nature portfolio

Peer Review File

CANCER CELL STIFFENING VIA CoQ10 AND UBIAD1 REGULATES ECM SIGNALING AND FERROPTOSIS IN BREAST CANCER

Editorial Note: Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The role of ubiquinone-10 (CoQ10) in mitochondrial respiration and its lipid antioxidant functions are well characterized; in addition, the role of CoQ10 in cancer cell resistance to ferroptosis, particularly in an GSH-independent manner, has been recently reported. However, the effects of CoQ10 on the biophysical properties of the plasma membrane is poorly understood; and the potential effects of such membrane alterations on breast cancer metastatic progression are unknown and understanding these effects would be a major advancement in better understanding cancer progression.
Tosi et al show that CoQ10 and its synthesizing enzyme, UBIAD1, are important modulators of membrane fluidity and overall cell stiffness. They show that low UBIAD1 levels are associated with poor prognosis, particularly, in patients with TNBC. Mechanistically, they propose that UBIAD1 plays the role of a tumor suppressor, by increasing CoQ10 synthesis, leading to increased levels of CoQ10 at the membrane, which disrupts lipid rafts and specifically suppresses PI3K-dependent signaling. Further, UBIAD1 overexpression suppressed adhesion to laminin, potentially, via altering global gene expression and suppressing the levels of alpha-6 integrin and laminin-511 (the laminin isoform associated with advanced breast cancer). The authors make the connection between high UBIAD1 and ferroptosis resistance, consistent with the involvement of CoQ10 in the process. Further, the phenotypic changes induced by UBIAD1 are associated with slower tumor growth, decreased CTCs, and suppressed metastasis, specifically to the lung, of TNBC cells.
There are several pieces of exciting evidence in this study pointing toward a role for CoQ10 mediated membrane alteration on metastatic progression; however, the work does not present a coherent story, but rather a fragmented collection of discoveries that fail to support the working model. Providing further mechanistic investigation of the central novel concepts would strengthen the model and increase the impact of the work.

General comments:
The connection between the CoQ10 induced biophysical alteration of the membrane and cell stiffness is novel and the potential connection of these changes with metastasis is a significant advance. However, the investigations do not go beyond a correlative analysis. Can the authors separate the lipid antioxidant role of CoQ from its role in modulating the biophysical properties of the membrane? Could they examine the effects of specifically altering the latter on metastasis? It is hard to answer these questions with the presented data since the authors are either altering the synthesis of CoQ10 or directly altering its levels; both would influence lipid oxidation, the effects of which on tumor progression is established.

The mechanistic connection between altering the abundance of lipid rafts and changing cortical actin might be an interesting angle to follow and might help with understanding the antioxidant-distinct role of CoQ10. More specifically, targeting an intermediate step that promotes actin cytoskeletal remodeling (for instance, recruiting or excluding actin regulators from the membrane) might interfere with cell stiffening but not alter the oxidative stress response at the membrane. Potentially, such mechanism will be completely novel.

Cortical tension is important for allowing cells to survive under high shear stress, such as the pressure experienced by CTCs after dissemination from the primary tumor. How do the authors explain the higher number of CTCs when UBIAD1 is low (cortical stiffness is low)? Is this number just reflective of bigger tumors?

The role of UBIAD1 as a tumor suppressor has not been previously explored in breast cancer. To further investigate the effects of UBIAD1/CoQ10 on breast cancer progression, the authors could further examine the interesting lung-organotropism phenotype observed when UBIAD1 is overexpressed. Is this phenotype a feature of TNBC tumors? Similarly to the comment on CTCs above, were the values of the lung mets normalized over tumor size?

The model of how altering the biophysical properties of the membrane by UBIAD1/CoQ10 influences PI3K signaling could be further explored. Is it related to integrin clustering as suggested by the authors? are focal adhesions different in cells loaded with CoQ10 or overexpressing UBIAD1?

It is interesting differences in UBIAD1 in patient samples are more relevant to TNBC. ER+ tumors exhibit higher rates of activating mutations in PIK3CA. And in fact, the majority of the ER+ cell lines used in this study have PIK3CA mutations. It is tempting to postulate that these mutations might overcome the regulation of PI3K by CoQ10, making tumor able to progress even when UBIAD1 is highly expressed. It would be interesting to check such model since it would shed more light on the clinical significance of the work.

Minor comments:

It would be beneficial to examine survival in the METBRIC dataset using UBIAD1 mRNA expression to cluster patients within each subtype.

I suggest moving the breakdown of tumor subtype within the METABRIC patient cohort from Figure 2m to Figure 1e.

Is there a difference between luminal A and luminal B with respect to UBIAD1 expression?

The introduction section needs improvement to better convey the novelty of the work and better put the study in the context of the field.

On line 757, it is not appropriate to refer to the samples from the TMA (vs the METABRIC dataset) as originating from “real-life BC patients”. All samples are donated by real patients and are equally valuable to the research community (whether they are used for mRNA microarray or TMA).

Reviewer #2 (Remarks to the Author):

This study focused on the role of UBIAD1 (an enzyme that generate non-mitochondrial CoQ) in breast cancer, and showed that UBIAD1 functions as a tumor suppressor in breast cancer and UBIAD1 deletion promotes breast cancer development and metastasis. Mechanistically, they proposed that its tumor suppressor function in breast cancer relates to its role in regulating cortical stiffness, ECM/integrin signaling, suppressing AKT2 signaling, and promoting ferroptosis (as summarized in Fig. 6).

The major strength of this study is the multiple in vivo studies (GEMM and xenograft models with knockout or overexpression) that establish a novel role of UBIAD1 as a tumor

suppressor in breast cancer. However, the major weakness of the current manuscript is the lack of strong functional evidence to CAUSALLY link their proposed mechanisms to UBIAD1's tumor suppressor function in breast cancer.

Essentially all the functional data only provide correlative evidence to support UBIAD1's tumor suppressor function in breast cancer, but additional functional studies are needed to establish the causality. For example, the authors concluded that "UBIAD1 expression limits significantly CTCs and metastasis in xenograft models of BC and that this is due to its ability to enhance sensitivity to ferroptosis" (page 31). To establish this point, the authors need to show UBIAD1 KO tumors have decreased lipid peroxidation levels (by MDA staining etc) than their KO counterparts, or UBIAD1 overexpressing tumors have increased lipid peroxidation levels than corresponding control tumors, and importantly, treatment of UBIAD1 KO tumors with the ferroptosis inhibitor (such as liproxstatin-1) should restore tumor growth or metastasis in UBIAD1 KO tumors (therefore, establishing the causality).

Likewise, to establish AKT2's role in this context, they need to manipulate AKT2's levels and examine whether knocking down AKT2 in UBIAD1 KO or overexpressing constitutively active form of AKT2 in UBIAD1-overexpressing context can rescue some of the phenotypes induced by UBIAD1 KO or overexpression.

Another major issue of the current study is that the identified pro-ferroptosis function of UBIAD1 is counterintuitive and lacks clear mechanistic explanation. UBIAD1 is known to promote the biosynthesis of non-mitochondrial CoQ, which is then reduced by FSP1 to CoQH2, and CoQH2 acts as a radical trapping antioxidant to suppress ferroptosis. Based on this, it is expected that UBIAD1 should suppress lipid peroxidation and ferroptosis. Therefore, it is hard to explain their findings that UBIAD1 and CoQ function to promote lipid peroxidation and ferroptosis, which is also opposite to their own recent publication (PMID: 35255427).

The authors suggest that UBIAD1 promotes ferroptosis by decreasing GPX4 expression and increasing ACSL4 levels (specifically under ferroptosis inducer treatment conditions), but there is no data to explore how UBIAD1 regulates GPX4 or ACSL4 levels under these conditions. It is also difficult to reconcile why they observed that RSL3 treatment increases GPX4 expression in some (Fig. S5f) but not in other experiments (Fig. 5g).

The authors suggest that UBIAD1 regulates ferroptosis by altering PM mechanical properties and stiffness as well as ECM/AKT2 signaling (the last sentence in page 30). However, this statement lacks relevant supporting data. For example, based on this claim, altering PM mechanical properties and stiffness or ECM/AKT2 signaling should affect ferroptosis sensitivity.

Fig. 2i-k, does Ubiad1 KO affects the animal survival of KBP models? (as they showed in MMTV-NeuT models, Fig. 2b)

Can the authors comment on why only AKT2 (but not AKT1) activation is affected by UBIAD1 and CoQ?

Page 29 "ACSL4 is an enzyme that modifies monounsaturated fatty acids into polyunsaturated fatty acids, the main target of lipid peroxidation." This statement is wrong. ACSL4 functions to catalyze the ligation of CoA to free PUFAs to generate PUFA-CoA.

Reviewer #3 (Remarks to the Author):

In this study, Tosi and colleagues show that Ubiad1 and CoQ10 are involved in cancer cell stiffening in breast cancer. This is important both mechanistically and therapeutically. Using a variety of models (from newly generated mouse models to human cell lines and data from patients), the authors demonstrate that the increase in membrane fluidity, the inhibition of the PI3K/AKT signaling and the reduction of ferroptosis resistance are some of the mechanisms involved in the final effect over cancer cells. Overall, the study is well conducted, both conceptually and methodologically, and the data are very robust and convincing. However, the authors should make additional efforts to better integrate the described mechanisms of actions and identify some connections between them. Also, it is not clear enough if the observed effects are due to Ubiad1, CoQ10 or both. This is especially important considering that Ubiad1 is not only involved in CoQ biosynthesis but also in vit k2 biosynthesis. In that sense, the authors must complete some experiments.

