion led to the dissociation of HK2 from VDAC1(***Revised Figure 6M, N***), we speculate that the narrowed pore of VDAC1 can shorten the physical distance between VSTM2L and HK2 on the mitochondria and maintain the stability of the HK2/VSTM2L/VDAC1 complex. Thus, we speculated that VSTM2L mediates the

interaction between VDAC1 and HK2, making VDAC1 keeps a stable three-dimensional structure with the help of VSTM2L and HK2 to maintain mitochondrial homeostasis, protects prostate cancer cells against ferroptosis, thereby promoting the development of prostate cancer. However, the detailed conformation of HK2/VSTM2L/VDAC1 complex on mitochondria needs to be further investigated in PCa. We have commented on this in the part of discussion of the manuscript from **the last paragraph of Page 21 to Page 23**.

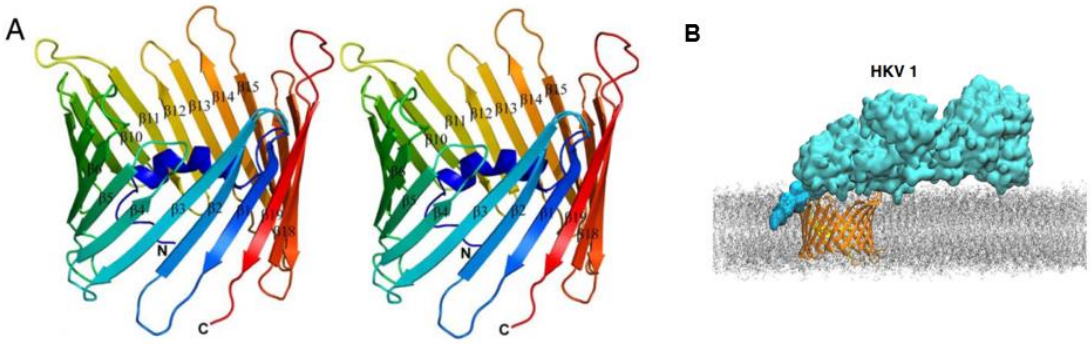

Figure 4 3D structure of VDAC1 and membrane-embedded HKII/VDAC1 complex. A Stereo representation of the VDAC1 structure in a ribbon presentation color coded from the N to the C terminus (N and C). Some β-strands are marked with their respective numbers[4]. **B** A snapshot (at t = 350 ns) of membrane-embedded HKII/VDAC1 complex 1 (HKV1) during the full-membrane MD simulation[8].

In addition, we have performed the semi-endogenous and endogenous IP assays, the results showed that the complex of VDAC1/VSTM2L/HK2 existed in normal prostate epithelial RWPE-1 cells (Figure 5).

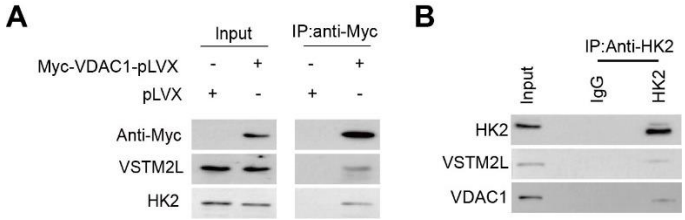

Figure 5 VSTM2L, VDAC1 and HK2 forms complex in normal prostate epithelial cell line. A Semi-endogenous IP assay. RWPE-1 cells overexpressed Myc-VDAC1-pLVX or control cells were subjected to immunoprecipitation with anti-Myc antibody and followed by western blot analysis. **B** Endogenous IP assay. The whole cell lysates were prepared from RWPE-1 cells transfected with VSTM2L shRNA lentivirus or control shRNA lentivirus, and immunoprecipitations were performed with anti-HK2 antibodies, followed by western blot with indicated antibodies.

15. The authors should acknowledge alternative mechanisms whereby VBIT4 could reverse the impact of VSTM2L depletion in addition to VDAC1 oligomerisation in vivo, given that VBIT4 can also influence type I interferon signaling and neutrophils.

