nature portfolio

Peer Review File

Targeting IRE1α reprograms the tumor microenvironment and enhances anti-tumor immunity in prostate cancer

REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author): with expertise in ER stress, cancer immunology

In their manuscript, Unal et al show that IRE1a-deficient prostate cancer cells implanted in immunocompetent mice alters the immune landscape of the tumor microenvironment. Their model suggests that the loss of IRE1a in cancer cells leads to improved anti-tumor immunity and survival, but the manuscript ultimately fails to deliver a cohesive explanation of the observations. This manuscript needs a more mechanistic approach to justify some experiments. Why focus specifically on the IFN-gamma signature and completely ignore the IFN-alpha/beta signature? What is the significance of the changing macrophage population beyond a weak association with adaptive immune responses? What is the justification for using cell lines interchangeably in various experiments?

The multiomic dataset generated by the authors could be highly valuable, but the presentation of this data undermines its potential usefulness due to major flaws in the experimental design and a lack of attention in the manuscript text.

Critically, all mouse experiments are carried out by implanting prostate tumors in the flank, which is an irrelevant anatomical site. Confirmatory experiments using orthotopic models of prostate cancer are essential but lacking. Furthermore, the in vivo experiments are not properly controlled as the authors compared tumor growth using WT vs IRE1-deficient prostate cancer cells generated using CRISPR-Cas9 and a vector that expresses GFP. The data and methods, as presented, show that WT cancer cells did not undergo the same treatment and were not transfected with the same GFP-expressing vector carrying non-targeting sgRNAs. This is a major experimental flaw as GFP may be operating as a very strong neo-antigen solely expressed by IRE1 KO cells, and not their WT counterparts, thus confounding the results and their interpretation.

The conceptual innovation of this study is very limited since a recent paper published at Nature Comm (Crowley et al, 2023) already demonstrated that IRE1 expression in the cancer cell controls the immune tumor microenvironment and limits protective anti-tumor immunity.

Major comments:

Ext Fig1 F-I: In lines 162-168 the authors describe these cell line viability/growth experiments as having no significant changes, but the actual figures indicate a statistical test was performed that determined a significant outcome. Overall, these experiments are poorly described, and this section should be rewritten.

Fig 2C: The authors excluded KO cell line #2 from the nude mouse experiment without explanation. The authors should include this data or provide an explanation for leaving out KO#2. In this experiment KO#1 showed a significant delay in the tumor growth, but then in Fig 3E-F they exclude that clone from the proteomic assay (line 1135-1136) without any justification. Also, why did the authors reduce the number of mice in this experiment to n=3 while a similar experiment in Fig 2A used an n=5?

In line 195-197, the authors concluded the substantial impact of adaptive immunity in their tumor model. However, there is an absence of supporting data confirming intratumoral T cell phenotypes (activity markers, cytokine expression, memory formation) within the tumor microenvironment. Thus, there is too little evidence to draw conclusions.
Fig 3E-G: KO cell line #1 is excluded from the proteomic analysis but it isn't clear why. What is the justification for comparing the proteomic and RNA-SEQ data in Fig3G when they don't include the same cell lines?

Fig2-3: The RNA-SEQ performed on tumor samples is identified as a whole tumor lysate (lines 647-653), and then used to hypothesize that there is increased immune activity in the IRE1-KO tumors. However, because this dataset samples from a heterogenous population likely dominated by cancer cells and non-immune cells these conclusions are difficult to accept. This data is further limited by including IRE1a expressing cells in the IRE1a-KO samples. The authors should better support these ideas with complementary experiments to describe the activity of immune cells in the cancer model. It's also unclear what timepoint in the tumor growth model was selected for these experiments or why they were selected.

Fig 3A-C: In lines 216-223 the author notes the expression of IFN-gamma is increased in

IRE1-KO samples and hypothesizes that T and NK cells are the most likely source, but this is very poorly explored. Later in the single cell data, the authors identify Macrophages as the main producers of IFN-gamma, while mentioning T cells and NK cells don't have an IFN-gamma signature. Overall, the focus of the analysis appears to switch from cancer cells to T cells to macrophages without much explanation.

Fig 4: The authors chose KO cell line #2 to perform the single cell sequencing analysis but they never address why they include or exclude each cell line in different experiments. From the data presented, KO#2 does not have the strongest response among the three cell lines in the tumor growth curve, is not included in the nude mouse experiment, and weakly upregulates the IFN-gamma response signature (Fig 2A-C, Fig 3C). Meanwhile, KO cell line #1 appears to perform best in the tumor growth experiment and with the upregulation of the IFN-gamma response signature in (Fig 2A-B, Fig 3C). Why choose KO#2 if another cell line had a more drastic phenotype?

Ext Fig 3E: This figure further highlights how the selection of cell line #2 is not well justified. The downregulation of UPR associated genes appears far stronger in cell line #1.

Fig 5: The justification for this experiment is very poor. The PD-1 therapy makes sense if there is a strong T cell phenotype and upregulation of this immune checkpoint or its ligand in the TME of mice bearing the KO tumors; however, the data presented does very little to convince us that this model induces a strong T cell response.

How do we know that the success of PD-1 does not rely on the direct effect of MKC8866 on T cells or macrophages instead of a tumor intrinsic cause? The effects of the inhibitor on intratumoral T cell and myeloid cell biology should be thoroughly addressed. Is MKC8866 really alleviating immunosuppression in the prostate cancer microenvironment? Functional experiments in this regard are crucial but lacking.

Additional comments:

1) The manuscript is difficult to read in some sections:

a. Line 200. Authors should write down which tissue they used for RNAseq.

b. Line 201-202. ".GSEA on the RNA-seq data confirmed downregulation of UPR target genes (Extended Data Fig. 3A) and classical IRE1α target pathways…". In which setting?

c. The use of the hyphenated words should be checked

d. Line 170-173. It's not clear whether the genes they are analyzing are downregulated in WT or KO cells.

e. Line 216-217. "… even though hallmark UPR genes were downregulated, expression of IFN-γ transcriptional response genes were significantly enhanced…". This sentence is not clear

f. Line 225. Which samples did the authors used for proteomic analysis? It should be written down at the end of the sentence.

g. The authors should also describe in the legend the time point when they performed the experiments. Also, is there a difference in tumor burden?

2) Fig. 4AC. The N is misleading. The figure legends says "…cell lines were subcutaneously injected into two flanks of FVB mice".

a. Fig 4A. Do the authors show the sum of both tumors or are the tumors independent as in Fig 4B?

b. Is Figure 4C made with the same mice? If so, why is the n different?

3) Fig. 4F. Why there is no difference in the number of cancer cells? Is this analysis done at a time point with similar tumor size? Why tumor growth of clone #2 is different from Fig 2A?

4) What's the rational for combining IRE1 inhibitor with anti-PD1? From Ext. Data Fig 8A, IRE1a KO tumors have lower expression of PD1.

5) Line 373-374. The authors suddenly describe an activation of NK cells in the scRNAseq, but it was never mentioned before and the reference to the figure is missing.

6) Fig 6A-B. The R value is very low, indicating a weak linear relationship, so I'm not entirely convinced that IRE1a positively correlate with M2 macrophage markers.

7) Ext Fig 7A. It seems that also other population express PD-L1 besides macrophages. Can the authors show a quantification?

8) The entire Ext Data Fig 8 is not described in the "result" section, but it is described towards the end of the "discussion" section. Why? This makes the initial part of the discussion difficult to read (from line 441), as, for example, the effects on Tregs were not described in the results

9) Line 567. The authors do not show any "… immunosuppressive phenotype in the TME.".

What are the authors referring to?

10) In several instances, the authors describe their experiment with nude mice as "xenografts". As the species of the donor and host is the same (mouse), this is not a xenograft. Authors should correct this.

Reviewer #2 (Remarks to the Author): with expertise in prostate cancer, immunotherapy

Unal et al. report on the role of the IRE1α-XBP1s pathway, a key modulator of the unfolded protein response (UPR), in regulating the tumor immune microenvironment of prostate cancer. The authors used the Myc-CaP model in which they generated IRE1α knockouts to investigate effects on tumor growth and the tumor immune microenvironment. Bulk RNA-seq and proteomics as well as scRNA-seq wereapplied to identify an association between IRE1α loss and increased inflammatory response as well as a reduction in tumor-associated macrophage populations. A first-in-class IRE1α inhibitor was applied to the Myc-CaP tumor model and shown to synergize with anti-PD-1 therapy. The TCGA primary prostate cancer dataset was used to make associations between 1) IRE1α expression and reduced CD8+ T cells and NK cell infiltration and 2) XBP1s expression and macrophage infiltration. A TAM gene signature was thereafter constructed using genes reduced in IRE1α knockout tumors and shown to associate with Gleason score and progression/disease-free survival in human prostate cancer datasets. While the premise of the study is interesting, the experimental data presented do not adequately support the general conclusions of the manuscript and could benefit from additional mouse models, further mechanistic interrogation, and orthogonal validation.

Major points:

1. Bulk analysis of the Myc-CaP tumors indicated an upregulation of immune-related signatures related to IRE1α knockout. The authors indicate that Myc-CaP IRE1α knockout cells do not demonstrate an IFN-g transcriptional response in vitro (lines 219-221) which suggests that the response is from the tumor microenvironment. However, Extended Data Fig. 4D appears to show enriched IFN-a and IFN-g from the cancer cells in the scRNA-seq

analysis of Myc-CaP IRE1α knockout vs. Myc-CaP wildtype tumors. This discrepancy should be addressed/discussed.

2. The conclusions may be overstated as they are based on manipulation of a single model (Myc-CaP) on one mouse genetic background (FVB). This needs to be strengthened by complementary experiments in other mouse prostate cancer models on other mouse genetic backgrounds (i.e., RM9 or TRAMPC2 on BL6).

3. In Fig. 4F and 4H, normalization of the proportions of immune cells in including macrophages to total Cd45+ cells (rather than to total cells) should be considered especially because the absolute differences are small and could be impacted by changes in other cell populations.

4. Mechanistic investigation of why concurrent MKC8866 and anti-PD-1 therapy induces substantial therapeutic responses as shown in Fig. 5E is lacking. What happens to the tumor immune microenvironment in this context?

5. There is a disconnect between the absence of differences found in NK and T cell frequencies between Myc-CaP wildtype and Myc-CaP IRE1α knockout tumors relative to what is suggested as a correlation between IRE1α expression levels and NK and T cell infiltration in human prostate cancer patient data.

6. Related to point #5, the authors are reliant on various inferred data analyses of bulk RNA-seq from human prostate cancer patient data to argue a variety of associations. This would be significantly strengthened by validation of these associations by IHC.

7. It is unclear why the authors use TCGA (primary prostate cancer) to develop/apply a TAM gene signature and how this relates to the evaluation of MKC8866 and anti-PD-1 therapy. It would be much more appropriate and informative to develop this in the context of advanced prostate cancer where these therapies would reasonably be applied.