Major issues:

- Fig 1i. The authors show a reduction in Ubiad1 expression in TNBC lines. Does Ubiad1 expression correlate with CoQ10 levels? Can the authors measure CoQ10 levels in those cell lines to see the correlation (if any)? That is important in order to understand if the effects of Ubiad1 depend on its function in CoQ biosynthesis.

- As a biological tool to develop this study, the authors have generated an Ubiad1 KO mouse model, which shows embryonic lethality, apparently due to CoQ deficiency. However, CoQ levels were only measured in heterozygous embryos. Can the authors extend those measurements to homozygous mutant mice? Also, the authors briefly described the phenotype of heterozygous mice, but they do not mention the phenotype of homozygous mutant mice. Do they also show hemorrhagic phenotype? A similar model was previously generated by Nakagawa and colleagues (doi:10.1371/journal.pone.0104078). In that study, the authors claimed that Ubiad1 is not involved in CoQ biosynthesis in mouse. Could the authors comment the differences in the conclusions from both studies? Is Ubiad1 involved in CoQ biosynthesis in mouse? What about in human?

- Fig 2f. The authors show a reduction in Ubiad1 expression in the mouse derived tumors. How are the levels of CoQ9 in those tumors? Again, those data are important in order to understand if the effects of Ubiad1 depend on its function in CoQ biosynthesis.

- Fig 3f and Supplementary Fig 3d,e. If the observed effects of Ubiad1 overexpression are due to CoQ10-dependent mechanisms, can the authors achieve similar results with CoQ10 supplementation?

- The results about ferroptosis are confusing. Both FSP1 (through CoQ redox state) and GPx4 (through GSH redox state) prevent ferroptosis by reducing lipid peroxidation. However, Ubiad1 overexpression, which may increase CoQ in plasma membrane, reduces the resistance to ferroptosis; and cancer cells with low Ubiad1 levels, which may have decreased CoQ in plasma membrane, have more resistance to ferroptosis. These data seem to contradict the mechanism of ferroptosis involving CoQ. Apparently, those results can be explained by an inverse correlation between Ubiad1 levels and Gpx4 levels. Is that the explanation that the authors propose? If yes, how can Ubiad1 levels influence GPx4

levels? Could CoQ10 supplementation induce similar effects on GPx4 and ferroptosis? Perhaps, GPx4-dependent ferroptosis should be added in Fig 6 (bottom picture). Moreover, a schematic supplementary figure about the different components of ferroptosis (iron, ROS, lipid peroxidation, GPx4, GSH, FSP1 and CoQ10) and the proposed involvement of Ubiad1 would help the reader to better understand the results.

Minor issues:

- Supplementary Fig 2e. The figure indicates -/-. I guess it must be +/- because CoQ deficiency is not very severe. In that case, which are the CoQ9 levels in the -/- mice?

REVIEWER COMMENTS

Reviewer #1:

The connection between the CoQ10 induced biophysical alteration of the membrane and cell stiffness is novel and the potential connection of these changes with metastasis is a significant advance. However, the investigations do not go beyond a correlative analysis. Can the authors separate the lipid antioxidant role of CoQ from its role in modulating the biophysical properties of the membrane? Could they examine the effects of specifically altering the latter on metastasis? It is hard to answer these questions with the presented data since the authors are either altering the synthesis of CoQ10 or directly altering its levels; both would influence lipid oxidation, the effects of which on tumor progression is established.

We thank the Reviewer for this comment. We have now provided novel mechanistic insights to strengthen the model and increase the impact of the work. Please see new data Figures and rebuttal letter data.

CoQ_{10} is a unique molecule holding both antioxidant functions, through its quinone ring undergoing redox cycling, and biophysical properties through the insertion/intercalation in the plasma membrane of its polyprenyl isoprenoid moiety. To prove that and to try to separate the lipid antioxidant role of CoQ_{10} from its role in modulating the biophysical properties of the membrane we have tested in Atomic Force Microscopy (AFM) different molecules, some structural analogues of CoQ_{10} but with a shorter isoprenoid moiety (CoQ_6 and CoQ_2) and other with antioxidant function but lacking the structural features of CoQ_{10} (idebenone and NAC). Interestingly, we observed that antioxidant compounds like idebenone and NAC do not alter cellular stiffness. Interestingly, CoQ_2 containing a short side chain of 2 isoprenoid units also does not alter cell stiffness, while CoQ_6 containing a side chain of 6 isoprenoid units significantly increases stiffness although not at the same levels of CoQ_{10} (Fig. A.a,b). Therefore, these data showed that it is conceivable to separate the lipid antioxidant role of CoQ_{10} (linked to the redox ubiquinone ring) and the plasma membrane mechanical role (linked to the polyprenyl tail).
The connection between CoQ_{10} and increased cellular stiffness is kept in different BC cell lines (e.g. MCF7, MDA-MB-468, and MDA-MB-231), but not in non-tumorigenic MCF10A (Fig. A.c). This suggests that the effect of CoQ_{10} on stiffness is related to the tumorigenic state of breast cancer cells. Interestingly, this effect partially correlates with the total amount of CoQ_{10} expressed by these cell lines (Fig. A.d) (see R#3.1). To further elaborate with the ability of CoQ_{10} to regulate cellular stiffness, we have treated MDA-MB-231 cells with CoQ_{10} and a combination of the ROCK inhibitor Y27632 and the MLCK inhibitor ML7 known to make BC soft (https://www.nature.com/articles/s41556-018-0270-5) (Fig. A.e). We showed that Y27632 + ML7 inhibitors promote softness in MDA-MB-231 cells as expected. However, in presence of CoQ_{10} this effect is blunted.

When testing the invasion potential of MDA-MB-231 $UBIAD1^{OE}$ cells, we observed that the diminished number of invading cells due to UBIAD1 overexpression could be partially restored by either

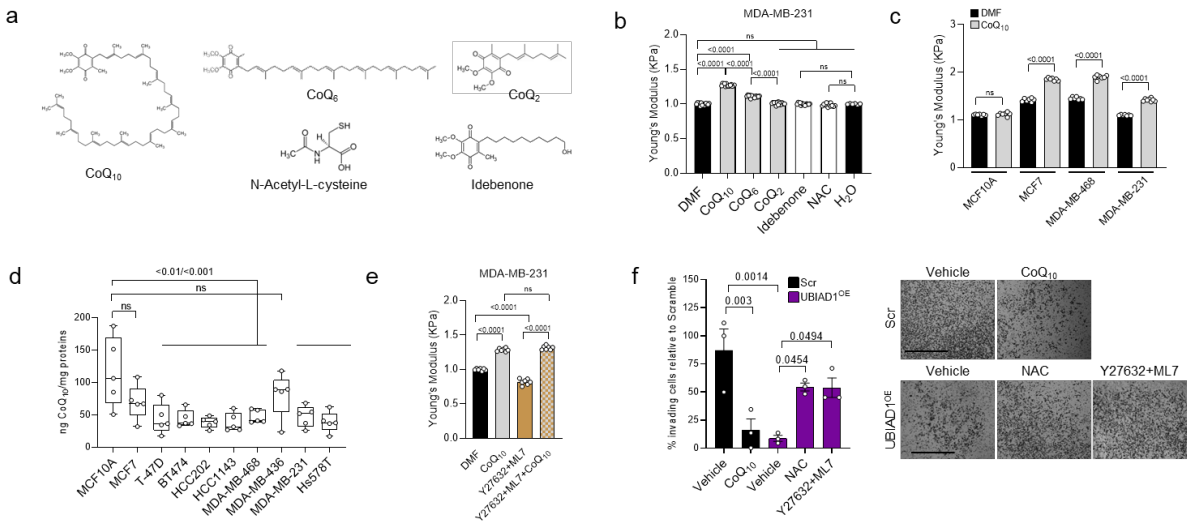


***Figure A**: **a** Chemical structure of different CoQs and antioxidant compounds. **b** Atomic Force Microscopy (AFM) to measure stiffness of MDA-MB-231 cells upon treatment with 20μM CoQ_{10}, 20μM CoQ_6, 20μM CoQ_2, 20μM Idebenone, 10mM N-Acetyl-L-Cysteine (NAC) for 24h. Either DMF (Dimethylformamide) or H_2O were used as Vehicle. Measurements were performed on two independent replicates and each dot represents the average of two technical replicates on the same cell. Mean ± SEM. Adjusted p-value was calculated using one-way Anova followed by Tukey's multiple comparisons test. **c** AFM to measure stiffness of non-tumorigenic breast line MCF10A and three breast cancer (BC) cell lines upon treatment with 20μM CoQ_{10} for one week. DMF was used as Vehicle. Measurements were performed on two independent replicates and each dot represents the average of two technical replicates on the same cell. Mean ± SEM. P-value was calculated using two-tailed un-paired t-test. **d** Mass spectrometry analysis to evaluate total CoQ_{10} levels normalized over mg of proteins in the non-tumorigenic breast line MCF10A and a panel of BC cell lines. Each dot represents a biological replicate. Mean ± SEM. Adjusted p-value was calculated using one-way Anova followed by Tukey's multiple comparisons test. **e** AFM to measure stiffness of MDA-MB-231 cells upon treatment with 20μM CoQ_{10}, the combination of the ROCK inhibitor Y27632 20μM and MLCK inhibitor ML7 20μM, and the cotreatment. DMF was used as Vehicle. Measurements were performed on two independent replicates and each dot represents the average of two technical replicates on the same cell. Mean ± SEM. Adjusted p-value was calculated using one-way Anova followed by Tukey's multiple comparisons test. **f** Invasion assay of MDA-MB-231 Scr vs. $UBIAD1^{OE}$ cells through a Matrigel-coated membrane. Cells were alternatively treated for 24h with 20μM CoQ_{10}, 10mM N-Acetyl-L-Cysteine (NAC) and the combination of the ROCK inhibitor Y27632 20μM and MLCK inhibitor ML7 20μM. Data are expressed as percentage of invading cells compare to Vehicle-treated Scr (set at 100%). Each dot represents a biological replicate. Data are shown as mean ± SEM. Adjusted p-value was calculated using one-way Anova followed by Sidak's multiple comparisons test. On the right, representative images of invading cells. Scale bar, 100μm.*

rendering the cells softer through treatment with Y27632 + ML7 inhibitors or by reducing cellular oxidative stress via NAC treatment (Fig. A.f).
These data support a model where both stiffness and lack of antioxidant power explain the reduced invasion properties achieved by enhancing UBIAD1/CoQ_{10} levels.