Response:

We thank the Reviewer for this insightful suggestion. We have tested the expression level of the genes related with type I interferon signaling (IFN) as previously described, in xenograft tumors[9]. The results showed that VSTM2L depletion did not affect the mRNA levels of genes related with IFN, such as interferon-stimulated gene 15 (ISG15), interferon alpha-4 (IFNA4), interferon-induced protein with tetratricopeptide repeats 1 (IFIT1) and interferon-induced protein 44 (IFIT44) treated with or without VBIT4 (Figure 6A, B). To evaluate the impact of VSTM2L on neutrophils, we stained the xenograft tumors with Ly-6G[10], which is the marker of neutrophils. The results indicated that knockdown of VSTM2L has no impact on the amounts of neutrophils in tumors in the presence or absence of VBIT4 (Figure 6C, D). These results indicated that VBIT4 reversed the impact of VSTM2L depletion by inhibiting VDAC1 oligomerisation other than type I interferon signaling and neutrophils *in vivo* in PCa.

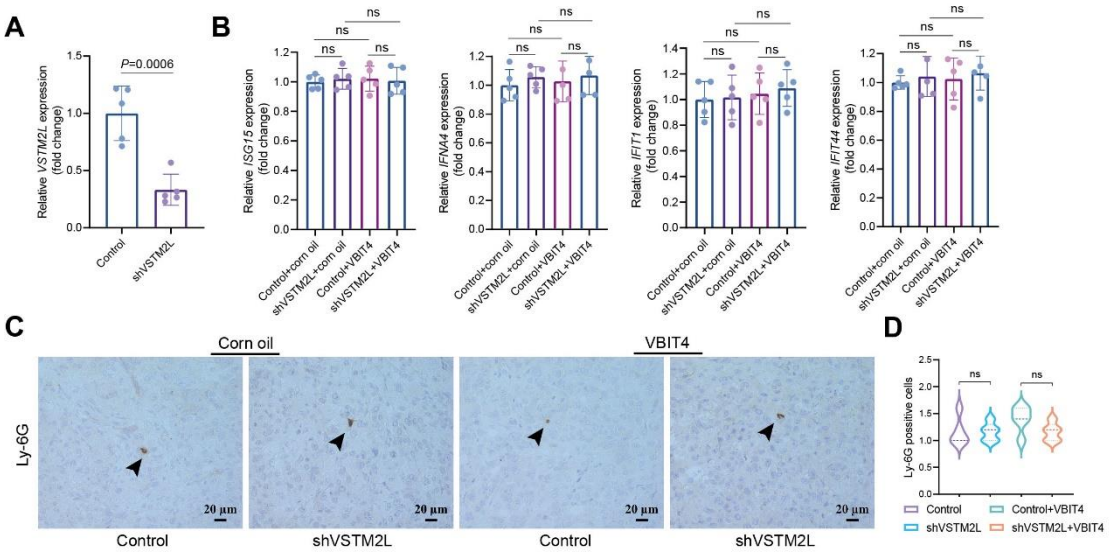

Figure 6 VSTM2L or VBIT4 have no relationship with type I interferon signalling and neutrophils in PCa. A Relative *VSTM2L* expression of xenograft tumors in the main manuscript Figure 8. Data shown as mean ± SD, n=5. **B** Relative expression of IFN stimulated genes, including *ISG15*, *IFNA4*, *IFIT1* and *IFIT44* in tumors administrated with or without VBIT4. Data shown as mean ± SD, n=5. **C** Immunohistochemistry (IHC) staining for Ly-6G were performed in isolated tumor tissues in the main manuscript figure 8. **D** quantification of **C**. Data shown as mean ± SD, n=5, 5 randomly selected magnification fields per slide, Scale bar =20 μm. *P* value was determined by unpaired two-tailed Student's *t*-test.

16. This reviewer is not convinced that VBIT4 is colony forming assays, scratch assays and transwell assay representative images are quantitated accurately in Figure 8, as the quantitation does not appear to correlate with the images. For instance, the colony forming assays shown in figure 8C do not appear to be representative of the colony quantitation shown in Fig 8D for the DMSO control. There appears to be a higher seeding density in the DMSO shRNA control setting. The authors should

confirm if the assay was performed at a similar seeding density for each treatment arm and plot the % colony forming efficiency, by normalizing the data to the number of cells plated. The VBIT4 control plate also appears to have less colony formation relative to the DMSO control in Fig 8C, yet this is not observed in the quantitation in Fig 8D. Do the VBIT4 treated arms correlate with increased stem cell activity in vivo?