Minor points:

1. In Fig. 1C, it is unclear how many samples are Gleason 6 vs. Gleason 7-10.

2. In Fig. 4E, the authors should note the number of tumors and number of cells displayed within the UMAP plots.

3. In line 337 of the text, the authors describe “xenografted tumor growth” but this should be “syngeneic tumor growth.”

Reviewer #3 (Remarks to the Author): with expertise in prostate cancer

The manuscript, “Targeting IRE1α alleviates the immunosuppressive tumor microenvironment in prostate cancer” by Unal et al, addresses mechanisms of activation of the unfolded protein response pathway (UPR) on immunologic features of prostate cancer and develop therapeutic strategies. Authors show that IRE1α protein was upregulated in prostate tumors in comparison to benign prostate gland and increased levels of the IRE1α proteins associated with higher Gleason score. Overexpression of IRE1α and XBP1 was also shown in TCGA-prostate cancer dataset and higher XBP1 expression correlated with increased expression of cancer hallmarks genes. The IRE1α knock-out in a prostate cancer cell line derived from prostate targeted C-Myc-transgenic mice and syngeneic prostate cancer mouse model from this cell line was extensively used to evaluate both tumor biology and immunology. The IRE1α KO/Myc-CaP cells showed markedly reduced tumor growth only in immune competent mice as compared to growth in nude mice or in cell culture. RNA-Seq data from the IRE1α-KO/Myc-CaP prostate tumors in immune competent mice showed down regulation of UPR gene expression signature and increased expression of genes associating with IFN-γ signaling and immune functions related to B cells, T cells and NK cells. Single cell RNA-seq data from these tumors showed marked reduction in tumor associated macrophages (TAM), T-regs and cancer associated fibroblasts. In TCGA prostate cancer dataset increased IRE1α expression correlated with increased TAM gene expression markers. The evaluation of a TAM gene expression signature defined from this study showed a strong association with poor survival and disease progression in the TCGA-

prostate cancer dataset. In the Myc-CaP prostate tumor mouse model IRE1α inhibitor, MKC8866 showed synergy with anti-PD1 therapy.

Overall, this manuscript presents compelling data for the role of IRE1α in suppressing prostate tumor immune microenvironment from a complementary set of experiments utilizing mouse model and human prostate cancer. These findings are concordant with suggested roles of UPR/IRE1α -XBP1 pathway in modulating immune functions.

Questions/clarification

While the IRE1α-KO/Myc-CaP prostate tumor model used in this report has provided promising data, the cancer genomic and immunologic contexts are expected to be lot more complex and heterogenous in human prostate cancer, especially in metastatic prostate cancer context. Although the PD1/PDL1 blockade therapy has not been successful in human prostate cancer, the data shown in the mouse model exhibits significant antitumor activity (Figure 6F). The authors may discuss possible limitations while translating these data for human prostate cancer treatment.

Is IRE1α increased expression restricted to epithelial cells in human prostate tumors? What percent of Gleason 7-10 prostate tumors show high expression of IRE1α (Figure 1D)? Similar results may be provided for CD68 and IRE1α in Figure 6.

TAM prognostic gene signature appears to be robust. An IHC assay may be more useful given the complexity of TME evaluations in clinical specimens.

Reviewer #4 (Remarks to the Author): with expertise in IRE1α, ER stress

In this manuscript Unal and colleagues very elegantly document the role of IRE1 signaling in prostate cancer cells and the impact on the tumor microenvironment (in particular the immune infiltrate) and evaluate the impact of IRE1 pharmacological inhibition on the response of those tumors to immunotherapy. This is a very solid work that will be of great

value to the prostate cancer (and more extensively to the cancer) community.

Comments on the manuscript:

1) The authors justify their study by the fact that IRE1 expression is elevated in prostate tumors. However, the expression of IRE1 (evaluated by immunohistochemistry (IHC)) appears to be highly heterogeneous.
a. Is there any clinical feature associated with IRE1high tumors vs. IRE1low tumors? (e.g. expression of hormone receptors etc…)
b. Does IRE1 protein expression correlates with IRE1 mRNA expression? If so the authors could identify genes exhibiting similar or opposite expression patterns than IRE1 and evaluate the functional enrichments in those gene sets.
c. What are the characteristics of IRE1 expression in tumor cells (for instance markers of hallmarks of cancer
d. The expression of IRE1 appears undetectable in normal prostate tissue, does that mean that prostate cells (epithelial) do not express IRE1? Or is that just that IHC is not sensitive enough to detect low levels of IRE1.
e. IRE1 expression levels have been shown to be linked to its signaling outcomes depending either on catalytic or non-catalytic (scaffold) mechanisms, have the authors considered those aspects?

2) To further document the signaling mechanisms triggered in prostate cancer cells in correlation with IRE1 overexpression.
a. The authors rely on publicly available datasets to evaluate an UPR signature (the list of genes composing this signature should be provided) and jump from these results to XBP1 mRNA spliced forms without clear articulation. To document the link between IRE1 high expression and XBP1 mRNA splicing the authors should i) evaluate the expression of XBP1s protein in their prostate cancer sections, ii) if IRE1 protein and mRNA expression is correlated, then show the correlation between XBP1s mRNA and IRE1 mRNA in public datasets.
b. Again, based on this, the expression of XBP1s target genes (see https://chip-atlas.org/) could easily be correlated.

c. In the UPR signature used in fig 1D, what is the IRE1 specific component (and by extend, what are the PERK and ATF6 components)?
d. If the activation of both PERK and ATF6 components are also found elevated in prostate tumors, what is the reason for the authors to privilege IRE1 signaling over the two other UPR arms?
e. From the data presented in this manuscript, the authors solely focus on the IRE1/XBP1s axis, however, it is relatively well documented that IRE1 overexpression is sufficient to also yield activation of the RIDD pathway. As such, it would be of great interest to evaluate how RIDD targets behave in prostate cancer cells (wt or ko for IRE1 or treated with the IRE1 RNase inhibitor) and how this could actually impact on the behavior of those cells – in the same line of idea, the expression of identified RIDD targets could be tested in silico in publicly available datasets.
f. It appears from gene and protein expression profiles that the expression of several components of the Integrated Stress Response is altered upon blunting IRE1 expression, how does this impact on tumor cells' phenotypes?
g. IRE1 phosphorylation is key for the modulation of IRE1 signaling, do the authors have any information regarding IRE1 phosphorylation status in prostate tumors?
h. At last, scaffolding functions of IRE1 appear to contribute to IRE1 unconventional signaling, do the authors have any information that could document this aspect in prostate tumors?

3) Since the rational for this study relies on the expression levels (and subsequent signaling of IRE1), the authors should provide information on their choices regarding the selection of the studied cell lines. Were "basal" expression levels of IRE1 documented for those lines? It would be interesting to identify whether low and high expression lines do exist. In the present study the authors have blunted IRE1 expression or activity, what about overexpressing IRE1 and evaluating whether the observed phenotype is in line with what is found in tumor samples.

4) Mouse models – the authors have used both syngeneic and xenogenic models to evaluate the impact of IRE1 signaling alteration on their phenotypes, however, the implantation system selected is heterotopic which might be of importance regarding the nature of the

immune infiltrate. To demonstrate that the same effects are observed in orthotopic models, the authors could, instead of repeating the same experiments but in another model, test the hypothesis (e.g. using qPCR, IHC or FACS analyses) in a prostate cancer orthotopic model. In these models it might also be worth it to test other IRE1 inhibitors that do not covalently bind to IRE1 which could then represent a strong confirmatory approach for the preclinical proof of concept.

Response to Reviewers' comments:

Reviewer #1: with expertise in ER stress, cancer immunology
General comment:

In their manuscript, Unal et al show that IRE1a-deficient prostate cancer cells implanted in immunocompetent mice alters the immune landscape of the tumor microenvironment. Their model suggests that the loss of IRE1a in cancer cells leads to improved anti-tumor immunity and survival, but the manuscript ultimately fails to deliver a cohesive explanation of the observations. This manuscript needs a more mechanistic approach to justify some experiments.

We thank the Reviewer for this feedback. In the revised manuscript, we have added significant new data that we feel greatly improved our manuscript, including uncovering of potential mechanisms of our observations.

Why focus specifically on the IFN-gamma signature and completely ignore the IFN-alpha/beta signature?

Our decision in this regard was based on the well-established role of IFN-gamma (IFN-γ) in anti-tumor immune responses, particularly in the context of T cell-mediated immunity and antigen presentation. While we recognize the importance of IFN-alpha/beta, we chose to prioritize IFN-γ due to the specific findings in our model. We clarified this in our revised manuscript and ensured that the relevance of IFN-alpha/beta is appropriately acknowledged.

What is the significance of the changing macrophage population beyond a weak association with adaptive immune responses?

Macrophages represent a key component of the tumor microenvironment (TME) and play a multifaceted role in cancer progression. Macrophages in the TME, particularly tumor-associated macrophages (TAMs), can exhibit a spectrum of phenotypes ranging from pro-inflammatory (M1-like) to immunosuppressive (M2-like), each contributing differently to tumor progression. The shift in macrophage population dynamics could indicate a change in the immune landscape of the tumor, potentially influencing tumor growth, metastasis, and response to therapy. Additionally, the interaction between macrophages and other immune cells, such as T cells, is critical in shaping the immune response to tumors. A change in the macrophage population could, therefore, have significant implications for the effectiveness of immunotherapies, which are increasingly important in cancer treatment. These points are made in the revised manuscript.

What is the justification for using cell lines interchangeably in various experiments?

Please see our response below in the major comments section.

The multiomic dataset generated by the authors could be highly valuable, but the presentation of this data undermines its potential usefulness due to major flaws in the experimental design and a lack of attention in the manuscript text.

We appreciate the Reviewer's comments on the potential value of our multiomic dataset. Regarding the mention of 'a major flaw' in our experimental design, we

understand this to be related to the concerns about the GFP expression in our cell line generation and its potential impact on our data, from the other comments made below. However, we believe that there is an important misunderstanding in this regard as in our experiments, GFP is not expressed constitutively; it is transiently expressed during cell line generation. Therefore, it would not be a source of neo-antigens that could potentially influence the experimental outcomes. Please see our answer below.

Critically, all mouse experiments are carried out by implanting prostate tumors in the flank, which is an irrelevant anatomical site. Confirmatory experiments using orthotopic models of prostate cancer are essential but lacking.

We agree with the Reviewer on the importance of orthotopic models for studying the TME and disease progression in cancer models, including prostate cancer (PCa). In the revised manuscript, we now show that the growth of IRE1α KO Myc-CaP tumors was also significantly hindered in the orthotopic setting compared to wild type Myc-CaP tumors **(Extended Data Fig. 3E, F)**, similar to significant inhibition of growth in the subcutaneous Myc-CaP model **(Fig. 2A and B)**.

Furthermore, the in vivo experiments are not properly controlled as the authors compared tumor growth using WT vs IRE1-deficient prostate cancer cells generated using CRISPR-Cas9 and a vector that expresses GFP. The data and methods, as presented, show that WT cancer cells did not undergo the same treatment and were not transfected with the same GFP-expressing vector carrying non-targeting sgRNAs. This is a major experimental flaw as GFP may be operating as a very strong neo-antigen solely expressed by IRE1 KO cells, and not their WT counterparts, thus confounding the results and their interpretation.