These data have been now included in the new Fig. 1e-i and Fig. 5k.

See also reply to Ref. 3 comments.

#1.1. The mechanistic connection between altering the abundance of lipid rafts and changing cortical actin might be an interesting angle to follow and might help with understanding the antioxidant-distinct role of CoQ10. More specifically, targeting an intermediate step that promotes actin cytoskeletal remodeling (for instance, recruiting or excluding actin regulators from the membrane) might interfere with cell stiffening but not alter the oxidative stress response at the membrane. Potentially, such mechanism will be completely novel.

R#1.1. We would like to express our gratitude to Reviewer #1 for the insightful comment. To address this comment we have exploited the use of the combination of ROCK inhibitor Y27632 and MLCK inhibitor ML7 as a way to induce actin cytoskeletal remodeling, in particular simulating softness conditions (https://www.nature.com/articles/s41556-018-0270-5). We used this set up to interfere with the stiffness vs. softness of BC in this new version of the manuscript (see also previous reply). Please see also additional data in RL and revised manuscript.

#1.2. Cortical tension is important for allowing cells to survive under high shear stress, such as the pressure experienced by CTCs after dissemination from the primary tumor. How do the authors explain the higher number of CTCs when UBIAD1 is low (cortical stiffness is low)? Is this number just reflective of bigger tumors?

R#1.2. We appreciate the Reviewer's comment. The observed reduction in the number of circulating tumor cells (CTCs) in UBIAD1^{OE} cells cannot be attributed to a decrease in primary tumor volume. In fact, Fig 4d and Suppl. Fig 4r represent the percentage of CTCs in mice after 21 or 35 days post intracardiac injection of MDA-MB-231 or JIMT-1, respectively. Hence, no primary tumor was generated in this experimental setup as illustrated in Fig 4c and Suppl. Fig. 4o. We have now provided clearer specifications in the main text.
To normalize the number of CTCs over the total tumor/metastasis burden, we opted to normalize the percentages obtained against the total luminescence signal obtained on the day of the terminal blood collection from the whole body of the animal. Even with this normalization, the difference remains statistically significant for the MDA-MB-231 model. For JIMT-1, the trend is maintained, although with a p-value > 0.05 (Fig. B).
The reviewer rightly notes that higher cortical stiffness provides an advantage in withstanding the high shear stress encountered in the bloodstream (PMID: 34168685). However, it is noteworthy that more aggressive breast cancer cells, such as MDA-MB-231, often exhibit softer characteristics compared to less aggressive ones (PMID: 32548118 and Fig. A.c). Softer cells are known to have an increased likelihood of colonizing lung tissues (PMID: 32463148) and therefore the reduced number of CTCs may be attributed to less soft conditions of MDA-MB-231 UBIAD1^{OE} cells (due to increased stiffness by CoQ_{10} synthesis as indicated in Fig. A).

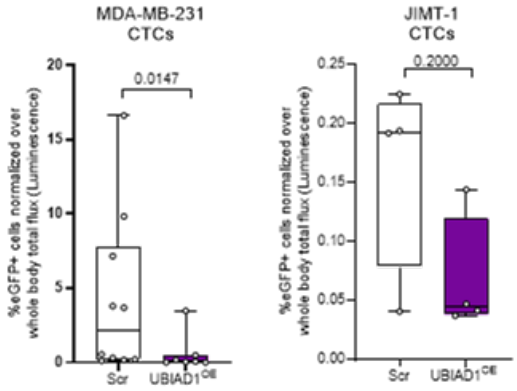


***Figure B**: Percentage of Circulated Tumor Cells (CTCs) eGFP+ over total cells in mouse whole blood. The percentage is normalized considering total flux (Luminescence) from whole mouse body. Each dot represents a single blood sample. Data are shown as mean ± SEM. P-value was calculated using Mann Whitney test.*

#1.3. The role of UBIAD1 as a tumor suppressor has not been previously explored in breast cancer. To further investigate the effects of UBIAD1/CoQ10 on breast cancer progression, the authors could further examine the interesting lung-organotropism phenotype observed when UBIAD1 is overexpressed. Is this phenotype a feature of TNBC tumors? Similarly to the comment on CTCs above, were the values of the lung mets normalized over tumor size?

R#1.3. We would like to express our gratitude to the Reviewer for their question. We have observed a similar lung-organotropism phenotype in the TNBC model MDA-MB-231, as well as in the HER2-enriched model JIMT-1 cells upon UBIAD1 overexpression (Suppl. Figure 4 o-r). This suggests that this phenomenon may be applicable to other breast cancer (BC) subtypes. It is important to note that the percentage of eGFP+ cells in the lungs has not been normalized based on the primary tumor volume. As explained in #1.2, these results were obtained from mice without a primary tumor but post intracardiac injection of tumor cells. Nevertheless, we present in Fig.C previous results in which we measured the percentage of eGFP+ cells in the lung tissue obtained 28 days after an injection of MDA-MB-231 cells Scr vs. UBIAD1^{OE} only into the mammary fat pad. In this case, we have normalized these percentages based on the primary tumor volume at sacrifice. It is evident that the effect remains appreciable even in these experimental conditions, although the reduced number of mice renders the statistics not significant. Nevertheless, this suggests that the size of the primary tumor alone is not sufficient to explain the observed reduction in the number of lung metastases

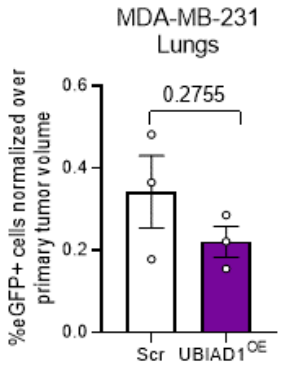


***Figure C**: Percentage of eGFP+ over total cells in mouse lungs. The percentage is normalized over primary tumor volume. Each dot represents a single mouse. Data are shown as mean ± SEM. P-value was calculated using two-tailed un-paired t-test.*

induced by UBIAD1-overexpressing cells.

#1.4. The model of how altering the biophysical properties of the membrane by UBIAD1/CoQ10 influences PI3K signaling could be further explored. Is it related to integrin clustering as suggested by the authors? are focal adhesions different in cells loaded with CoQ10 or overexpressing UBIAD1?

R#1.4. In response to these questions, using MDA-MB-231 cells loaded with CoQ_{10} or overexpressing UBIAD1 we examined focal adhesions as crucial components playing a central role in connecting integrin signaling with actin filament remodeling and cell invasion (PMID: 35450290). Paxillin and FAK are proteins associated with focal adhesions, potentially influencing various signaling pathways and frequently used to study the structure of cellular focal adhesion. Confocal imaging analyses of Paxillin and FAK distribution clearly demonstrates alterations in focal adhesions in MDA-MB-231 cells treated with CoQ_{10} or overexpressing UBIAD1 compared to controls. Notably, these alterations manifest as multiple blebs at the plasma membrane where focal adhesions should be localized. In contrast, control cells exhibit a more elongated morphology with visible lamellipodia and typical focal adhesions, characteristic of mesenchymal cells (Fig. D).
This observation aligns with previous findings in MDA-MB-231 cells, where cells with less ordered and more fluid membranes, and increased intracellular pressure (as observed after CoQ_{10} treatment or in UBIAD1^{OE} cells, Fig. 1a-c and 5g-j) display more blebs than lamellipodia (PMID: 33298514).
The role of blebs in the invasion of mesenchymal-like cancer cells, such as MDA-MB-231, remains insufficiently explored, and further studies are necessary to determine their contribution to metastasis formation in the context of aggressive breast cancer.