Response:

We thank the reviewer for point out these issues. To address the reviewers' queries, we have repeated the colony forming assay, scratch assays and trans-well assay with the same seeding density and plot the % colony forming efficiency as the reviewer kindly suggested. As the results shown in ***Revised Figure 8C-H***, VBIT4 could reverse the weakened colony forming efficiency and migration ability caused by VSTM2L knockdown.

In addition, we also evaluated the expression level of the genes related with stem cell activity, the results showed that VSTM2L depletion did not affect the mRNA levels of *EZH2* and *ABCG2* in the presence or absence of VBIT4, *in vivo*. Please find them in the following figure 7.

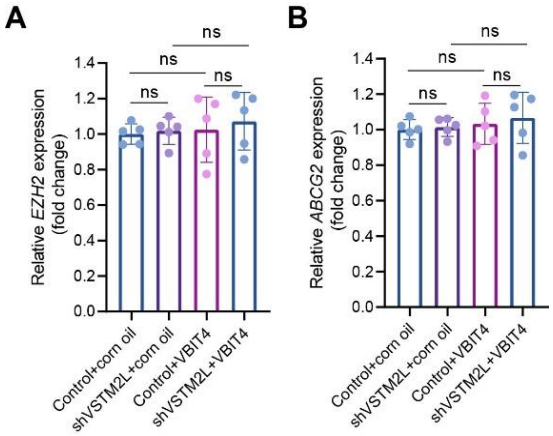

Figure 7 VSTM2L has no relationship with stem cell ability *in vivo*. A Relative expression of *EZH2* in xenograft tumors. **B** Relative expression of *ABCG2* in xenograft tumors. *P* value was determined by unpaired two-tailed Student's *t*-test.

17. Were there any histopathological differences between the different experimental arms shown in Fig 8M?

Response:

We thank the reviewer for pointing out this issue. We have added the description of the histopathological differences in the manuscript **Page 18, Line 8-11**.

18. The authors claim "the IHC and H&E staining results further indicated that the administration of VBIT4 to experimental animals notably reversed the ferroptotic cells death within the tumor tissues caused by VSTM2L suppression (Fig. 8M-N)." however cell death has not been assessed. Does VBIT4 treatment effect the number of cells undergoing apoptosis/ferroptosis in addition to altering proliferation?

Response:

We thank the Reviewer for this insightful suggestion. We have tested the ferroptosis marker GPX4 and apoptosis marker Cleaved-Caspase 3 in these tumor tissues (***Revised Figure 8M, P, Q***). The results indicated that VSTM2L knockdown influence the level of ferroptosis marker GPX4 rather than the apoptosis marker Cleaved-Caspase 3 (**Page 18, Line 11-20**).

Minor points:

- Intro line 2 – please amend the wording: prostate cancer is one of the most "common" (not "popular") cancer types

Response:

Thanks for pointing out this mistake, and we have changed the word "popular" to "common" (**Page 2, Line 7**).

- Please add citation for "VSTM2L is the newly identified VDAC1-interacting protein" in the introduction and comment on where VSTM2L is reported to bind to VDAC1.

Response:

Thanks for pointing out this issue, and we have deleted this sentence since it is not a proper description in this part.

- Page 6 – last paragraph refers to VSTM2L inhibited 22Rv1 or Du145 cells – this is scientifically incorrect terminology, please amend "inhibited" to "depleted".

Response:

Thanks for pointing out this mistake. We have revised the mistake of this sentence in the manuscript (**Page 8, Line 9**).

- Page 8, line 17-18 – the authors should indicate the 22Rv1 cell line was used to perform the xenograft study.

Response:

We thank the reviewer for pointing out this issue. We have clarified that 22Rv1 cell line was used to perform the xenograft study in the manuscript as the reviewer kindly reminded in **Page 10, Line 13**.

- Page 12 -line 2-4 – please amend wording to improve clarity. "is involved in" should be amended to "may involve the".

Response:

We thank the reviewer for pointing out this issue. We have changed the "is involved in" to "may involve the" in the manuscript (**Page 14, Line 11**).

- Figure 1H Venn diagram is referred to as proteins in the text and genes in the figure legend – please correct. Same issue with Figure 1I

Response:

We thank the reviewer for pointing out this issue. We have changed the "genes" into "proteins" in the figure legend (**Page 44, Line 4-5**).

- Methodology: Please indicate if cell lines were cell authenticated. How was the RNA for qRT-PCR quality checked? What parameters were used to generate the Kaplan-Meier plots in c-BioPortal (i.e. what is high and low referring to, was a z-score threshold used?), and what software was used to plot the data from cBioPortal?