As described in the Methods, and pointed out above, GFP expression is transient during cell line generation and does not persist in the cell lines used for in vivo experiments. This precludes the formation of neo-antigens, and thus would not confound our results.

The conceptual innovation of this study is very limited since a recent paper published at Nature Comm (Crowley et al, 2023) already demonstrated that IRE1 expression in the cancer cell controls the immune tumor microenvironment and limits protective anti-tumor immunity.

We acknowledge the significance of the study by Crowley et al. who showed that IRE1α signaling is important in a Kras model of NSCLC, referenced in our original submission. We would like to suggest that our study makes important and novel contributions to the field significantly beyond the findings of Crowley et al. First, we have identified IRE1α inhibition as a means to sensitize PCa, an immunologically ‘cold’ cancer type, to immune checkpoint inhibition; this is not a topic in the NSCLC paper. In the revised manuscript, we also confirmed our results in two additional syngeneic PCa models: Myc-CaP PTEN KO and the RM-1. Our findings demonstrate that MKC8866 synergizes with anti-PD-1 immunotherapy in these two models as well, suggesting that IRE1α inhibition can enhance the therapeutic efficacy of anti-PD-1-based immune checkpoint blockade (ICB) in PCa.

Second, the NSCLC study used targeted flow cytometry to probe the changes in TME upon loss of IRE1α, whereas we have used scRNA-seq that provides a greatly increased depth in the data on the cell composition and their activities within the TME. In the revised manuscript, we now provide an additional set of scRNA-seq data in an independent model to probe as to how the MKC8866 + anti-PD-1 combination therapy reprograms the TME and enhances anti-tumor immune responses **(Fig. 7)**. Additionally, we find that PTEN loss in Myc-CaP cells in the tumors significantly alters the TME and generates a more immunosuppressive TME **(Extended data Fig. 8)** which is reversed by MKC8866 + anti-PD-1 therapy **(Fig. 7 and Extended data Fig. 7)**. In our study, we also identified a 'TAM signature' associated with unfavorable PCa prognosis, which showed increased expression in the macrophage cluster of PTEN KO tumors. **(Extended Data Fig. 10C)**. Interestingly, in PTEN KO tumors, MKC8866 + anti-PD-1 therapy reduced the expression of TAM signature genes **(Extended Data Fig. 10D)**. These results are important since MKC8866 is now in clinical trials (NCT03950570) in advanced cancer patients; our results suggest that new clinical trials can be designed for PCa where immunotherapy approaches are coupled to IRE1α targeting. This kind of information is missing in the NSCLC study.

Third, the mechanism with which IRE1α functions in NSCLC and PCa appear to be quite different: whereas in NSCLC it appears to involve changes in DCs, CD4+ and CD8+ cell numbers, we have found that beside CD8+ T cells, Tumor-Associated Macrophages (TAMs), Cancer-Associated Fibroblasts (CAFs), pericytes, and Natural Killer (NK) cells are the most significantly altered populations in the TME upon IRE1α loss in PCa cells. In addition, whereas in the NSCLC study mPGES1 expression and ensuing PGE2 production is presented as the mechanism of action, we do not observe changes in this pathway in PCa. We thus believe that our study offers distinct and novel insights that are significantly different and beyond those presented by Crowley et al. on the role of IRE1α in TME dynamics and cancer.

Major comments:

Ext Fig1 F-I: In lines 162-168 the authors describe these cell line viability/growth experiments as having no significant changes, but the actual figures indicate a statistical test was performed that determined a significant outcome. Overall, these experiments are poorly described, and this section should be rewritten.

We thank the Reviewer for pointing this out. We have adjusted the text to indicate this. Although the change in apoptosis for clones #1 and #3 showed some differences that reached significance, please note that the extent of this is still very small; in addition, in all the cell growth/viability experiments **(Ext Fig. 1C, D, and E)**, there is no statistical differences between the clones. This is now more clearly indicated in the text.

The following three questions relate to the same point and addressed together: ***What is the justification for using cell lines interchangeably in various experiments?***

Fig 2C: The authors excluded KO cell line #2 from the nude mouse experiment without explanation. The authors should include this data or provide an explanation for leaving out KO#2. In this experiment, KO#1 showed a significant delay in the tumor growth, but then in Fig 3E-F they exclude that clone from the proteomic assay (line 1135-1136) without any justification. Also, why did the authors reduce the number of mice in this experiment to n=3 while a similar experiment in Fig 2A used an n=5?

Fig 3E-G: KO cell line #1 is excluded from the proteomic analysis but it isn't clear why. What is the justification for comparing the proteomic and RNA-SEQ data in Fig3G when they don't include the same cell lines?

We thank the Reviewer for bringing up these points. At the time of conducting these experiments, we had initially planned to work with two clones as biological replicates due to resource constraints. The selection of clones was made randomly. For the nude mice experiments, the take rate is 100% and there is very little variability, based on our previous experience; we therefore reasoned that six tumors per group would provide reliable data while optimizing the use of resources. In Figure 2A, for the experiment in syngeneic mice, we used higher number of mice since based on our experience the variability is intrinsically high in these experiments, including the frequent need for early sacrifice of some mice due to aggression in this genetic background.

Regarding the exclusion of KO cell line #1 in Figure 3E-F, as can be seen in Figure 2A, the tumor growth for #1 line was very minimal and thus very little material was left by the end of the experiment; since all this material was used for the RNA-Seq experiment, there was no material left for proteomics. We also want to emphasize that in our RNA-Seq analysis, there is very good correlation ($r > 0.56$) in the data obtained by the three clones, indicating that data from the two clones should be representative.

We now provide this information in the revised manuscript to clarify the experimental design and its rationale, ensuring that readers can better understand the reasons for these decisions **(Extended Data 3A)**.

In line 195-197, the authors concluded the substantial impact of adaptive immunity in their tumor model. However, there is an absence of supporting data confirming intratumoral T cell phenotypes (activity markers, cytokine expression, memory formation) within the tumor microenvironment. Thus, there is too little evidence to draw conclusions.

We thank the Reviewer for pointing this out. We have revised the text to moderate our conclusions about the impact of adaptive immunity, given the current evidence. The revision reflects a more cautious interpretation, in line with the data available.

Fig2-3: The RNA-Seq performed on tumor samples is identified as a whole tumor lysate (lines 647-653), and then used to hypothesize that there is increased immune activity in the IRE1-KO tumors. However, because this dataset samples from a heterogenous population likely dominated by cancer cells and non-

immune cells these conclusions are difficult to accept. This data is further limited by including IRE1a expressing cells in the IRE1a-KO samples. The authors should better support these ideas with complementary experiments to describe the activity of immune cells in the cancer model. It's also unclear what time point in the tumor growth model was selected for these experiments or why they were selected.

We agree with the Reviewer that the heterogeneity of the tumor samples instill potential limitations for drawing conclusions about immune activity from whole tumor lysate data. For this reason, at that point in the manuscript, we state that the data only "suggest", rather than conclusively demonstrate, increased immune activity.

However, as you know, we subsequently present single-cell RNA-Seq (scRNA-Seq) analysis in the manuscript, which provides an in-depth and cell-specific view of gene expression changes specifically within the TME immune compartment.

Regarding the time point for the tumor growth analyses in mice, samples for various analyses were collected at the endpoint of tumor growth. This time point was chosen based on ethical considerations to ensure that the tumors did not exceed the maximum permissible size in the control group. We could potentially collect tumors earlier, e.g. in the middle of this time frame, but then we would have had very small tumors in the KO lines for downstream analyses as they grow slowly.

Fig 3A-C: In lines 216-223 the author notes the expression of IFN-gamma is increased in IRE1-KO samples and hypothesizes that T and NK cells are the most likely source, but this is very poorly explored. Later in the single cell data, the authors identify Macrophages as the main producers of IFN-gamma, while mentioning T cells and NK cells don't have an IFN-gamma signature. Overall, the focus of the analysis appears to switch from cancer cells to T cells to macrophages without much explanation.

We thank the Reviewer for this comment. We merely suggested a possibility in this section, but to enhance clarity and not to cause misunderstanding, we have now revised this text. Specifically, we have removed the sentence that suggested a possibility for specific cell types as the source of IFN-γ production.

Fig 4: The authors chose KO cell line #2 to perform the single cell sequencing analysis but they never address why they include or exclude each cell line in different experiments. From the data presented, KO#2 does not have the strongest response among the three cell lines in the tumor growth curve, is not included in the nude mouse experiment, and weakly upregulates the IFN-gamma response signature (Fig 2A-C, Fig 3C). Meanwhile, KO cell line #1 appears to perform best in the tumor growth experiment and with the upregulation of the IFN-gamma response signature in (Fig 2A-B, Fig 3C). Why choose KO#2 if another cell line had a more drastic phenotype?

Ext Fig 3E: This figure further highlights how the selection of cell line #2 is not well justified. The downregulation of UPR associated genes appears far stronger in cell line #1.

The scRNA-Seq experiment is very costly and unfortunately, we did not have the resources to perform this experiment on all three clones. We did not want to choose #1 since the effect was most dramatic and the tumor growth is very poor providing very little amount of material. If we chose clone #1 for the scRNA-Seq analysis, we anticipated that it might raise questions as to why we selected the clone with the most pronounced effect, especially when the other two clones exhibited more similar results to each other in terms of their tumor growth profile. However, we now show that ectopic expression of XBP1s in IRE1α KO clone #1 rescues tumor growth *in vivo* **(Extended Data Fig. 3G, H)**. Furthermore, we observed similar tumor growth kinetics, as measured by tumor mass data, in clones #1 and #2 from the orthotopic model **(Extended Data Fig. 3E, F)** as in the subcutaneous PCa model **(Fig. 2A, B)**. Our goal was to provide a representative analysis while considering both the resource limitations and the need to ensure a robust dataset. We thus decided between #2 and #3 clones, which had similar tumor growth profiles. As we pointed out above **(Extended Data Fig. 3A)**, please note that our bulk RNA-Seq data indicate very similar expression profiles of the three clones indicating their similar biology.

Fig 5: The justification for this experiment is very poor. The PD-1 therapy makes sense if there is a strong T cell phenotype and upregulation of this immune checkpoint or its ligand in the TME of mice bearing the KO tumors; however, the data presented does very little to convince us that this model induces a strong T cell response. Can be also supported from literature.

We thank the Reviewer for this feedback. The decision to explore the combination of an IRE1α inhibitor with anti-PD1 therapy was based on a multifaceted understanding of the TME and immune response modulation. In fact, TAMs, the most abundant immune cell type in PCa tumors, are known to significantly influence PD-1/PD-L1 mediated immunosuppression. They achieve this through various mechanisms, including cytokine secretion, expression of immune checkpoint ligands, and the release of exosomes. Notably, targeting TAMs has been reported to bolster the anti-cancer effects of PD-1/PD-L1 inhibitors. For instance, several reports suggested that depletion of TREM2 expression on macrophages enhances response to anti-PD-1 immunotherapy [2, 3, 4].