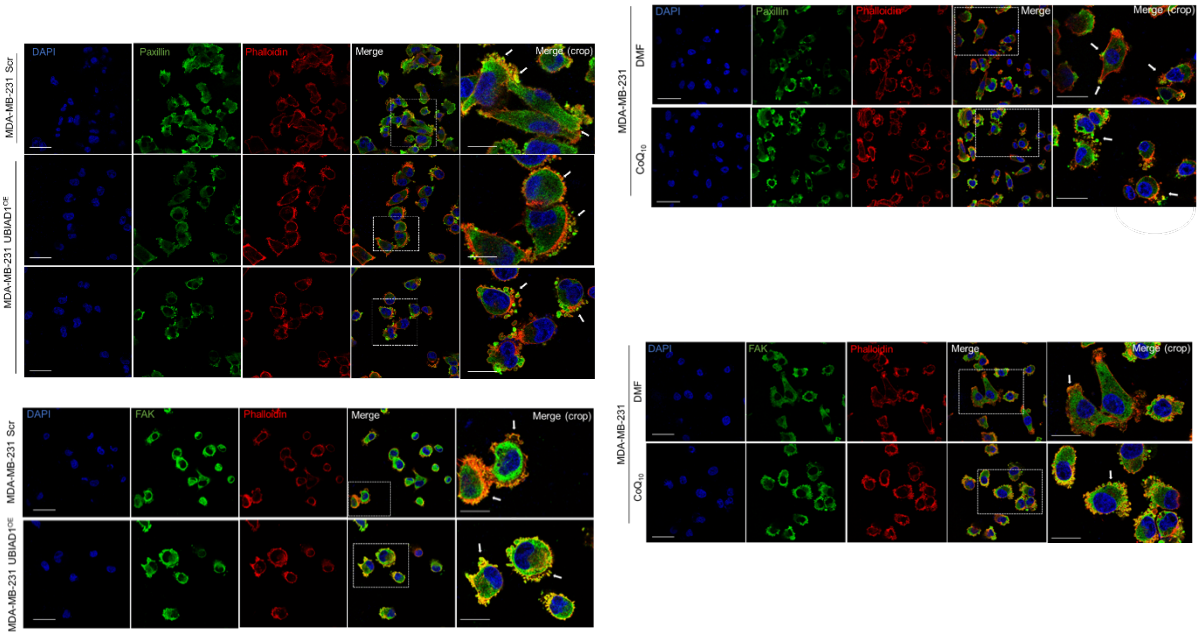


Figure D: *Immunofluorescence analysis of MDA-MB-231 Scr vs. MDA-MB-231 UBIAD1^{OE} and MDA-MB-231 treated with DMF (Vehicle) vs. 20μM CoQ_{10} for 24h. Staining was performed using the indicated antibodies, DAPI (for nuclei) and Phalloidin (for F-actin). Arrows indicates focal adhesion structures. Scale bar, 60 mm. Scale bar Crop, 30 mm.*

These data have been now added to Fig. 5f, Suppl. Fig. 1e,f, and Suppl. Fig. 5i.

#1.5. It is interesting differences in UBIAD1 in patient samples are more relevant to TNBC. ER+ tumors exhibit higher rates of activating mutations in PIK3CA. And in fact, the majority of the ER+ cell lines used in this study have PIK3CA mutations. It is tempting to postulate that these mutations might overcome the regulation of PI3K by CoQ10, making tumor able to progress even when UBIAD1 is highly expressed. It would be interesting to check such model since it would shed more light on the clinical significance of the work.

R#1.5. We thank the Reviewer for this insightful comment. While a systematic analysis of the prognostic value of high vs. low UBIAD1 mRNA expression in the entire METABRIC cohort of breast cancer patients did not show any significant difference (Fig. E.a), performing stratified analyses based on their intrinsic PI3K/PTEN mutational status highlighted some interesting points. We observed a significantly worse prognosis associated with low UBIAD1 expression exclusively in the subgroup without PIK3CA/PTEN alterations (Fig. E.b). This difference is even more pronounced in the Luminal subtype (Fig. E.c).
These findings align with the Reviewer's observation that the presence of PIK3CA/PTEN mutations appear to override the tumor suppression function of UBIAD1 and CoQ_{10} in breast cancer, thereby further confirming the role of UBIAD1/CoQ_{10} as an upstream regulator of the AKT pathway. This is in agreement with data obtained in reply to #2.2.

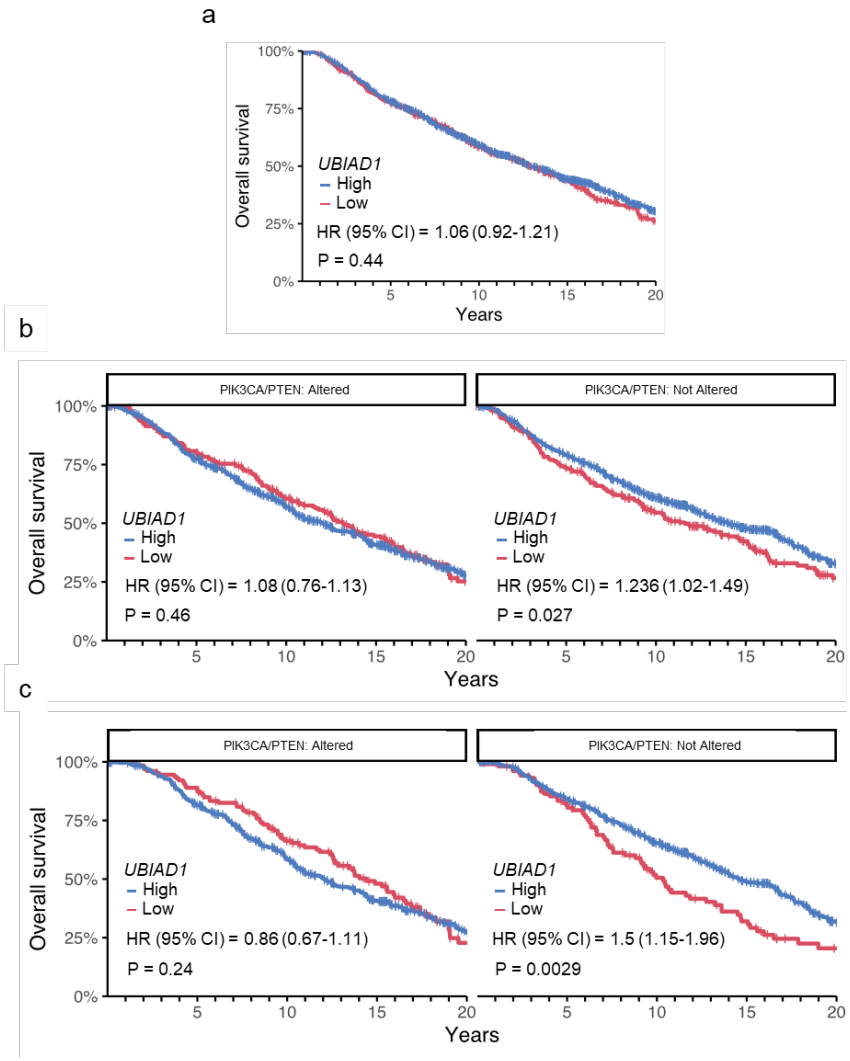


Figure E. a *Kaplan-Meier analysis of overall survival in METABRIC breast cancer patients stratified according to UBIAD1 mRNA expression levels (Low: <25th percentile; High: ≥25th percentile). HR, hazard ratio; CI, confidence intervals. P, Log-rank test p-value.* ***b*** *Kaplan-Meier analysis of overall survival in METABRIC breast cancer patients subgrouped based on their mutational status of PIK3CA and PTEN and stratified according to UBIAD1 mRNA expression levels (Low: <25th percentile; High: ≥25th percentile). HR, hazard ratio; CI, confidence intervals. P, Log-rank test p-value.* ***c*** *Similar analysis to that in (**b**) was performed on patients with Luminal Breast Cancer only. HR, hazard ratio; CI, confidence intervals. P, Log-rank test p-value.*

These data have been now added to Suppl. Fig. 5h

Minor comments:

#1.6. It would be beneficial to examining survival in the METABRIC dataset using UBIAD1 mRNA expression to cluster patients within each subtype would be beneficial.

R#1.6. According to the Reviewer's request, we have performed a systematic analysis of the prognostic value of high vs. low UBIAD1 mRNA expression in the METABRIC cohort of breast cancer (Fig. F). The mRNA stratification did not show any significant prognostic stratification in the individual subtypes.

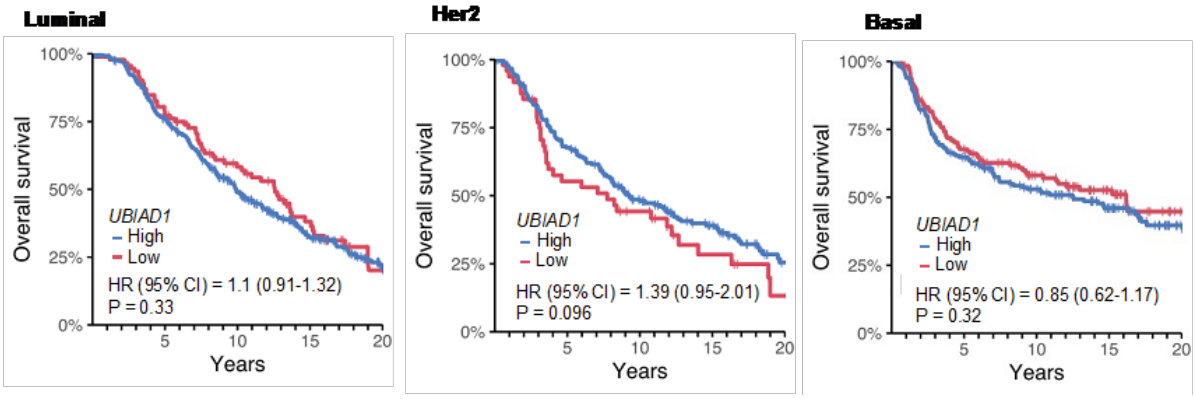


***Figure F**: Kaplan-Meier analysis of overall survival in METABRIC breast cancer patients stratified according to UBIAD1 mRNA expression levels (Low: <25th percentile; High: ≥25th percentile) among the 3 main PAM50 subtypes. HR, hazard ratio; CI, confidence intervals. P, Log-rank test p-value.*

#1.7. I suggest moving the breakdown of tumor subtype within the METABRIC patient cohort from Figure 2m to Figure 1e.

R#1.7. We agree and rearranged figures as suggested (see new Fig. 2b).

#1.8. Is there a difference between luminal A and luminal B with respect to UBIAD1 expression?

R#1.8. In the analysis of the METABRIC cohort, UBIAD1 deletion appears to be highly significantly enriched in luminal B *vs.* Luminal A patients (OR = 3.33, p<0.0001). These analyses are now included in the new Figure 2b. Consistently, in our TMA cohort, the immunohistochemical analysis of UBIAD1 protein expression reveals a higher proportion of patients with impaired UBIAD1 expression in the Luminal B compared to the Luminal A subtype patients (OR = 1.57; CI, 1.0-2.5), although the p value did not reach statistical significance (p = 0.073). See revised new Fig. 2e.