Response:

We thank the reviewer for pointing out these questions.

The LNCaP (Lot#62402565), 22Rv1(Lot#60437301), Du145(Lot#61761869), PC3 (Lot#63031796) and RWPE1(lot#63226349, account number: 176701) cells were purchased from ATCC, the prostate cancer cell lines were authenticated by the commercial suppliers before purchase using short tandem repeat (STR) profiling, which can be checked using the lot numbers. Moreover, we have clarified this in the method part, **Page 24, Line 13-14**: "The prostate cancer cell lines were authenticated by the commercial suppliers before purchase using short tandem repeat (STR) profiling."

In addition, we used QubitRNA BR kit to check the quality of the RNA for qRT-PCR. We have clarified this in the method part of the manuscript at **Page 28, Line 18-19**: "RNA concentration and purity was analyzed with QubitRNA BR kit (Invitrogen, #Q10211)".

The parameters and software used to generate the Kaplan-Meier plots in c-BioPortal were added in the method part at **Page 38, Line 2-4**: "GraphPad Prism 9 was used to plot the Kaplan-Meier plots and z-score threshold parameters were used to generate the Kaplan-Meier plots in c-BioPortal."

Response to Reviewer #2:

Reviewer #2 (Remarks to the Author):

1. It is a bit confusing that cell survival data shown in Figure 3I (22Rv1 cells displayed drastic cell death upon VSTM2L KD) and in Figure 5B (DMSO group, minimal cell death detected upon VSTM2L KD also in the same cell line). The authors should explain why.

Response:

We thank the Reviewer for pointing out this mistake. We have misused the data of 22Rv1 cells treated with drugs. We have repeated this experiment, and corrected this result in ***Revised Figure 3G, I***. The death rate is consistent with the result in ***Figure 5D***.

2. VDAC1 has long been connected with apoptosis regulation. The authors should examine apoptotic activity in cells received short hairpins against VSTM2L.

Response:

We thank the Reviewer for this insightful suggestion. We fully agree that it is important to examine the apoptotic activity considering that VDAC1 has been widely

reported with apoptosis. Thus, we detected the Annexin-V level of the cells with or without the shRNA targeting VSTM2L, the results showed that knockdown of VSTM2L has no effect on the exposure of phosphatidylserine on the cell membrane (***Revised Supplementary Figure 3D-E***) (**Page 9, Line 10-12**). Next, we examined the apoptosis markers (Bax, Bcl2 and Cleaved-Caspase 3) at cellular level in control and VSTM2L knockdown PCa cells. The results indicated the VSTM2L did not affect the protein level of these apoptosis markers (***Revised Supplementary Figure 3F***) (**Page 9, Line 20-22**). Then, we also evaluated the apoptosis marker Cleaved-Caspase 3 in the xenograft tumors, the result showed that there is no obvious difference between these four groups, which further confirmed that RSL3 triggers ferroptosis in this setting not other forms of apoptosis (***Revised figure 4 G, K***) (**Page 11, Line 17-21**). These results proved that knockdown of VSTM2L mainly triggered the ferroptosis instead of apoptosis.

3. Related to (2), the authors should examine if the cell death phenotype observed in Figure 3I can be reversed by Fer-1 or Lip-1. (The following figures show Fer-1 rescue in the context of RSL3 treatment, but this is different.

Response:

We thank the Reviewer for this important suggestion, we have performed this experiment and the result indicated that Fer-1 could reverse the cell death in VSTM2L depleted 22Rv1 or Du145 cells (***Revised supplementary figure 3G-I***) (**Page 9, Line 21-22 to Page 10, Line 1**).

4. In Figure 1B-1E, the authors should include IgG controls in the immunoprecipitation assays.

Response:

We thank the Reviewer for pointing out this issue. We apologized that we did not describe clearly of these experiment in the figure legend. ***Figure 1B-C*** is the exogenous IP experiment, and the tag antibody was used for the IP assay. ***Figure 1D-E*** is the semi - endogenous IP experiment, we overexpressed MYC tagged VDAC1 in cells and performed the IP experiment using anti-MYC antibody. Thus, we utilized the tag antibody MYC or Flag as control in these experiments.