In our revised manuscript, we now show that MKC8866 treatment alone increased the expression of PD-1 and PD-L1 in CD8+ T cells and TAMs, respectively. Moreover, combining MKC8866 with anti-PD-1 further increased the expression of PD-1 and PD-L1 in these corresponding cell types. These data are presented in **(Figure 7C, H, and Extended Data Fig. 7F)**. We believe that these results now clearly explain the rationale for combining MKC8866 with anti-PD-1 therapy.

How do we know that the success of PD-1 does not rely on the direct effect of MKC8866 on T cells or macrophages instead of a tumor intrinsic cause? The effects of the inhibitor on intratumoral T cell and myeloid cell biology should be thoroughly addressed. Is MKC8866 really alleviating immunosuppression in the prostate cancer microenvironment? Functional experiments in this regard are crucial but lacking.

Since both MKC8866 and anti-PD-1 are systemically administered, they can have effects in different cells in the TME. In the literature, it is established that IRE1α inhibition directly affects a number of immune cell types, as we describe in the Introduction. In the revised manuscript, we now also provide scRNA-seq data upon MKC8866 + anti-PD-1 treatment in an advanced PCa model (PTEN KO). Interestingly, the MKC8866 + anti-PD-1 therapy shifted the transcriptional profile of all TAM sub-types towards an M1-like phenotype by upregulating the expression of genes involved in MHC-II components, IFN responses (Ligp1, Gbp2b, and Cxcl9), and inflammatory response and activation (AW112010, Nos2, Cd86) **(Fig. 7E and Extended Data Fig. 7E)**. However, the expression of genes associated with M2 macrophages such as Cd63, Spp1, Lyz2, and Pf4 was downregulated in the TAMs upon combination therapy **(Fig. 7E and Extended Data Fig. 7F)**. Treatment with either MKC8866 or anti-PD-1 alone resulted in a marked increase in the abundance of CD8+ T cells and NK cells **(Fig. 7B).** Remarkably, MKC8866 + anti-PD-1 therapy further boosted the representation of these cells in the TME **(Fig. 7B)**. Analysis of differentially expressed genes in the CD8+ T cell cluster revealed that MKC8866 treatment alone increased the expression of T cell activation markers such as Gzmb, Prf1, Ccl5 and Nkg7 genes **(Fig. 7H)**. Interestingly, MKC8866 + anti-PD-1 therapy further boosted the expression of these markers in CD8+ T cells **(Fig. 7H).** However, extensive experiments are required to determine whether these changes depend on the direct effects of MKC8866 on cancer cells or immune cells. We are currently exploring these possibilities for another study.

Additional comments:

1) The manuscript is difficult to read in some sections:

We thank the Reviewer for this feedback. We have now significantly revised the text in the manuscript as a whole, which has improved the clarity.

a. Line 200. Authors should write down which tissue they used for RNAseq.

We thank the Reviewer for this point. The data are the average from all three Myc-CaP IRE1α KO clones, which is now indicated more clearly in the text.

b. Line 201-202. "GSEA on the RNA-seq data confirmed downregulation of UPR target genes (Extended Data Fig. 3A) and classical IRE1α target pathways…". In which setting?

These are from the Myc-CaP tumors. It is now more clearly stated in the text.

c. The use of hyphenated words should be checked.

We thank the Reviewer for this feedback. All of these have now been checked and corrected as needed.

d. Line 170-173. It's not clear whether the genes they are analyzing are downregulated in WT or KO cells.

The analysis is on KO cells and so the WT specification is removed.

e. Line 216-217. "… even though hallmark UPR genes were downregulated, expression of IFN-γ transcriptional response genes were significantly enhanced…". This sentence is not clear.

We now altered this sentence to make it clear that there is no direct relationship between the expression of UPR and IFN-γ gene sets.

f. Line 225. Which samples did the authors used for proteomic analysis? It should be written down at the end of the sentence.

We used clones #2 and #3 for this analysis, as explained above; this is now included in the text.

g. The authors should also describe in the legend the time point when they performed the experiments. Also, is there a difference in tumor burden?

The samples that were submitted for the proteomics experiments were the samples collected at the end of the experiment presented in Figure 2A (at day 24). This is now more clearly indicated in the text.

2) Fig. 4AC. The N is misleading. The figure legends says "…cell lines were subcutaneously injected into two flanks of FVB mice".

We thank the Reviewer for pointing this out. The figure legend is updated to indicate that n indicates the number of animals used per group. Please note that in the revised manuscript these figures are moved to Extended Data Fig. 5A-C.

a. Fig 4A. Do the authors show the sum of both tumors or are the tumors independent as in Fig 4B?

The graph does not show the sum of both tumors; instead, it represents the average tumor size per group, with error bars indicating the standard error of the mean (SEM). This is now clarified in the text.

b. Is Figure 4C made with the same mice? If so, why is the n different?

The animals in Figure 4A/B were sacrificed at the end of the study. This is an independent experiment where the number of mice between WT and IRE1 KO groups were different, as we had to sacrifice one mouse due to fighting related injury during the experiment. No data were excluded.

3) Fig. 4F. Why there is no difference in the number of cancer cells? Is this analysis done at a time point with similar tumor size? Why tumor growth of clone #2 is different from Fig 2A?

The tumors were collected at the end of the experiment depicted in Figure 4A. Since for scRNAseq, after digestion of the tumor tissue, relatively equal number of cells are sequenced for the control and IRE1 KO, one would not expect to see a significant change in terms of number of tumor cells, although the tumor size is approximately 90% smaller.

Regarding the variability in tumor growth in our IRE1α KO animal experiments: variability of tumor size in mouse experiments is common. Such variability can arise from a multitude of inherent biological/mouse specific as well as external factors. Although we took measures to minimize variations in external effects, some degree of variability is inevitable, especially for models with an intact immune system, and is a well-recognized aspect of in vivo biological research. We believe that our data demonstrate a consistent picture in tumor growth in the IRE1α KO groups when compared to the wild type.

4) What's the rational for combining IRE1 inhibitor with anti-PD1? From Ext. Data Fig 8A, IRE1a KO tumors have lower expression of PD1.

The rationale and scientific underpinnings for our approach has been addressed above in response to question 2 on page 7.

5) Line 373-374. The authors suddenly describe an activation of NK cells in the scRNAseq, but it was never mentioned before and the reference to the figure is missing.

We thank the Reviewer for pointing this out. We have now noted NK cells in the table in Figure 4C. In addition, these data are included in the Results and noted in the Discussion.

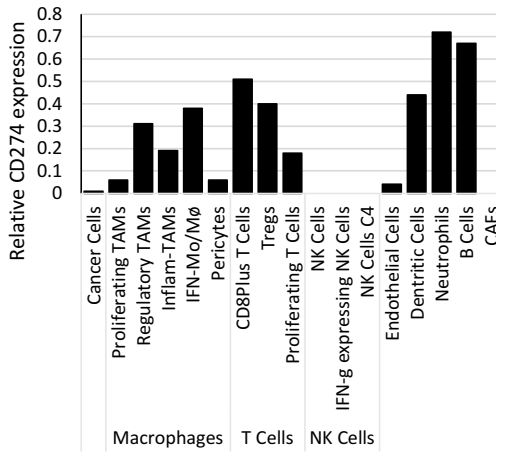
6) Fig 6A-B. The R value is very low, indicating a weak linear relationship, so I'm not entirely convinced that IRE1a positively correlates with M2 macrophage markers.

We thank the Reviewer for this feedback. We would like to point out that in complex biological systems, especially within the TME, the relationships between different entities can be non-linear and influenced by a multitude of factors. While the R value indicates a relatively weak relationship, it does not necessarily negate a biologically relevant association, especially in the context of other supporting experimental data. For example, previous studies have indicated that IRE1α can influence macrophage polarization [5, 6, 7], and our findings are consistent with these reports. In the revised manuscript, we also demonstrate that MKC8866 + anti-PD-1 therapy shifted the transcriptional profile of all TAM sub-types towards an M1-like phenotype **(Fig. 7E and**

Extended Data Fig. 7E) and reduced the expression of M2 macrophage markers **(Fig. 7E and Extended Data Fig. 7F)**.

7) Ext Fig 7A. It seems that also other population express PD-L1 besides macrophages. Can the authors show a quantification?

CD274 (encoding PD-L1) is indeed expressed across various cell types in the TME **(Rebuttal Letter Figure 1)**. These cell types include T Cells, Neutrophils, B Cells, Dendritic Cells, and Macrophages. Conversely, it is either not detected or minimally expressed in Cancer-Associated Fibroblasts, Pericytes, Cancer Cells, Endothelial Cells, and NK Cells. This information is not included in the revised manuscript as we chose not to discuss these data.



Rebuttal Letter Figure 1

Expression of PD-L1 in various cell types identified in the TME from WT Myc-CaP tumors scRNA-Seq data.

8) The entire Ext Data Fig 8 is not described in the "result" section, but it is described towards the end of the "discussion" section. Why? This makes the initial part of the discussion difficult to read (from line 441), as, for example, the effects on Tregs were not described in the results.

We thank the Reviewer for this feedback. The placement of Extended Data Figure 8 in the Discussion was intended to contextualize the results within a broader perspective. However, we recognize that this may have impacted the readability and flow of our manuscript; we thus moved some of these data to Figure 4 and introduced these findings in the Results section for ease of following the text.

9) Line 567. The authors do not show any "… immunosuppressive phenotype in the TME.". What are the authors referring to?

We thank the Reviewer for this point. We have now re-written the statement as '…but it also reprograms the TME by modulating anti-tumor immune responses'. We think it is now clear.

10) In several instances, the authors describe their experiment with nude mice as "xenografts". As the species of the donor and host is the same (mouse), this is not a xenograft. Authors should correct this.

We thank the Reviewer for this feedback; we have now corrected this in the revised manuscript.

***Reviewer #2**: with expertise in prostate cancer, immunotherapy*

Unal et al. report on the role of the IRE1α-XBP1s pathway, a key modulator of the unfolded protein response (UPR), in regulating the tumor immune microenvironment of prostate cancer. The authors used the Myc-CaP model in which they generated IRE1α knockouts to investigate effects on tumor growth and the tumor immune microenvironment. Bulk RNA-seq and proteomics as well as scRNA-seq were applied to identify an association between IRE1α loss and increased inflammatory response as well as a reduction in tumor-associated macrophage populations. A first-in-class IRE1α inhibitor was applied to the Myc-CaP tumor model and shown to synergize with anti-PD-1 therapy. The TCGA primary prostate cancer dataset was used to make associations between 1) IRE1α expression and reduced CD8+ T cells and NK cell infiltration and 2) XBP1s expression and macrophage infiltration. A TAM gene signature was thereafter constructed using genes reduced in IRE1α knockout tumors and shown to associate with Gleason score and progression/disease-free survival in human prostate cancer datasets.

While the premise of the study is interesting, the experimental data presented do not adequately support the general conclusions of the manuscript and could benefit from additional mouse models, further mechanistic interrogation, and orthogonal validation.