#1.9. The introduction section needs improvement to better convey the novelty of the work and better put the study in the context of the field.

R#1.9. Thank you for the suggestion. We add a paragraph in the introduction section to covey the novelty of the work and put the study in the context of the field.

#1.10. On line 757, it is not appropriate to refer to the samples from the TMA (vs the METABRIC dataset) as originating from “real-life BC patients”. All samples are donated by real patients and are equally valuable to the research community (whether they are used for mRNA microarray or TMA).

R#1.10. We acknowledge the relevance of this Reviewer’s point, and we changed the text accordingly.

Reviewer #2:

#2.1) Essentially all the functional data only provide correlative evidence to support UBIAD1's tumor suppressor function in breast cancer, but additional functional studies are needed to establish the causality. The authors concluded that "UBIAD1 expression limits significantly CTCs and metastasis in xenograft models of BC and that this is due to its ability to enhance sensitivity to ferroptosis" (page 31). To establish this point, the authors need to show UBIAD1 KO tumors have decreased lipid peroxidation levels (by MDA staining etc) than their WT counterparts, or UBIAD1 overexpressing tumors have increased lipid peroxidation levels than corresponding control tumors, and importantly, treatment of UBIAD1 OE tumors with the ferroptosis inhibitor (such as liproxstatin-1) should restore tumor growth or metastasis in UBIAD1 OE tumors (therefore, establishing the causality).

We believe that our new experiments and conclusions provide now enough evidence of direct involvement of UBIAD1/CoQ_{10} axis at the crosstalk between the regulation of ferroptosis and mechano-properties in BC. Below are our replies to each single claim.

R#2.1. We appreciate the insightful comments from the Reviewer, which we find both reasonable and of great interest. The Reviewer has raised the question of detecting ferroptosis (or lipid peroxidation) in *in vivo* settings using our animal models. To address this, we conducted MDA staining to measure lipid peroxidation levels in UBIAD1 KO (*Ubiad1*$^{+/-}$) tumors compared to their WT counterparts (*Ubiad1*$^{+/+}$) for MMTV-NeuT and MMTV-PyMT genetic models. However, we did not observe a positive reliable signal (Fig. G.a-b). This lack of detection may be attributed to challenges in processing samples while maintaining lipid oxidation intact during fixation and following staining procedure.

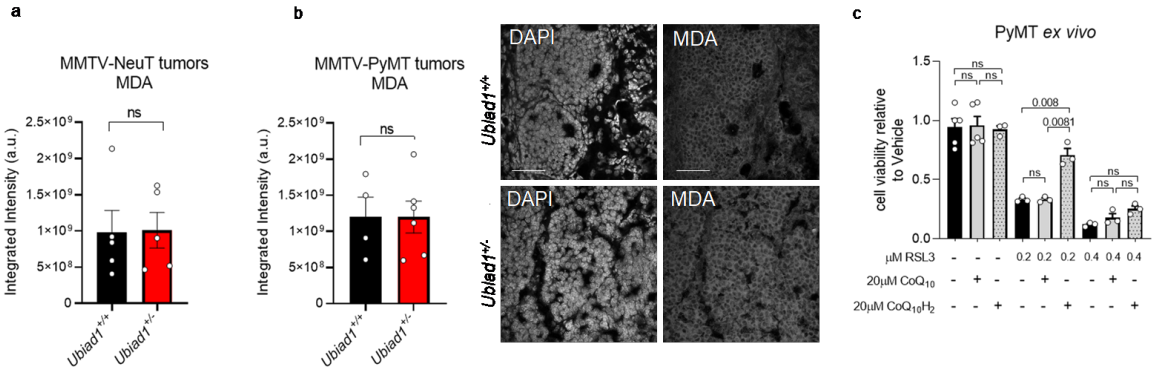


Figure G: a-b *Immunofluorescence analysis of Malondialdehyde (MDA) levels in tumors collected from (**a**) MMTV-NeuT; Ubiad1*$^{+/-}$ *vs. MMTV-NeuT; Ubiad1*$^{+/+}$ *and (**b**) from MMTV-PyMT; Ubiad1*$^{+/-}$ *vs. MMTV-PyMT; Ubiad1*$^{+/+}$*, 14 or 8 weeks after tumor development, respectively. Each dot represents a single tumor. Data are shown as mean ± SEM. P-value was calculated using two-tailed un-paired t-test. Scale bar, 70μm.* ***c*** *Cell viability assay measures cell sensitivity to combinational treatment with* CoQ_{10}*,* $CoQ_{10}H_2$ *and ferroptosis inducer RSL3. MDA-MB-231 cells were treated for 24h with different concentration of RSL3 alone, or in combination. Cell viability was assessed through crystal violet staining and expressed as viability relative to vehicle-treated cells. Each dot represents a biological replicate. Mean ± SEM. Adjusted p-value was calculated using one-way Anova followed by Tukey's multiple comparisons test. ns, not significant.*

The tumor microenvironment is complex in nature, making it difficult to isolate the tumor mass component from other cells such as immune cells, cancer-associated fibroblasts, and endothelial cells. Additionally, differences in lipid peroxidation might occur during early stages of tumor development and could contribute to the different onset of tumors in *Ubiad1*$^{+/-}$ mice compared to controls (Fig. 3b and Suppl. Fig. 3h). Such difference might diminish at later stages (when our samples have been processed) due to adaptation mechanisms. Therefore, it would make challenging to detect differences in tumors at, and after, "palpable stage".

Performing experiments to detect lipid peroxidation in freshly isolated tumor cells is challenging, because using the current state-of-the-art methods (BODIPY-C11 followed by FACS) would have required intact freshly isolated cells from tumors. The process of isolating tumor cells requires extensive manipulation for a long time that would not have allowed to maintain intact the peroxidation states of the tumor cells. Instead, we measured ferroptosis/lipid peroxidation *ex vivo* by examining sensitivity to ferroptosis inducers (RSL3, FIN56), as well as *Acsl4* and *Gpx4* expression levels in cancer cells (Fig. 6e-g; Suppl. Fig. 6e,f). We also conducted new experiments *ex vivo*, introducing a

reduced form of CoQ_{10}, $CoQ_{10}H_2$ (or ubiquinol), which demonstrated the ability to rescue ferroptosis in breast tumor cells, while the oxidized form of CoQ_{10} (or ubiquinone) did not exhibit the same effect (Fig. G.c). We conclude that the role of UBIAD1/CoQ_{10} in protecting from ferroptosis is directly related to the cells' capacity to reduce the molecule to exert its antioxidant effect, protecting from ferroptosis. When cells are loaded with CoQ_{10} or overproduce CoQ_{10} through UBIAD1 overexpression, the result is not protection from oxidative stress but an enhanced sensitivity to ferroptosis.

Unfortunately, law and ethical regulations at our University of Padua prevented us to perform *in vivo* experiments with ferroptosis inhibitors like liproxstatin-1. Specific authorizations from the Italian Ministry of Health are required to perform such treatments. Obtaining such approval would have taken more than six months, along with additional time to set up the correct experimental conditions (i.d., dose and route of administration), plus performing the experiments. Altogether, this would be impossible within the editorial timeline for the revision.

We revised our statement at page 31 as "UBIAD1 expression limits significantly CTCs and metastasis in xenograft models of BC and that this is *possibly related* to its ability to enhance sensitivity to ferroptosis".

#2.2. Likewise, to establish AKT2's role in this context, they need to manipulate AKT2's levels and examine whether knocking down AKT2 in UBIAD1 KO or overexpressing constitutively active form of AKT2 in UBIAD1-overexpressing context can rescue some of the phenotypes induced by UBIAD1 KO or overexpression.

R#2.2 We thank the Reviewer for the suggestion. We therefore overexpressed the *wildtype* form of AKT2 and the constitutively active form AKT2-Myr in MDA-MB-231 UBIAD1^{OE} cells and we evaluate their ability to invade through a Matrigel-coated membrane. We found that the overexpression of AKT2 *wildtype* did not alter the cells invasion ability, confirming the upstream action of UBIAD1/CoQ_{10} in inhibiting AKT2 pathway, while the constitutively active form rescues the reduced number of invading cells observed upon UBIAD1 overexpression (Fig. H.a,b). These data have now been included in the manuscript (see new Suppl. Fig. 5g). See also reply to #1.5.