5. Page 12, Line 8-10; Figure 6J, K. It will be highly informative and necessary to provide data on the tripartite complex comprising VSTM2L, VDAC1, and HK2 in a semi-endogenous assay using PCa cells besides the exogenous data in 293T cells in Figure 6J. Or, is VSTM2L co-immunoprecipitated following anti-HK2 immunoprecipitation in the shCtrl DU145 cell lysates showed in Figure 6K?

Response:

We thank and agree with the reviewer. We have performed exogenous and semi-endogenous IP assay in HEK293T and Du145 cells and confirmed that VSTM2L, VDAC1 and HK2 formed complex (***Revised Figure 6J-L***) (**Page 14, Line 16-18**). In addition, we checked the endogenous interaction between HK2 and VSTM2L after VSTM2L knockdown in DU145 cell (***Revised Figure 6M***) (**Page 14, Line 20-22 to**

Page 15, Line 1-2). The result showed that VSTM2L can be co-immunoprecipitated following anti-HK2 immunoprecipitation in Du145 cells. Moreover, we conduct the endogenous IF experiment with VDAC1 and HK2 specific antibodies. The results clearly showed that knockdown of VSTM2L facilitated the dissociation of HK2 from VDAC1 (***Revised Figure 6N***) (**Page 15, Line 2-4**).

6. Page 13, Line 6-8; Figure 7A. For the left representative image, the mitochondrial ROS ratios for shVSTM2L-1 and shVSTM2L-2 seem mislabeled and inconsistent between the actual areas (~50%) and indicated numbers (13.3%, 17.3%).

Response:

We thank the Reviewer for pointing out this mistake. We have revised this mistake as the reviewer kindly reminded (***Revised Figure 7A***).

7. Page 14, Line 3-4; Figure 7I. The description "The results illustrated an elevation in MMP levels following the suppression of VSTM2L" is not correct and illogical for the data showed in Figure 7I if using JC-1 aggregates/JC-1 monomers ratio to indicate MMP levels.

Response:

We thank the Reviewer for pointing out this mistake. We have revised this mistake as the reviewer kindly reminded in **Page 16, Line 15**: "The results illustrated the decreased MMP levels following the suppression of VSTM2L, and this effect was counteracted by VBIT4 (Fig. 7I)"

Response to Reviewer #3:

Reviewer #3 (Remarks to the Author):

1. Recently, Lee et al. (2024) [11] published that VSTM2L protects against cancer cell death in cholangiocarcinoma, reducing the novelty of this paper which reports that VSTM2L protects against prostate ca cell death. Therefore, one questions the novelty of this paper.

Response:

We thank the Reviewer for pointing out this issue.

Firstly, this paper mentioned the role of VSTM2L in cholangiocarcinoma rather than prostate cancer.

Secondly, in the method part "Measurement of cell viability and death" in this paper, they examined the viability and survival of cholangiocarcinoma cells after VSTM2L knockdown just using the **WST-1 viability assay** and **colony-formation assay**, respectively. In fact, these two methods were mainly used for testing **cell viability/proliferation** other than cell death.

Thirdly, this paper did not report the relationship between VSTM2L and ferroptosis, which is one of the most important novelties of our work.

So, we believe that the paper from Lee et al did not diminish the novelty of our work. In contrast, it highlighted the significance and necessity of our research to thoroughly investigate the molecular mechanism of VSTM2L in prostate cancer,

given that VSTM2L has critical functions in various types of cancer.

2. The most novel part of this paper is the claim that VSTM2L interacts with and stabilizes the VDAC1-HK2 interaction, which prevents VDAC1 oligomerization on mitochondrial outer membrane. This is cited as the mechanism by which VSTM2L prevents cancer cell death. However, previous reports indicate that VSTM2L is not localized to mitochondria but is rather a secreted protein. Moreover, according to Mitocarta, which exhaustively identified mitochondria-localized proteins, VSTM2L is not localized to mitochondria. Therefore, it is possible that the effect of VSTM2L on the VDAC1-HK2 interaction is not direct but is indirect, which makes this paper less interesting. The authors need to prove that endogenous VSTM2L is a mitochondrial protein.

Response:

We thank the Reviewer for pointing out this important issue. We agree that the localization of VSTM2L is important to support the conclusions of our manuscript. Although VSTM2L was a secreted protein, another two research groups confirmed that VSTM2L was also localized to cytosolic using cell line and tissues[12, 13].