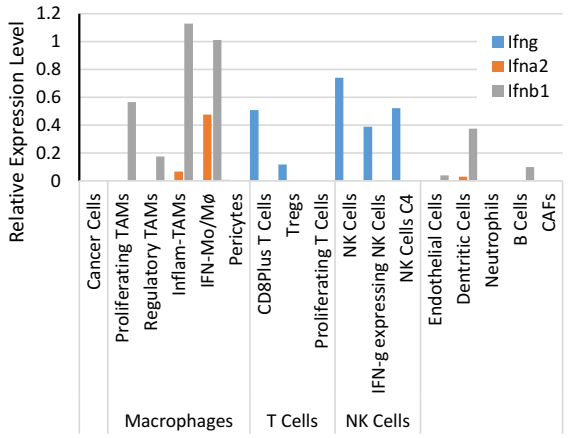
We thank the Reviewer for the feedback and finding our study of interest. We have included significant additional data in the revised manuscript that we feel addresses all concerns of the Reviewer. These are detailed below.

Major points:

1. Bulk analysis of the Myc-CaP tumors indicated an upregulation of immune-related signatures related to IRE1α knockout. The authors indicate that Myc-CaP IRE1α knockout cells do not demonstrate an IFN-g transcriptional response in vitro (lines 219-221) which suggests that the response is from the tumor microenvironment. However, Extended Data Fig. 4D appears to show enriched IFN-a and IFN-g from the cancer cells in the scRNA-seq analysis of Myc-CaP IRE1α knockout vs. Myc-CaP wildtype tumors. This discrepancy should be addressed/discussed.

In Extended Data Fig. 4D (In the revised manuscript Extended Data Fig. 6B), we presented an analysis showing the enrichment of signaling pathways in various cell types within the TME. While this analysis indicated a deregulation of IFN-γ signaling in cancer cells, it is crucial to clarify that this does not necessarily imply that these cancer cells are the primary source of IFN-γ.

Our scRNA-Seq data provide a more detailed perspective on this issue. According to these data, IFN-γ expression was not detected in most cell types within the TME, including the cancer cells. Instead, the primary sources of IFN-γ expression were NK cells and CD8+ T cells. This finding aligns with the known biology of the antitumor immune response, where NK cells and CD8+ T cells are typically the primary producers of IFN-γ in the TME. Furthermore, cancer cells did not express IFN genes **(Rebuttal Letter Figure 2)**.



Rebuttal Letter Figure 2
Expression of IFN genes in various cell types identified in the TME from WT Myc-CaP tumors scRNA-Seq data.

Therefore, the observed IFN signaling deregulation in cancer cells, as seen in the scRNA-Seq analysis, could be a response to IFNs produced by other immune cells rather than a direct transcriptional activity of the cancer cells themselves.

2. The conclusions may be overstated as they are based on manipulation of a single model (Myc-CaP) on one mouse genetic background (FVB). This needs to be strengthened by complementary experiments in other mouse prostate cancer models on other mouse genetic backgrounds (i.e., RM9 or TRAMPC2 on BL6).

We thank the Reviewer for this feedback. In the revised manuscript, we have now used two additional syngeneic mouse models, one in the FVB background (Myc-CaP PTEN KO) and another in the BALB/c background (RM-1) and obtained similar results on the efficacy of IRE1α inhibition and synergy with anti-PD-1 blockade on tumor growth **(Fig. 5 and 6)**. In addition, we now provide scRNA-Seq data from the Myc-CaP PTEN KO model for the significant therapeutic responses induced by MKC8866 + anti-PD-1 combination **(Fig. 7)**. Inclusion of these additional data significantly strengthened our conclusions on the role of IRE1α in shaping the PCa TME.

3. In Fig. 4F and 4H, normalization of the proportions of immune cells in including macrophages to total Cd45+ cells (rather than to total cells) should be considered especially because the absolute differences are small and could be impacted by changes in other cell populations.

We thank the Reviewer for this suggestion. After careful consideration, we would like to maintain the normalization to the total number of cells. The reason for this is that normalizing the counts to total CD45+ cells could potentially lead to misinterpretation of the data, especially given the relatively small absolute differences in immune cell populations. Such normalization might inadvertently give the impression that the abundance of other immune cells has significantly increased, which would not

accurately reflect the actual changes within the TME. We believe that normalization to the total number of cells provides a more accurate representation of the relative proportions and changes in immune cell populations for a clear and precise understanding of the data.

4. Mechanistic investigation of why concurrent MKC8866 and anti-PD-1 therapy induces substantial therapeutic responses as shown in Fig. 5E is lacking. What happens to the tumor immune microenvironment in this context? IHC from tumors.

In the revised manuscript, we now provide scRNA-seq data for the MKC8866 + anti-PD1 therapy **(Figure 7)**. Also, please see our response above to a similar comment from Reviewer 1 on this point.

5. There is a disconnect between the absence of differences found in NK and T cell frequencies between Myc-CaP wildtype and Myc-CaP IRE1α knockout tumors relative to what is suggested as a correlation between IRE1α expression levels and NK and T cell infiltration in human prostate cancer patient data. Can be further supported with IHC.

We thank the Reviewer for this question. In our scRNA-seq data, we indeed identified a cluster that contained both T and NK cells. The percentage of this cluster did not show significant differences between knockout and wild type tumors. However, upon subclustering of this cluster, we identified six distinct cell-type populations. Among these populations, three were T cell subtypes, where the relative percentage of CD8+ T cells and NK cells increased, while Tregs and proliferating T cells decreased in KO tumors.

In the revised manuscript, we have now included another scRNA-Seq dataset, on Myc-CaP PTEN KO model, where we also observed an increase in the abundance of NK Cells and CD8+ T Cells upon MKC8866 treatment. The abundance of these cells was further increased when MKC8866 was combined with anti-PD-1 treatment **(Fig.7)**.

These observations suggest that our scRNA-seq data are consistent with human PCa patient data regarding the presence of NK and T cell subtypes.

6. Related to point #5, the authors are reliant on various inferred data analyses of bulk RNA-seq from human prostate cancer patient data to argue a variety of associations. This would be significantly strengthened by validation of these associations by IHC.

We agree with the Reviewer that this would be a very good validation of our current results. We have initiated these experiments, but due to technical issues we do not have reliable data at present. Nevertheless, this is an important experiment for the future.

7. It is unclear why the authors use TCGA (primary prostate cancer) to develop/apply a TAM gene signature and how this relates to the evaluation of MKC8866, and anti-PD-1 therapy. It would be much more appropriate and informative to develop this in the context of advanced prostate cancer where these therapies would reasonably be applied.

We thank the Reviewer for this feedback. While the ideal scenario would be to utilize datasets from advanced PCa, practical limitations exist in terms of availability and comprehensiveness of these datasets, which often have a smaller sample size and lack detailed clinical annotations, such as survival data. Furthermore, samples from advanced stages, typically from distant metastases, may show varying signaling pathway activities due to the influence of the metastatic site.

Given these challenges, we opted for the TCGA dataset for its larger sample size and well-annotated clinical information, allowing for more robust analyses. We correlated our TAM signature with increasing Gleason scores and survival outcomes in primary PCa cases (**Fig. 8F and G**).

Recognizing the importance of examining advanced PCa stages, we now extended our analysis to the PCa Transcription Atlas dataset, which includes castration resistant PCa (CRPC) and neuroendocrine PCa (NEPC) cases. This analysis indicated an increase in the TAM signature during PCa progression, with CRPC samples exhibiting a higher TAM signature than primary samples, whereas in NEPC there was no significant difference **(Fig. 8E)**. These data are now incorporated into the revised manuscript.

Additionally, we have now incorporated scRNA-seq data from MKC8866 + anti-PD-1 combination treatment in the tumors from PTEN KO advanced PCa model. These data show that the expression of TAM signature genes was increased in the macrophage cluster in PTEN KO tumors compared to wild type **(Extended Data Fig. 10C)**. Interestingly, in these tumors, combination therapy reduced the expression of TAM signature genes **(Extended Data Fig. 10D)**.

Minor points:

In Fig. 1C, it is unclear how many samples are Gleason 6 vs. Gleason 7-10.

We thank the Reviewer for pointing this out. The figure legend has now been updated to clearly indicate the number of samples that are Gleason 6 versus Gleason 7-10.

2. In Fig. 4E, the authors should note the number of tumors and number of cells displayed within the UMAP plots.

This is now done. We thank the Reviewer for pointing this out.

3. In line 337 of the text, the authors describe "xenografted tumor growth" but this should be "syngeneic tumor growth."

This is now corrected. We thank the Reviewer for pointing this out.

***Reviewer #3**: with expertise in prostate cancer*

The manuscript, "Targeting IRE1α alleviates the immunosuppressive tumor microenvironment in prostate cancer" by Unal et al, addresses mechanisms of activation of the unfolded protein response pathway (UPR) on immunologic features of prostate cancer and develop therapeutic strategies. Authors show that IRE1α protein was upregulated in prostate tumors in comparison to benign prostate gland and increased levels of the IRE1α proteins associated with higher Gleason score. Overexpression of IRE1α and XBP1 was also shown in TCGA-prostate cancer dataset and higher XBP1 expression correlated with increased expression of cancer hallmarks genes. The IRE1α knock-out in a prostate cancer cell line derived from prostate targeted C-Myc-transgenic mice and syngeneic prostate cancer mouse model from this cell line was extensively used to evaluate both tumor biology and immunology. The IRE1α KO/Myc-CaP cells showed markedly reduced tumor growth only in immune competent mice as compared to growth in nude mice or in cell culture. RNA-Seq data from the IRE1α-KO/Myc-CaP prostate tumors in immune competent mice showed down regulation of UPR gene expression signature and increased expression of genes associating with IFN-γ signaling and immune functions related to B cells, T cells and NK cells. Single cell RNA-seq data from these tumors showed marked reduction in tumor associated macrophages (TAM), T-regs and cancer associated fibroblasts. In TCGA prostate cancer dataset increased IRE1α expression correlated with increased TAM gene expression markers. The evaluation of a TAM gene expression signature defined from this study showed a strong association with poor survival and disease progression in the TCGA- prostate cancer dataset. In the Myc-CaP prostate tumor mouse model IRE1α inhibitor, MKC8866 showed synergy with anti-PD1 therapy.

Overall, this manuscript presents compelling data for the role of IRE1α in suppressing prostate tumor immune microenvironment from a complementary set of experiments utilizing mouse model and human prostate cancer. These findings are concordant with suggested roles of UPR/IRE1α -XBP1 pathway in modulating immune functions.

Response: We thank the Reviewer for the overall positive feedback on our manuscript and finding our data compelling.

Questions/clarification

While the IRE1α-KO/Myc-CaP prostate tumor model used in this report has provided promising data, the cancer genomic and immunologic contexts are expected to be lot more complex and heterogenous in human prostate cancer, especially in metastatic prostate cancer context. Although the PD1/PDL1 blockade therapy has not been successful in human prostate cancer, the data shown in the mouse model exhibits significant antitumor activity (Figure 6F). The authors may discuss possible limitations while translating these data for human prostate cancer treatment.