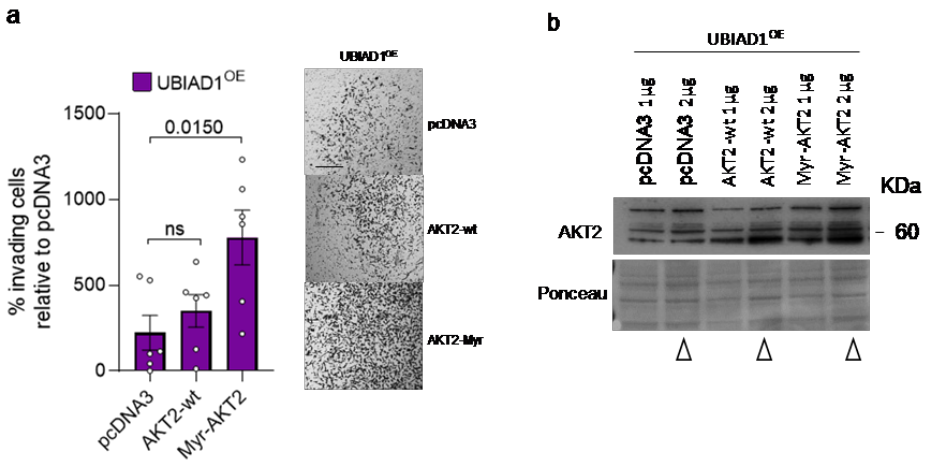


Figure H: a *Invasion assay of MDA-MB-231 UBIAD1^{OE} cells through a Matrigel-coated membrane. Cells were alternatively transfected with empty plasmid (pcDNA3), plasmid encoding AKT2-wildtype (AKT2-wt), and plasmid encoding the constitutively active form Myristylated-AKT2 (Myr-AKT2). Data are expressed as percentage of invading cells compare to pcDNA3 (set at 100%). Each dot represents a biological replicate. Data are shown as mean ± SEM. Adjusted p-value was calculated using one-way Anova followed by Tukey's multiple comparisons test. On the right, representative images of invading cells. Scale bar, 300µm.* ***b*** *Western blot analysis of AKT2 in MDA-MB-231 UBIAD1^{OE} cells to confirm AKT2 wt or Myr overexpression. Cells were transfected with two increasing amounts of plasmid (1µg and 2µg). Ponceau is showed as loading control. The conditions highlighted by the arrowheads (2µg of plasmid) were chosen and used to obtain the results showed in panel (**a**). Uncropped blots at the end of the rebuttal letter.*

#2.3. Another major issue of the current study is that the identified pro-ferroptosis function of UBIAD1 is counterintuitive and lacks clear mechanistic explanation. UBIAD1 is known to promote the

biosynthesis of non-mitochondrial CoQ, which is then reduced by FSP1 to CoQH2, and CoQH2 acts as a radical trapping antioxidant to suppress ferroptosis. Based on this, it is expected that UBIAD1 should suppress lipid peroxidation and ferroptosis. Therefore, it is hard to explain their findings that UBIAD1 and CoQ function to promote lipid peroxidation and ferroptosis, which is also opposite to their own recent publication (PMID: 35255427).

R#2.3 We thank the Reviewer for their observation. In this paper we are claiming and showing that CoQ_{10} has two distinct functions being both a radical-trapping antioxidant when coupled to an oxidoreductase enzyme and an agent able to alter membrane fluidity towards stifness.

To act as antioxidant it needs to be converted to $CoQ_{10}H_2$ by FSP1. Therefore, we evaluated FSP1 expression in MDA-MB-231 cells and in our panel of BC cell lines (Fig. I.a, and new Suppl. Fig. 6h). Western blot analyses showed that FSP1 is absent or expressed at an extremely low level in MDA-MB-231 cells compared to other BC, which can impact the ability of CoQ_{10} and UBIAD1-derived CoQ_{10} to be converted into $CoQ_{10}H_2$ and act as a scavenger of lipid-radicals. Accordingly, also the exogenously administered CoQ_{10} must be slowly converted to $CoQ_{10}H_2$ by the limited amount of FSP1 present in MDA-MB-231 cells. Instead when we provided $CoQ_{10}H_2$, CoQ is able to rapidly act as an antioxidant (Fig. G.c).

[Redacted]

Figure I: a *Western blot analysis of FSP1 expression in a panel of BC cell lines and MCF10A. ACTIN is showed as loading control. Uncropped blots at the end of the rebuttal letter.* ***b*** *Cytofluorimetric analysis of cellular lipid peroxidation level using BODIPY C11 staining. MDA-MB-231 cells were treated with 20μM of CoQ_{10}, RSL3 0.45μM, and the combination for 12-16 hours (short-treatment) or 24-36 hours (long-treatment). Cotreatment with NAC 10mM and RSL3 has been used as positive control of protection from an antioxidant, while Cumene (Cumene Hydroperoxide) has been used as positive control of lipid peroxidation. The fluorescent intensity signal was quantified as ratio between oxidized and reduced BODIPY C11. Data are expressed as fold change over vehicle-treated MDA-MB-231 cells. Each dot represents a biological replicate. Mean ± SEM. Adjusted p-value was calculated using one-way Anova followed by Tukey's multiple comparisons test. ns, not significant.*

Therefore, it looks like CoQ_{10} needs to be reduced to $CoQ_{10}H_2$ by the FSP1 oxido-reductases to become protective against lipid peroxidation. Interestingly, we confirm experimentally this hypothesis by performing time-course experiments by looking at the effect of CoQ_{10} in ferroptosis resistance at different time points (Fig. I.b). The treatment with CoQ_{10} fails to have any protective activity in RSL3-mediated lipid peroxidation in the first 12-16h of treatments while it can protect starting after 24-36h. We can speculate that possibly some oxidoreductase will eventually convert CoQ_{10} to $CoQ_{10}H_2$, but this will take some sort “adaptation” to these conditions. Thus, it is conceivable to believe that in the presence of continuous synthesis of CoQ_{10}, as in UBIAD1^{OE} cells, the continous CoQ_{10} synthesis (without the rapid possibility to be converted by FSP1) counteracts the protective action of the reduced form, masking its antioxidant role. Accordingly, UBIAD1-derived CoQ_{10} fails to decrease cell death induced upon RSL3 treatment like $CoQ_{10}H_2$ (Fig. G.c) and in some circumstances it even reduces the cellular viability of RSL3-treated cells (Fig. 6h).

Measuring the reduced vs. oxidized form of CoQ_{10} in cells with UBIAD1^{OE} would be interesting and possibly solving the enigma but it is also quite challenging from an experimental point of view. We contacted and discussed performing mass spectrometric assays for CoQ pool redox state *in vivo* and *in vitro* in collaboration with Dr. Mike Murphy, MRC, UK (PMID: 31811922). Dr. Murphy explained the critical and technical challenge of maintaining the reduced form of CoQ_{10} after cell lysis during the shipment of the samples from Italy to the UK, suggesting the impracticality of obtaining reliable measurements.

In addition, published data and unpublished observations from our lab led us also to speculate that when UBIAD1 is expressed (high level conditions), this rewires mevalonate intermediates from selenoproteins/GPX4 synthesis to CoQ_{10} synthesis lowering ferroptosis defence in BC (see new Fig. 7 and reply to #3.5).

Finally, it is not so surprising that the effect of UBIAD1 overexpression and the increase in CoQ_{10} levels can lead to two opposite outcomes in such different tumor types such as melanoma or breast cancer. This is especially true considering that UBIAD1 is overexpressed in melanoma compared to healthy tissue (melanocytes), while in breast cancer, we observed the opposite tendency. It is indeed likely to speculate that besides FSP1, other CoQ_{10} oxidoreductases could be functional in breast cancer cells and other in melanoma cells (e.g., NQO1; as shown in Arlsanbaeva et al., 2020, PMID: 35255427).

#2.4. The authors suggest that UBIAD1 promotes ferroptosis by decreasing GPX4 expression and increasing ACSL4 levels (specifically under ferroptosis inducer treatment conditions), but there is no data to explore how UBIAD1 regulates GPX4 or ACSL4 levels under these conditions. It is also difficult to reconcile why they observed that RSL3 treatment increases GPX4 expression in some (Fig. S5f) but not in other experiments (Fig. 5g).

R#2.4. We thank the reviewer for this comment. In order to address the question of how CoQ_{10} mediates ferroptosis activation by increasing ACSL4 expression, we evaluate STAT3 activation. Indeed, it has been shown that STAT3 activation and translocation into the nucleus can either increase ACSL4 expression in adherent cells (https://doi.org/10.1016/j.celrep.2022.110870) or decrease it in detached conditions (PMID: 28972104). Given that, and considering the increased ACSL4 gene expression observed in UBIAD1-overexpressing cells compared to their control upon detachment (condition known to be associated with increased oxidative stress) shown in new Suppl. Fig. 6e,f for *ex vivo* PyMT cells and now replicated in Fig. J.a (new Suppl. Fig. 6g) for MDA-MB-231 cells, we analyzed the activation of STAT3 looking at the phosphorylation status of the Tyrosine 705 (PMID: 8197455, PMID: 7566171). We observed that MDA-MB-231 $UBIAD1^{OE}$ cells in adhesion showed higher activation of STAT3 compared to Scr cells, while upon detachment Scr cells tends to increase STAT3 phosphorylation more than $UBIAD1^{OE}$ cells (Fig. J.b).This is in line with our proposed model that links an higher ACSL4 expression with an increased sensitivity to lipid peroxidation and suggests a possible role of UBIAD1 in regulating ACSL4 gene expression via STAT3 activation/inhibition. Moreover, the effect is more prominent on detached cells suggesting that also the ECM interaction and mechano-transduction could influence how cells are sensing UBIAD1/CoQ_{10} related to ferroptosis conditions.

We also compared STAT3 activation based on cellular stiffness, by treating the MDA-MB-231 cells with CoQ_{10} or Y27632+ML7 and we found that softness (e.i., Y27632+ML7 inhibitors treatment) reduced phosphorylation of STAT3 while CoQ_{10}/stiffness conditions tends to increase it (Fig. J.c). Thus, we suggest that STAT3 signaling might be involved in connecting CoQ_{10}-mediated stiffness with ECM signaling and ferroptosis**.** This is further sustained by previous evidence showing that increased cellular stiffness is associated with ferroptosis through an increase of ACSL4 expression caused by matrix stiffness via YAP (https://doi.org/10.1002/adhm.202300458) and by recent findings about the role of Extracellular mechanical forces and ferroptosis in triple negative breast cancer (10.1200/JCO.2023.41.16_suppl.e13086).