In order to clarify the main localization of VSTM2L in the PCa cells, we have separately extracted the mitochondria and cytoplasmic proteins from three prostate cancer cell lines, respectively, and evaluated the protein levels of VSTM2L by western blot assay. As shown in the ***Revised Figure 1J***, VSTM2L is mainly localized to the mitochondria rather than cytoplasm in PC3, Du145 and 22Rv1 cells (**Page 5, Line 21-22 to Page 6, Line 1-4**). Moreover, Immunofluorescence (IF) staining also showed that endogenous VSTM2L was localized to mitochondria in PC3, Du145 and 22Rv1 cells (***Revised Figure 1K***) (**Page 6, Line 4-7**). Collectively, these results confirmed that VSTM2L is mainly localized to the mitochondria of prostate cancer cells. Our study reveals a previously unrecognized localization to mitochondrial for VSTM2L.

References

1. Banerjee P*, et al.* Therapeutic implications of cancer stem cells in prostate cancer. *Cancer Biology & Medicine*, 1-20 (2023).
2. Teijido O, Ujwal R, Hillerdal CO, Kullman L, Rostovtseva TK, Abramson J. Affixing N-terminal α-helix to the wall of the voltage-dependent anion channel does not prevent its voltage gating. *The Journal of biological chemistry* **287**, 11437-11445 (2012).
3. Preto J, Krimm I. The intrinsically disordered N-terminus of the voltage-dependent anion channel. *PLoS Comput Biol* **17**, e1008750 (2021).
4. Bayrhuber M, Meins, T., Habeck, M., Becker, S., Giller, K., Villinger, S., Vonrhein, C., Griesinger, C., Zweckstetter, M., & Zeth, K. Structure of the human voltage-dependent anion channel. *Proceedings of the National Academy of Sciences of the United States of America* **105(40)**, 15370–15375 (2008).
5. Sung Hoon Baik VKR, Courtney Becker, Sarah Fett, David M. Underhill,

Andrea J. Wolf. Hexokinase dissociation from mitochondria promotes oligomerization of VDAC that facilitates NLRP3 inflammasome assembly and activation. *SCIENCE IMMUNOLOGY* **2023;8(84)**, eade7652 (2023).
6. Hodge T, Colombini M. Regulation of metabolite flux through voltage-gating of VDAC channels. *The Journal of membrane biology* **157**, 271-279 (1997).
7. Gincel D, Silberberg SD, Shoshan-Barmatz V. Modulation of the voltage-dependent anion channel (VDAC) by glutamate. *Journal of bioenergetics and biomembranes* **32**, 571-583 (2000).
8. Haloi N*, et al.* Structural basis of complex formation between mitochondrial anion channel VDAC1 and Hexokinase-II. *Communications Biology* **4**, (2021).
9. Kim J*, et al.* VDAC oligomers form mitochondrial pores to release mtDNA fragments and promote lupus-like disease. *Science* **366**, 1531-1536 (2019).
10. Qi X*, et al.* Identification and characterization of neutrophil heterogeneity in sepsis. *Critical care (London, England)* **25**, 50 (2021).
11. Lee J, Sim W, Lee J, Kim JH. VSTM2L is a promising therapeutic target and a prognostic soluble-biomarker in cholangiocarcinoma. *BMB reports* **57**, 324-329 (2024).
12. Li Y*, et al.* VSTM2L contributes to anoikis resistance and acts as a novel biomarker for metastasis and clinical outcome in ovarian cancer. *Biochemical and biophysical research communications* **658**, 107-115 (2023).
13. Liu H, Zhang Z, Zhen P, Zhou M. High Expression of VSTM2L Induced Resistance to Chemoradiotherapy in Rectal Cancer through Downstream IL-4 Signaling. *Journal of immunology research* **2021**, 6657012 (2021).

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VSTM2L protects prostate cancer cells against ferroptosis via inhibiting VDAC1 oligomerization and maintaining mitochondria homeostasis in men

Reviewer #1 (Remarks to the Author):
I would like to congratulate the authors for addressing all of the reviewer comments in full, and believe that their article would be a valuable addition for the readership of Nature Communications.

Thanks for the acceptance.

Response to Reviewer #2:
Reviewer #2 (Remarks to the Author):
The authors have done a good job in addressing the concerns raised by this reviewer. The manuscript is now ready for the next stage of the publication process.

Thanks for the acceptance.

Response to Reviewer #3:
Reviewer #3 (Remarks to the Author):
I am satisfied with the revision.

Thanks for the acceptance.