We thank the Reviewer for bringing up this point. As described above, we have now validated our main observation in two additional syngeneic models (one in the FVB background, the Myc-CaP PTEN KO, and another in the BALB/c background, RM-1) **(Fig. 5 and 6)**, as well as an orthotopic model of PCa **(Extended Data Fig. 3E, F)**, as discussed above. Taken together, these data suggest that our findings are robust and relevant for different PCa contexts.

Is IRE1α increased expression restricted to epithelial cells in human prostate tumors? What percent of Gleason 7-10 prostate tumors show high expression of IRE1α (Figure 1D)? Similar results may be provided for CD68 and IRE1α in Figure 6.

We thank the Reviewer for these points. Regarding the specificity of IRE1α expression in epithelial cells of human prostate tumors, as shown in Figure 1A, IRE1α is predominantly in glandular cells. This observation aligns with the data from the Prostate Cell Atlas, which includes single-cell RNA sequencing (scRNA-Seq) data from both normal and cancerous prostate tissues (https://www.prostatecellatlas.org/). In this dataset, IRE1α was observed in 50 out of 1325 luminal cells in normal prostate tissue, compared to 161 out of 2500 luminal cells in PCa samples, indicating a notable increase in expression in cancerous luminal cells.

In Figure 1D (now Figure 1C in the revised manuscript), among the 64 Gleason score 7-10 tumor samples analyzed, approximately 47% (30 samples) exhibited high IRE1α expression. We have updated the figure legends to include specific quantitative data as requested.

TAM prognostic gene signature appears to be robust. An IHC assay may be more useful given the complexity of TME evaluations in clinical specimens.

We thank the Reviewer for this suggestion. An IHC assay would indeed be useful especially to interrogate the potential translational value of the TAM signature. This, however, depends on very specific and reliable antisera for all the proteins encoded by the genes in the TAM signature for multiplex IHC, which unfortunately is not available at present.

Reviewer #4 *(Remarks to the Author): with expertise in IRE1α, ER stress*

In this manuscript Unal and colleagues very elegantly document the role of IRE1 signaling in prostate cancer cells and the impact on the tumor microenvironment (in particular the immune infiltrate) and evaluate the impact of IRE1 pharmacological inhibition on the response of those tumors to immunotherapy. This is a very solid work that will be of great value to the prostate cancer (and more extensively to the cancer) community.

We thank the Reviewer for the positive comments on our manuscript.

Comments on the manuscript:

1) The authors justify their study by the fact that IRE1 expression is elevated in prostate tumors. However, the expression of IRE1 (evaluated by immunohistochemistry (IHC)) appears to be highly heterogeneous.
a. Is there any clinical feature associated with IRE1high tumors vs. IRE1low tumors? (e.g. expression of hormone receptors etc…)

We thank the Reviewer for this question. In Figure 1C, we have already demonstrated a correlation between IRE1α protein levels and the Gleason score of the samples, indicating a potential association with tumor aggressiveness. In addition, in the TCGA dataset, we have observed that androgen receptor (AR) expression is highly correlated with IRE1α expression (rs = 0.87) and AR protein levels were higher in high IRE1α expressing samples. This was consistent with our IHC results where we observed increased nuclear AR levels with increasing IRE1α levels; this is consistent with our observation that IRE1α expression was higher in AR expressing PCa cell lines (see our response to comment #3 below). Interestingly, expression of canonical AR target genes such as KLK3, TMPRSS2, and FKBP5, showed little to no correlation with IRE1α expression. In contrast, ERN1-correlated genes were highly enriched for mitochondria-localized and cellular respiration-associated genes. Further insights from the TCGA dataset, particularly from RPPA (Reverse Phase Protein Array) data, suggest that the phosphorylation levels of AKT and ERK increase with the mRNA levels of IRE1α. In addition, we also observed a high correlation between mRNA levels of ERN1 and HIF1A (the alpha subunit of transcription factor hypoxia-inducible factor-1), which may suggest a potential interplay between the IRE1 signaling pathway and cellular hypoxic responses in PCa. We now revised the results section based on the new findings (**Extended Data Fig. 1)**.

In summary, our findings highlight a complex interplay between IRE1α expression and various molecular pathways in PCa. While there is a clear association with the Gleason score and IRE1α expression, the exact role of IRE1α in modulating these pathways warrants further investigation.

b. Does IRE1 protein expression correlate with IRE1 mRNA expression? If so the authors could identify genes exhibiting similar or opposite expression patterns than IRE1 and evaluate the functional enrichments in those gene sets.

We thank the Reviewer for this comment. Unfortunately, there is a lack of publicly available PCa datasets that allow for a direct analysis of the correlation between mRNA and protein levels of IRE1α in patient samples. However, according to the cProSite database (Cancer Proteogenomic Data Analysis Site), we note that in breast and lung adenocarcinomas, there is a moderate correlation between ERN1 mRNA and IRE1α protein levels (r ~0.6 and r ~0.46, respectively), while this correlation is substantially weaker in pancreatic and kidney carcinoma (r ~0.07 and r ~0.18, respectively).

To explore this further in PCa, we have utilized a gene signature approach, identifying a set of genes that are upregulated by Thapsigargin or Tunicamycin (ER stress inducers) in an IRE1α-dependent manner, as reported in prior literature [8]. In the TCGA dataset, we observed that approximately half of these 18 genes positively correlate with ERN1 mRNA levels, while the rest show a negative correlation **(Rebuttal Letter Figure 3)**. Interestingly, in the SU2C dataset, which includes 150 metastatic CRPC samples, the majority of IRE1α downstream targets (13 out of 18) positively correlated with ERN1, with none showing a negative correlation [9]. This suggests that ERN1 mRNA expression may have a more pronounced influence on IRE1α activity in metastatic PCa compared to primary tumors. However, we must consider the variation in the TME in metastatic samples, based on the host tissue, as a potential confounding factor.

Rebuttal Letter Figure 3

IRE1α dependent genes	ERN1 Correlation	
	Primary	Metastatic
MTDH	0.57	0.40
TMED2	0.32	0.57
ERLEC1	0.31	0.43
SSR3	0.27	0.24
SSR1	0.24	0.45
ARMCX3	0.23	0.33
DNAJB9	0.22	0.39
SERP1	-0.01	0.53
SEC61A1	-0.03	0.56
OSTC	-0.13	0.04
TIMM17A	-0.25	0.06
SELENOS	-0.32	NA
NANS	-0.34	0.37
TMED9	-0.36	0.39
SSR2	-0.36	0.23
MYDGF	-0.42	0.35
PPIB	-0.45	0.10
SEC61B	-0.52	0.08

The table presents spearman correlation coefficient values between ERN1 and indicated IRE1α dependent genes in the TCGA (primary) or SU2C (metastatic CRPC) datasets.

Furthermore, we analyzed genes correlating with ERN1 in both the TCGA and SU2C datasets. The top 500 correlating genes in the TCGA dataset were primarily associated with mitochondrial pathways, whereas in the SU2C dataset, they were linked to stress-related pathways. Notably, 131 genes common to both datasets were enriched for stress-related signaling.

We believe all of these findings underscore the complex relationship between ERN1 mRNA and IRE1α protein expression and their potential impact on PCa pathophysiology. These observations warrant further investigation, particularly in the context of the diverse TMEs of metastatic PCa.

c. What are the characteristics of IRE1 expression in tumor cells (for instance markers of hallmarks of cancer)?

We thank the Reviewer for this question. Utilizing the PCa Transcription Atlas dataset (www.thepcta.org), which encompasses a diverse range of PCa samples including 174 normal, 714 primary PCa, 316 CRPC, and 19 NEPC samples, we observed that IRE1α mRNA levels did not show significant variation across most sample types. However, an exception was noted in NEPC samples, where there was an increase in IRE1α mRNA levels. On the other hand, expression of certain cancer associated genes and signaling pathways, such as AR and AKT signaling, were correlated with IRE1α levels. Some of these were discussed above as a response to 1a.

d. The expression of IRE1 appears undetectable in normal prostate tissue, does that mean that prostate cells (epithelial) do not express IRE1? Or is that just that IHC is not sensitive enough to detect low levels of IRE1.

We thank the Reviewer for this question. Since the prostate is a secretory organ, IRE1α is expressed and is functional. Previous studies have shown that androgen deprivation therapies downregulate the expression of IRE1α-XBP1s target genes indicating its role in normal functioning of the prostate gland [10, 11]. We therefore conclude that with the antiserum that we use and under these conditions, IHC is not sensitive enough to detect the IRE1α expression in normal prostate glands. Also, as we have noted before, publicly available scRNA-Seq datasets of prostate show expression of IRE1α in epithelial cells, which was increased in PCa. These data suggest that while IRE1α is expressed in normal prostate epithelial cells, its levels may be too low for detection through our current IHC methodology. In the revised version, we now further confirmed our observation in an independent PCa cohort where IRE1α expression was significantly increased in PCa samples compared to adjacent normal tissues **(Fig. 1D)**.

e. IRE1 expression levels have been shown to be linked to its signaling outcomes depending either on catalytic or non-catalytic (scaffold) mechanisms, have the authors considered those aspects?

We thank the Reviewer for this question. Studies have indeed revealed that IRE1α, beyond its well-known role in the unfolded protein response, possesses several non-catalytic functions [12]. This includes its function as a structural determinant of mitochondria-associated membranes, crucial for calcium transfer and energy metabolism [13]. Additionally, IRE1α's involvement in cytoskeleton dynamics and cell migration, independent of its canonical role, further underscores its diverse non-catalytic activities [14]. While these aspects are potentially of significance, our current focus has been on catalytic functions of IRE1a. Certainly, future research should consider the non-catalytic aspects, and this has now been pointed out in the Discussion.

2) To further document the signaling mechanisms triggered in prostate cancer cells in correlation with IRE1 overexpression.

The following three questions relate to the similar points and addressed together:

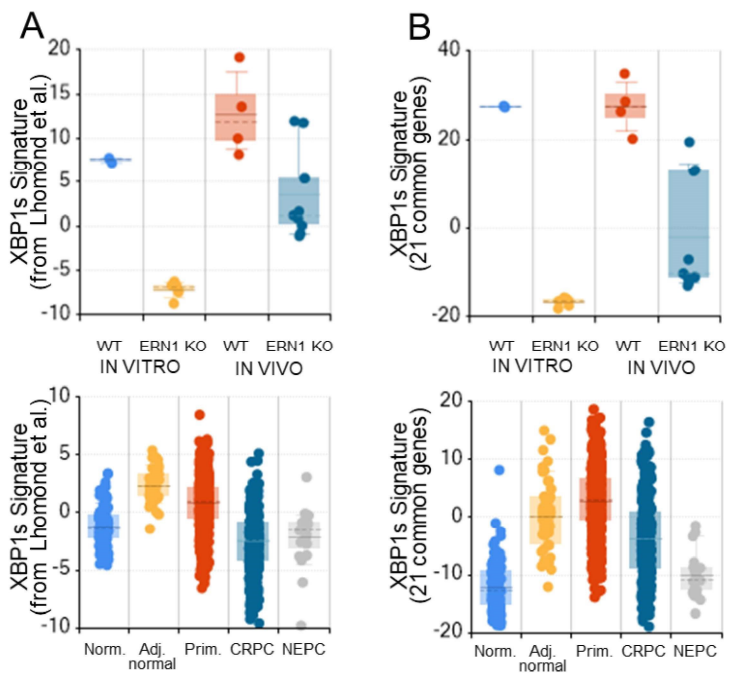
a. The authors rely on publicly available datasets to evaluate an UPR signature (the list of genes composing this signature should be provided) and jump from these results to XBP1 mRNA spliced forms without clear articulation. To document the link between IRE1 high expression and XBP1 mRNA splicing the authors should i) evaluate the expression of XBP1s protein in their prostate cancer sections, ii) if IRE1 protein and mRNA expression is correlated, then show the correlation between XBP1s mRNA and IRE1 mRNA in public datasets.

b. Again, based on this, the expression of XBP1s target genes (see https://chip-atlas.org/) could easily be correlated.

c. In the UPR signature used in fig 1D, what is the IRE1 specific component (and by extend, what are the PERK and ATF6 components)?