[Redacted]

Figure J: a *qPCR analysis for ACSL4 mRNA expression in adherent or 48h detached MDA-MB-231 Scr vs. UBIAD1^{OE} cells. Each dot represents a biological replicate. Mean ± SEM. Adjusted p-value was calculated using one-way Anova followed by Tukey's multiple comparisons test. ns, not significant.* ***b*** *Western blot analysis of STAT3 activating phosphorylation at residue Tyr-705 in adherent or 24h detached MDA-MB-231 Scr vs. UBIAD1^{OE} cells. Ponceau was used as loading control.* ***c*** *Western blot analysis of STAT3 activating phosphorylation at residue Tyr-705 in MDA-MB-231 Scr cells treated for 24h with 20μM CoQ_{10} or 20μM Y27632+ML7 inhibitors. ACTIN was used as loading control. Uncropped blots at the end of the rebuttal letter.*

These data makes the study even more complicated and intriguing that merit further investigation in particular looking at the function of Hippo pathway (https://onlinelibrary.wiley.com/doi/10.1002/adhm.202300458).
Overall, we do not have enough solid data to publish any mechanistic model explaining how UBIAD1 regulates ACSL4 levels under these conditions. We do have indeed an explanation on how UBIAD1 regulate GPX4 and ferroptosis (see R#3.5).

Regarding the apparent incongruency between Fig. 6g and new Suppl. Fig. 6d, it is common for different cell lines to regulate GPX4 expression with varying magnitudes upon treatment with ferroptosis inducers (e.g., https://www.mdpi.com/1422-0067/23/16/9014). We agree that it's unusual that GPx4 increases upon ferroptosis treatment in MDA-MB-231 cells, however, the observed increase of GPX4, could be probably related to compensatory mechanisms.

#2.5. The authors suggest that UBIAD1 regulates ferroptosis by altering PM mechanical properties and stiffness as well as ECM/AKT2 signaling (the last sentence in page 30). However, this statement lacks relevant supporting data. For example, based on this claim, altering PM mechanical properties and stiffness or ECM/AKT2 signaling should affect ferroptosis sensitivity.

R#2.5. We thank the Reviewer for this comment. This is our claim as it has been previously shown in PMID: 35165418, PMID: 29934307, PMID: 35165418, PMID: 33707306 and PMID: 37022980. This is now supported by data shown in Fig. A.f,, Fig. H, and Fig. J.c.

#2.6. Fig. 2i-k, does Ubiad1 KO affects the animal survival of KBP models? (as they showed in MMTV-NeuT models, Fig. 2b)

R#2.6. The Reviewer's comment highlights a significant challenge posed by the utilization of these mouse genetic models for studying breast cancer. Specifically, what we refer to as "animal survival" in Fig. 3b and Suppl. Fig. 3h actually pertains to "progression-free survival" or "tumor-free mouse survival." This term corresponds to the time required for the detection of the first palpable mammary tumor. Assessing survival in the context of MMTV-PyMT and MMTV-NeuT models would not yield additional insights since the animals, even with substantial tumors, displayed no indications of distress or discomfort.
Consequently, the concept of "animal survival" would rely solely on reaching a predetermined tumor volume. However, this information is already encompassed within the tumor growth curve depicted in Fig. 3c and Suppl. Fig. 3i. For the KBP model, it proved unfeasible to generate comparable graphs to

those in Fig. 3b and Suppl. Fig. 3h. This is because these mice develop detectable tumors with a T_{50} of 298 days (as per PMID: 11694875), which does not align with our research timeline. In light of this, we chose to harvest the mammary glands at a fixed time point and evaluate the dimensions of the neoplastic area before tumors become palpable. Other measurement methods would demand a significant investment of time and resources from both a temporal and animal welfare perspective yet would not contribute novel or crucial information to our study.

#2.7. Can the authors comment on why only AKT2 (but not AKT1) activation is affected by UBIAD1 and CoQ?

R#2.7. As pointed out by the Reviewer #2, we observed a more pronounced effect of CoQ_{10} overload on AKT2 activation rather than AKT1 in our MDA-MB-231 TNBC cell line. This observation aligns with evidence suggesting a predominant localization of AKT2, as opposed to AKT1, on the plasma membrane, particularly within lipid rafts (PMID: 31752925). This indicates that disrupting lipid rafts assembly, whether through CoQ_{10} administration or UBIAD1 overexpression, might have a more direct impact on AKT2 than on AKT1.
Furthermore, studies have shown that AKT2, rather than AKT1, is the main isoform active in breast cancer metastases (PMID: 33227065). The MDA-MB-231 cell line used in our study was indeed derived from a pleural effusion of a 51-year-old Caucasian female with metastatic mammary adenocarcinoma (source: https://www.atcc.org/products/htb-26).

#2.8. Page 29 “ACSL4 is an enzyme that modifies monounsaturated fatty acids into polyunsaturated fatty acids, the main target of lipid peroxidation.” This statement is wrong. ACSL4 functions to catalyze the ligation of CoA to free PUFAs to generate PUFA-CoA.

R#2.8. We thank the reviewer to point this out. We agree that the sentence is not correct. We have now changed the sentence accordingly.

Reviewer #3:

Major issues:

#3.1- Fig 1i. The authors show a reduction in Ubiad1 expression in TNBC lines. Does Ubiad1 expression correlate with CoQ10 levels? Can the authors measure CoQ10 levels in those cell lines to see the correlation (if any)? That is important in order to understand if the effects of Ubiad1 depend on its function in CoQ biosynthesis.

R#3.1. We thank the reviewer for this request since we did not consider providing such information before. We have now measured total CoQ_{10} levels in all BC cell lines (see below or Fig. A.d). We detected that MCF10A cells contain a statistically significant higher amount of CoQ_{10}, with respect to other tumorigenic cells lines (T47D, BT474, HCC202, HCC1143, MDA-MB-468, MDA-MB-231, Hs578T). These quantitative analyses have been done considering both mitochondrial and cytosolic CoQ_{10} pools since it is not possible to isolate separately mitochondria vs plasma membrane/Golgi and quantify their respective CoQ10 pool (one produced only by the plasma membrane and Golgi apparatus UBIAD1 or by the mitochondrial COQ2. These data have now been included in Suppl. Fig. 2e.

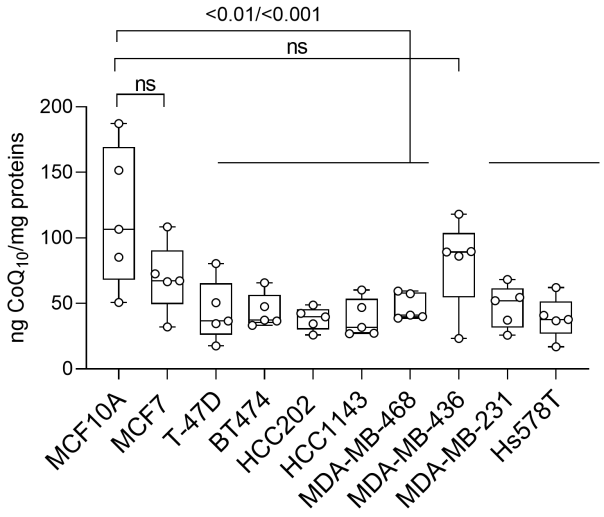


Figure K: *Mass spectrometry analysis to evaluate total CoQ_{10} levels normalized over mg of proteins in the non-tumorigenic breast line MCF10A and a panel of BC cell lines. Each dot represents a biological replicate. Mean ± SEM. Adjusted p-value was calculated using one-way Anova followed by Tukey's multiple comparisons test.*

#3.2- As a biological tool to develop this study, the authors have generated an Ubiad1 KO mouse model, which shows embryonic lethality, apparently due to CoQ deficiency. However, CoQ levels were only measured in heterozygous embryos. Can the authors extend those measurements to homozygous mutant mice? Also, the authors briefly described the phenotype of heterozygous mice, but they do not mention the phenotype of homozygous mutant mice. Do they also show hemorrhagic phenotype? A similar model was previously generated by Nakagawa and colleagues (doi: 10.1371/journal.pone.0104078). In that study, the authors claimed that Ubiad1 is not involved in CoQ biosynthesis in mouse. Could the authors comment the differences in the conclusions from both studies? Is Ubiad1 involved in CoQ biosynthesis in mouse? What about in human?

R#3.2. We express our gratitude to the Reviewer for raising this question. Unfortunately, we are unable to provide measurements of $CoQ_{9/10}$ in homozygous *Ubiad1* knockout mice since they die very early at E7.5-9.5 in the womb and at E8.5-10.5 *Ubiad1*$^{-/-}$ embryos are likely resorbed. Therefore, even when attempting to collect embryos at earlier stages, we were unable to confidently isolate any embryos without potential tissue contamination from the mother, that makes the analysis unreliable. We have taken into consideration the data generated by Nakagawa and colleagues. First of all, they used a different mouse model where *Ubiad1* has been conditionally deleted after crossed with a Cre-reporter line. We have instead generated a complete null allele (to our knowledge never published before). Although Nakagawa and colleagues declare that $CoQ_{9/10}$ levels do not differ between

Ubiad1$^{+/+}$ and *Ubiad1*$^{+/-}$ embryos in comparison to *Ubiad1*$^{-/-}$ embryos, they demonstrate a substantial rescue of embryonic lethality through administration of both MK-4 and CoQ_{10} to pregnant females (their Table 2). Furthermore, they do not observe any embryonic lethality in *Ubiad1*$^{+/-}$ embryos (their Table 1). In contrast, we note a distinct reduction in the number of *Ubiad1*$^{+/-}$ offspring compared to the number expected according to Mendelian inheritance laws (our Suppl. Fig. 3b). This observation, along with our inability to achieve any form of embryonic lethality rescue—whether through CoQ_{10} or MK-4 administration—in *Ubiad1*$^{-/-}$ embryos (only achieving such rescue in *Ubiad1*$^{+/-}$ embryos with CoQ_{10} and NAC, as depicted in our Suppl. Fig. 3f)—leads us to speculate that our mice exhibit a more pronounced phenotype. This difference in phenotype could be attributed to the divergent strategies employed: NAkagawa's model relies on Cre-loxP recombination (which may not achieve 100% efficiency), while our model centers on the disruption of exon 1 through the insertion of a stable cassette.
Finally, clear evidence from our previous publication has demonstrated that UBIAD1 functions as a CoQ_{10}-biosynthetic enzyme in *Danio rerio* and human primary endothelial cells (PMID: 23374346). In this study, we observed that *Ubiad1*$^{+/-}$ mice exhibit reduced levels of CoQ_9 in the whole-body embryo (Fig. Suppl. Fig. 3e), and similarly in the tumors of genetic models of breast cancer (Fig. L). Additionally, the overexpression of UBIAD1 results in an increased cellular content of $CoQ_{9/10}$ in both mouse and human cancer cells (Fig. 4l and Suppl. Fig. 4b). Therefore, we affirm that UBIAD1 functions as a CoQ_{10}-biosynthetic enzyme in both mice and humans.