We thank the Reviewer for this query. In this analysis, we used the Hallmark UPR gene set from MsigDB, comprising 113 genes identified as upregulated during UPR [15]. This set was derived from 22 founder gene lists from experiments involving all three UPR arms. Specifically, the IRE1α component was represented by several known XBP1s targets, including DNAJB9, SERP1, SSR1, EDEM1, PDIA5, PDIA6, HSPA5, and DNAJC3. In the PCa Transcription Atlas dataset, in contrast to normal prostate tissue, all of these genes were significantly upregulated in primary PCa samples, CRPC tumors as well as tumor adjacent normal tissues. Notably, there was no significant difference between primary PCa samples and adjacent normal tissue, but a slight decrease in CRPC samples compared to primary ones. Remarkably, expression in NEPC samples was significantly downregulated compared to primary or CRPC tumors.

Rebuttal Letter Figure 4



Two distinct XBP1s signature scores were analyzed for the PCa Transcription Atlas dataset and our RNA-Seq data from both in vitro cell cultures and in vivo tumor samples of wild-type and IRE1α knockout cells. The PCa transcription atlas dataset contains 116 normal, 52 adjacent normal, 714 primary PCa, 316 CRPC, and 19 NEPC samples.

Furthermore, we investigated a XBP1s signature based on a

previous publication [16] **(Rebuttal Letter Figure 4A)**. In our RNA-Seq data for wild-type and IRE1α knockout Myc-CaP cells, we observed significant downregulation of this signature *in vitro* in knock-out cells. Although most knockout tumors showed decreased XBP1s scores *in vivo*, two tumors did not exhibit a significant decrease. In the PCa patient samples, XBP1s signature was elevated in PCa tissue compared to normal counterparts, with a decreasing trend from tumor-adjacent normal tissue to primary tumors, and then to CRPC tumors. Given that these scores were initially generated using glioblastoma cells, we developed a PCa-specific XBP1s signature. This new signature was established based on three PCa datasets: genes downregulated upon XBP1 knockdown or MKC8866 treatment in the thapsigargin-treated LNCaP cells, genes down regulated in IRE1 KO Myc-CaP cells, and XBP1s binding sites from the ChIP Atlas database; the resulting XBP1s signature comprised 21 common genes. In our RNA-Seq data, this score was more strongly decreased in IRE1 KO samples, in both *in vitro* and *in vivo* experiments. In contrast to the previous XBP1s signature, scoring based on this new signature indicated that XBP1s signaling was upregulated in PCa, with an increase in primary tumors compared to adjacent normal tissue, and a slight decrease in mCRPC samples relative to primary tumors **(Rebuttal Letter Figure 4B)**. Since the differences between the two signatures need to be analyzed in more detail, we choose to not present these data in the revised manuscript.

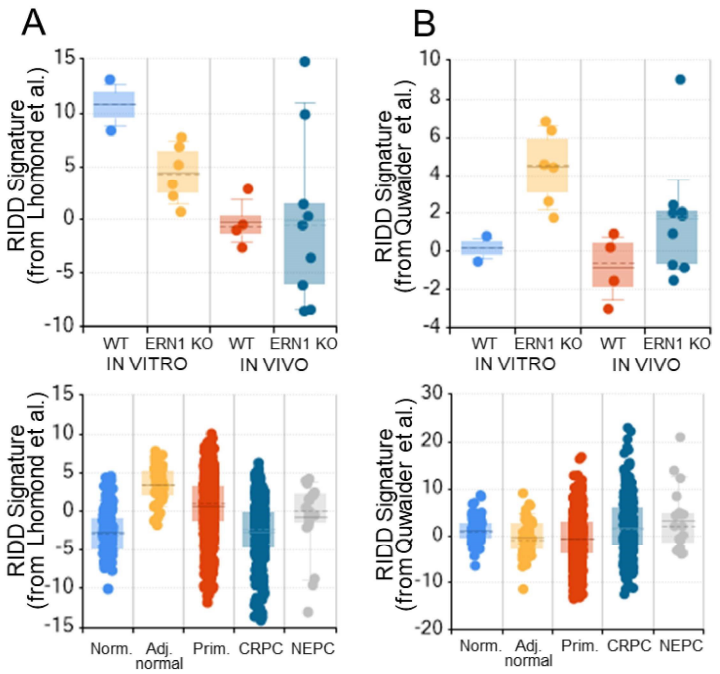
d. If the activation of both PERK and ATF6 components are also found elevated in prostate tumors, what is the reason for the authors to privilege IRE1 signaling over the two other UPR arms?

In our original evaluation of the UPR arms and their potential links to PCa, we found that only IRE1α and PERK arms were significantly deregulated [17]. Our subsequent work therefore focused on both of these pathways [18, 19, 20]. We are continuing to characterize the PERK pathway, similar to that for IRE1α signaling in this manuscript, and find that the PERK signaling also significantly affects the TME (unpublished data).

e. From the data presented in this manuscript, the authors solely focus on the IRE1/XBP1s axis, however, it is relatively well documented that IRE1 overexpression is sufficient to also yield activation of the RIDD pathway. As such, it would be of great interest to evaluate how RIDD targets behave in prostate cancer cells (wt or ko for IRE1 or treated with the IRE1 RNase inhibitor) and how this could actually impact on the behavior of those cells – in the same line of idea, the expression of identified RIDD targets could be tested in silico in publicly available datasets.

We thank the Reviewer for this suggestion. We utilized a RIDD gene signature [16] to assess possible changes in RIDD activity upon IRE1α targeting in both our RNA-seq datasets and publicly available PCa datasets **(Rebuttal Letter Figure 5A).** Surprisingly, we observed a decrease in RIDD activity in ERN1 knockout Myc-CaP cells compared to wild type cells *in vitro,* but there were no differences *in vivo*.

Rebuttal Letter Figure 5



Two distinct RIDD signature scores were analyzed for the PCa Transcription Atlas dataset and our RNA-Seq data from both in vitro cell cultures and in vivo tumor samples of wild-type and IRE1α knockout cells. The PCa transcription atlas dataset contains 116 normal, 52 adjacent normal, 714 primary PCa, 316 CRPC, and 19 NEPC samples.

In the PCa Transcription Atlas dataset, we observed an elevated RIDD signature in PCa compared to normal prostate, which then decreased in mCRPC samples. However, since this signature was originally designed for glioblastoma, we also evaluated an alternative RIDD signature developed for multiple myeloma **(Rebuttal Letter Figure 5B)** [21]. This gene signature showed an opposite pattern in our RNA-Seq data: in contrast to wild-type cells, scores were significantly higher in IRE1α KO cells, and a similar trend was observed in tumor samples. Notably, in PCa patient samples, this score did not exhibit a prominent change across different groups.

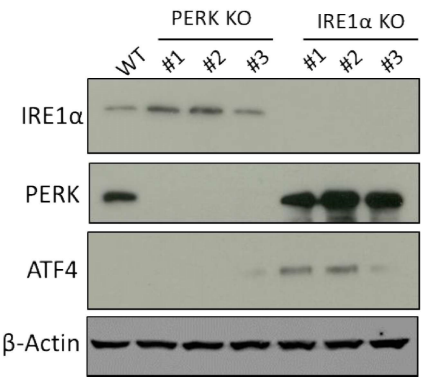
In light of these contrasting patterns in the data and the fact that these signatures were initially developed in different contexts, their applicability to PCa appear to be limited; we thus choose not to present these data in the manuscript but discussed these observations in the Discussion section. Further work is warranted, however, to evaluate the potential role of the RIDD pathway in PCa.

In the revised manuscript, we do present data which suggest that it is the IRE1α-XBP1s pathway, and not RIDD, which is of prime importance in PCa, at least in our model systems. To evaluate whether the reduction in tumor growth was attributable to the IRE1α-XBP1s signaling axis, we ectopically expressed XBP1s in Myc-CaP IRE1α KO #1 cells, which showed the greatest reduction in tumor growth. XBP1s overexpression in IRE1α KO cells rescued the expression of IRE1α-XBP1s target genes (EDEM1, p58IPK, and PDIA6) *in vitro* indicating its functionality **(Extended Data, Fig. 3G).** Importantly, ectopic XBP1s expression fully rescued the growth of IRE1α KO tumors *in vivo* **(Extended Data Fig. 3H).**

f. It appears from gene and protein expression profiles that the expression of several components of the Integrated Stress Response is altered upon blunting IRE1 expression, how does this impact on tumor cells' phenotypes?

We thank the Reviewer to bring this up. Indeed, there is compensatory crosstalk between the IRE1α and PERK arms of the UPR [22, 23, 24] and this is what we observe in our models, including the Myc-CaP model **(Rebuttal Letter Figure 6).** However, we feel that proper examination of the individual and collective contributions of the IRE1α and PERK arms of UPR requires an independent study, which is currently under way in our laboratory, and thus we prefer not to present these data in this manuscript.

Rebuttal Letter Figure 6



Western blot showing increased PERK expression in IRE1α knockout, and increased IRE1α expression in PERK knockout Myc-CaP cells.

g. IRE1 phosphorylation is key for the modulation of IRE1 signaling, do the authors have any information regarding IRE1 phosphorylation status in prostate tumors?

We checked IRE1α phosphorylation in protein samples from PCa patients. However, we did not observe any differences between PCa and benign matched adjacent tissues, suggesting that phospho-IRE1α itself may not have prognostic significance compared to total IRE1α. This requires further investigation in larger data sets and we thus prefer not to present these negative data in the manuscript.

h. At last, scaffolding functions of IRE1 appear to contribute to IRE1 unconventional signaling, do the authors have any information that could document this aspect in prostate tumors?

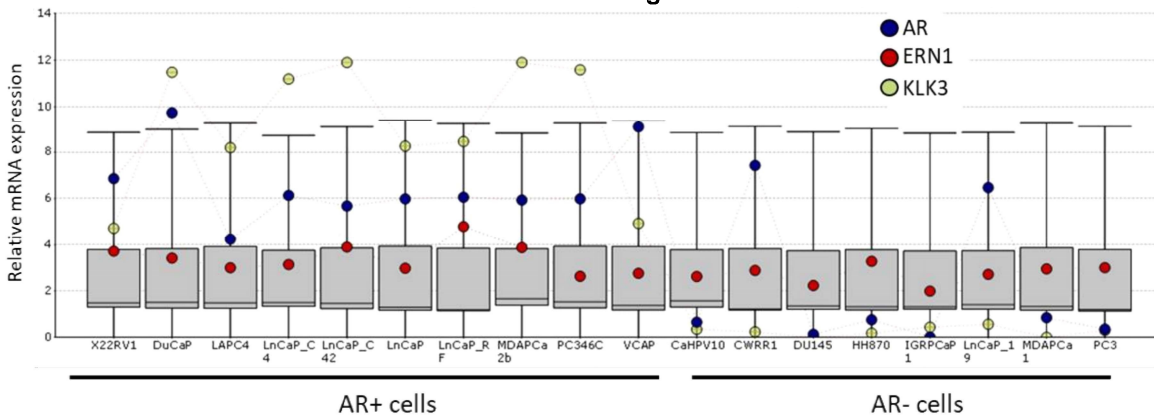
We thank the Reviewer for this pertinent question. As we partially addressed in response to comment 1e, IRE1α has been demonstrated to have cellular roles that extend beyond its enzymatic functions, operating via mechanisms involving interactions with other proteins. For example, mitochondria-associated membranes (MAMs) orchestrate bioenergetics through calcium transfer from the ER to mitochondria. Independent of its enzymatic activity, IRE1α was shown to act as a structural determinant within MAMs by interacting with inositol-1,4,5-trisphosphate (InsP3) receptors (InsP3Rs) to regulate mitochondrial calcium uptake [13]. Mitochondrial Ca^{2+} levels play a crucial role in regulating various aspects of mitochondrial function, including oxidative phosphorylation [25]. In IRE1 KO tumors and upon MKC8866 treatment, we observed that expression of genes regulating oxidative phosphorylation was significantly downregulated **(Figure 3F, Extend Data Fig. 4D, Fig. 7D)**, which could be due to dysregulated Ca^{2+} levels in the mitochondria. Moreover, we observed

that there was an increased NADH/NAD+ ratio in IRE1α KO tumors, which can inhibit the flow of electrons through the electron transport chain (ETC), potentially slowing down oxidative phosphorylation and ATP synthesis. An imbalanced NADH/NAD^+ ratio may increase the likelihood of electron leakage in the electron transport chain, leading to the generation of reactive oxygen species (ROS). Excessive ROS production can contribute to oxidative stress and mitochondrial damage. These effects could be also due to conventional roles of IRE1 signaling in UPR as well. We added some of these points to the Discussion and noted that this requires further investigation.

3) ***Since the rational for this study relies on the expression levels (and subsequent signaling of IRE1), the authors should provide information on their choices regarding the selection of the studied cell lines. Were "basal" expression levels of IRE1 documented for those lines? It would be interesting to identify whether low and high expression lines do exist. In the present study the authors have blunted IRE1 expression or activity, what about overexpressing IRE1 and evaluating whether the observed phenotype is in line with what is found in tumor samples.***

We thank the Reviewer for these points. IRE1α is ubiquitously expressed across a spectrum of human cell lines commonly utilized in PCa research without significant changes (no clearly low or high expressors) **(Rebuttal Letter Figure 7).** We agree that IRE1α ectopic expression, in addition to our XBP1s rescue experiments described above, would be a very good approach. We in fact tried to generate these lines, but for technical reasons failed to obtain them to date. However, we feel that the XBP1s rescue experiment is very convincing that the phenotype that we observe is related to the IRE1α-XBP1s signaling pathway.

Rebuttal Letter Figure 7



mRNA levels of ERN1, AR and KLK3 (a canonical AR target gene) is shown for various PCa cell lines. The histograms shows distribution of the expression of all detected genes in these cells. Note that, ERN1 is ubiquitously expressed across all cell lines, but relatively decreased in AR negative (in terms of activity) cell lines. Data obtained from Smith et al. [1].

4) ***Mouse models – the authors have used both syngeneic and xenogenic models to evaluate the impact of IRE1 signaling alteration on their phenotypes, however, the implantation system selected is heterotopic which might be of importance regarding the nature of the immune infiltrate. To demonstrate that the same effects are observed in orthotopic models, the authors could, instead of repeating the same experiments but in another model, test the hypothesis (e.g. using qPCR, IHC or FACS analyses) in a prostate cancer orthotopic model. In these models it might also be worth it to test other IRE1 inhibitors that do not covalently bind to IRE1 which could then represent a strong confirmatory approach for the preclinical proof of concept.***

We thank the Reviewer for these important points. We acknowledge that our current approach using heterotopic implantation systems may have limitations, particularly concerning the nature of the immune infiltrate and the TME. This is a critical aspect that we have discussed in our response to Reviewer 1. While we recognize the importance of testing IRE1α inhibitors that do not covalently bind to IRE1α, our current resources and access to such compounds are limited. This is an area we are actively exploring, as we agree that it could provide a strong confirmatory approach for the preclinical proof of concept. We would like to suggest that this should be the topic of future investigations.

REFERENCES

1. Smith R*, et al.* Enzalutamide response in a panel of prostate cancer cell lines reveals a role for glucocorticoid receptor in enzalutamide resistant disease. *Sci Rep* **10**, 21750 (2020).
2. Molgora M*, et al.* TREM2 Modulation Remodels the Tumor Myeloid Landscape Enhancing Anti-PD-1 Immunotherapy. *Cell* **182**, 886-900 e817 (2020).
3. Binnewies M*, et al.* Targeting TREM2 on tumor-associated macrophages enhances immunotherapy. *Cell Rep* **37**, 109844 (2021).
4. Nasir I, McGuinness C, Poh AR, Ernst M, Darcy PK, Britt KL. Tumor macrophage functional heterogeneity can inform the development of novel cancer therapies. *Trends Immunol* **44**, 971-985 (2023).
5. Batista A*, et al.* IRE1alpha regulates macrophage polarization, PD-L1 expression, and tumor survival. *PLoS Biol* **18**, e3000687 (2020).
6. Shan B*, et al.* The metabolic ER stress sensor IRE1alpha suppresses alternative activation of macrophages and impairs energy expenditure in obesity. *Nat Immunol* **18**, 519-529 (2017).
7. Soto-Pantoja DR, Wilson AS, Clear KY, Westwood B, Triozzi PL, Cook KL. Unfolded protein response signaling impacts macrophage polarity to modulate breast cancer cell clearance and melanoma immune checkpoint therapy responsiveness. *Oncotarget* **8**, 80545-80559 (2017).
8. Adamson B*, et al.* A Multiplexed Single-Cell CRISPR Screening Platform Enables Systematic Dissection of the Unfolded Protein Response. *Cell* **167**, 1867-1882 e1821 (2016).

9. Robinson D*, et al.* Integrative clinical genomics of advanced prostate cancer. *Cell* **161**, 1215-1228 (2015).

10. Yang F, Yuan C, Wu D, Zhang J, Zhou X. IRE1alpha Expedites the Progression of Castration-Resistant Prostate Cancers via the Positive Feedback Loop of IRE1alpha/IL-6/AR. *Front Oncol* **11**, 671141 (2021).

11. Doultsinos D, Mills I. The role of the androgen receptor as a driver and mitigator of cellular stress. *J Mol Endocrinol* **65**, R19-R33 (2020).

12. Le Goupil S, Laprade H, Aubry M, Chevet E. Exploring the IRE1 interactome: From canonical signaling functions to unexpected roles. *J Biol Chem* **300**, 107169 (2024).

13. Carreras-Sureda A*, et al.* Non-canonical function of IRE1alpha determines mitochondria-associated endoplasmic reticulum composition to control calcium transfer and bioenergetics. *Nat Cell Biol* **21**, 755-767 (2019).

14. Urra H*, et al.* IRE1alpha governs cytoskeleton remodelling and cell migration through a direct interaction with filamin A. *Nat Cell Biol* **20**, 942-953 (2018).

15. Liberzon A, Birger C, Thorvaldsdottir H, Ghandi M, Mesirov JP, Tamayo P. The Molecular Signatures Database (MSigDB) hallmark gene set collection. *Cell Syst* **1**, 417-425 (2015).

16. Lhomond S*, et al.* Dual IRE1 RNase functions dictate glioblastoma development. *EMBO Mol Med* **10**, (2018).

17. Sheng X*, et al.* Divergent androgen regulation of unfolded protein response pathways drives prostate cancer. *EMBO Mol Med* **7**, 788-801 (2015).

18. Pallmann N*, et al.* Regulation of the unfolded protein response through ATF4 and FAM129A in prostate cancer. *Oncogene* **38**, 6301-6318 (2019).

19. Pallmann N*, et al.* Stress-Mediated Reprogramming of Prostate Cancer One-Carbon Cycle Drives Disease Progression. *Cancer Res* **81**, 4066-4078 (2021).

20. Sheng X*, et al.* IRE1alpha-XBP1s pathway promotes prostate cancer by activating c-MYC signaling. *Nat Commun* **10**, 323 (2019).

21. Quwaider D*, et al.* RNA sequencing identifies novel regulated IRE1-dependent decay targets that affect multiple myeloma survival and proliferation. *Exp Hematol Oncol* **11**, 18 (2022).

22. Moore PC*, et al.* Parallel Signaling through IRE1alpha and PERK Regulates Pancreatic Neuroendocrine Tumor Growth and Survival. *Cancer Res* **79**, 6190-6203 (2019).

23. Chang TK*, et al.* Coordination between Two Branches of the Unfolded Protein Response Determines Apoptotic Cell Fate. *Mol Cell* **71**, 629-636 e625 (2018).

24. Yap WS, Thibault G. Human PERK rescues unfolded protein response-deficient yeast cells. *MicroPubl Biol* **2022**, (2022).

25. Vercesi AE*, et al.* Mitochondrial calcium transport and the redox nature of the calcium-induced membrane permeability transition. *Free Radic Biol Med* **129**, 1-24 (2018).

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have partially addressed my major concerns. Although they offer explanations for each point raised, the new data presented remains largely descriptive, and the overall narrative lacks substantial innovation. The clinical trials involving MKC8866 highlight the potential of targeting IRE1 as a viable strategy for enhancing prostate cancer treatment, particularly when combined with PD-1 blockade. This aspect of the paper is noteworthy. However, it is important to acknowledge that the concept of targeting the unfolded protein response to sensitize various cancer types to checkpoint inhibitors is already well-established, diminishing the overall novelty of the study.

Reviewer #2 (Remarks to the Author):

The authors have adequately addressed my concerns.

Reviewer #3 (Remarks to the Author):

[**editorial note**: box left blank.]

Reviewer #4 (Remarks to the Author):

The authors revised thoroughly their manuscript and addressed the major points raised in my previous critique of their work. I have no further comment and the manuscript is really improved in its revised version.