#3.3- Fig 2f. The authors show a reduction in Ubiad1 expression in the mouse derived tumors. How are the levels of CoQ9 in those tumors? Again, those data are important in order to understand if the effects of Ubiad1 depend on its function in CoQ biosynthesis.

R#3.3. To answer this question, we have measured by HPLC-MS CoQ_9 levels in tumors derived from MMTV-NeuT;*Ubiad1*$^{+/+}$ vs. MMTV-NeuT;*Ubiad1*$^{+/-}$ mice. We detected an important reduction of CoQ_9 when *Ubiad1* is partially deleted. Unfortunately, this difference does not appear to be statistically significant (0.056 vs. 0.050). Please see new Fig. 3g and Fig. L.

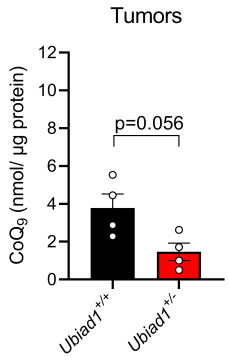
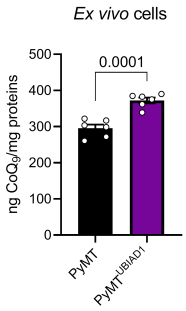


***Figure L**: Level of CoQ_9 was measured by Mass Spectrometry on whole tumor lysates and normalized to protein concentration. Each dot represents a single tumor. Mean ± SEM. P-value was calculated using Mann Whitney test.*

To further investigate this point, we also have measured CoQ_9 levels in PyMT^{UBIAD1} *ex vivo* cells and we here detected a statistically significant increase of CoQ_9 supporting the role of UBIAD1 in regulating the levels of $CoQ_{9/10}$ in mouse cancer cells (see graph below, from Fig. 4l).



#3.4- Fig 3f and Supplementary Fig 3d,e. If the observed effects of Ubiad1 overexpression are due to CoQ10-dependent mechanisms, can the authors achieve similar results with CoQ10 supplementation?

R#3.4. We appreciate the Reviewer's comments. To address this question, we initially tested the *in vitro* proliferation of MDA-MB-231 cells under treatment with 20µM of CoQ_{10}. No differences were detected in the number of cells between Vehicle- vs. CoQ_{10}-treated cells at all the time points tested (Fig. M.a, and new Suppl. Fig 1d). However, although no differences were observed in cell proliferation, we did observe a remarkable reduction in the number of MDA-MB-231 cells invading through a Matrigel-coated membrane upon treatment with CoQ_{10} (Fig. M.b).
Moving to *in vivo* experiments, we injected MDA-MB-231 cells into the mammary fat pad of NSG female mice and measured the percentage of eGFP+ circulating tumor cells (CTCs) in the bloodstream and the percentage of eGFP+ cells in the lungs of mice treated for 21 days with either Vehicle or CoQ_{10} in the drinking water at the concentration of 100µM (PMID: 36922562). By normalizing over the primary tumor volume, as suggested by Reviewer #2, either the percentage of eGFP+ cells found in the bloodstream or the percentage of eGFP+ cells upon dissociation of the lung tissue, we observed a decrease in CTCs and cancer cells in the lungs in the mice treated with CoQ_{10} (Fig. M.c). This corroborates the evidence obtained using the genetic approach regarding metastasis formation *in vivo*.
We are not comfortable to add these results to the main manuscript since we do not have a control on the amount of CoQ_{10} effectively absorbed by the animals in their tissues vs. tumor although from a rapid analysis of the mice blood it seems that CoQ_{10} treated mice have higher plasma CoQ_{10} concentration.

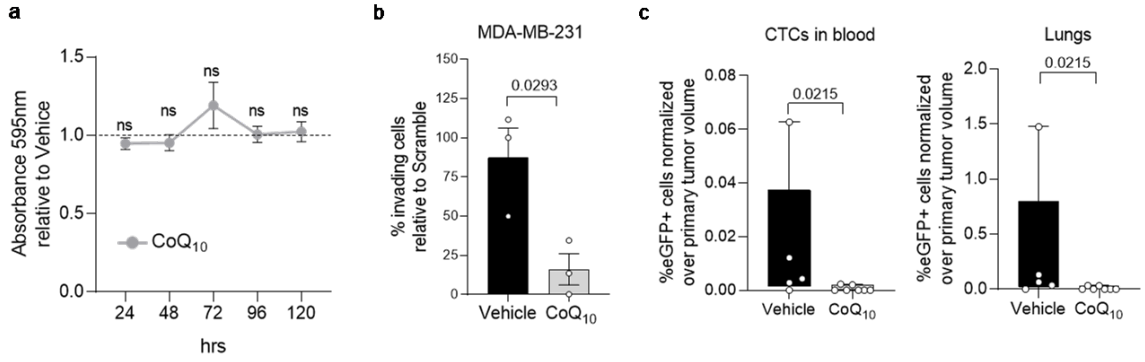


Figure M: a *Crystal violet assay to measure MDA-MB-231 cell number at different time points upon treatment with Vehicle or 20µM CoQ_{10}. Data are showed as absorbance at 595nm relative to Vehicle. Each point represents the mean ± SEM of three independent biological replicates. P-value was calculated using one-sample t-test to compare the mean of CoQ_{10}-treated per each time point with 1.0. ns, not significant.* ***b*** *Invasion assay of MDA-MB-231 cells through a Matrigel-coated membrane. Cells were allowed to invade for 24h in presence of either Vehicle or 20µM CoQ_{10}. Data are expressed as percentage of invading cells compare to Vehicle (set at 100%). Each dot represents a biological replicate. Data are shown as mean ± SEM. P-value was calculated using two-tailed un-paired t-test.* ***c*** *Percentage of Circulated Tumor Cells (CTCs) eGFP+ over total cells in mouse whole blood and % eGFP+ cells in mouse lungs. Animals were injected with $5x10^5$ MDA-MB-231 Luc-eGFP cells in the mammary fat pad and treated with 100µM of CoQ_{10} in the drinking water for 21 days. The percentage is normalized over primary tumor volume. Each dot represents a single mouse sample. Data are shown as mean ± SEM. P-value was calculated using Mann Whitney test.*

#3.5- The results about ferroptosis are confusing. Both FSP1 (through CoQ redox state) and GPx4 (through GSH redox state) prevent ferroptosis by reducing lipid peroxidation. However, Ubiad1 overexpression, which may increase CoQ10 in plasma membrane, reduces the resistance to ferroptosis; and cancer cells with low Ubiad1 levels, which may have decreased CoQ in plasma membrane, have more resistance to ferroptosis. These data seem to contradict the mechanism of ferroptosis involving CoQ. Those results can be explained by an inverse correlation between Ubiad1 levels and Gpx4 levels. Is that the explanation that the authors propose? If yes, how can Ubiad1 levels influence GPx4 levels? Could CoQ10 supplementation induce similar effects on GPx4 and ferroptosis? Perhaps, GPx4-dependent ferroptosis should be added in Fig 6 (bottom picture). Moreover, a schematic supplementary figure about the different components of ferroptosis (iron, ROS, lipid peroxidation, GPx4, GSH, FSP1 and CoQ10) and the proposed involvement of Ubiad1 would help the reader to better understand the results.

R#3.5. We thank the Reviewer for this point. This question is linked to the Reviewer's comment #2.3 and #2.4. It is indeed a little counteractive to understand the proposed model.
However, it is important to consider that previous published data on CoQ_{10} relies on indirect evidence of its function in ferroptosis. Indeed, from most of the published work on this topic, the results show the loading of cells with CoQ_{10} without information on the CoQ_{10} redox state, such as the amount of reduced vs. oxidized CoQ_{10} within the cells upon treatment. We think we have to distinguish between the endogenous synthesis of a metabolite by expressing its forming enzyme (e.g UBIAD1) and the exogenous loading of cells with the metabolite.
GPX4/GSH and FSP1/CoQ_{10} are two major anti-ferroptosis systems that prevent lipid ROS-induced cell death. Mevalonate pathway (MVA) protects cells from ferroptosis by two parallel mechanisms: 1) by providing the lipophilic antioxidant CoQ_{10} for FSP1; 2) by generating isopentenyl pyrophosphate (IPP), a substrate for Selenocysteine (Sec)-tRNA modification for selenoprotein GPX4 translation. Selenoproteins are a class of proteins composed of selenocysteine, the 21st amino acid not found in the codon table. Selenocysteine is decoded by the stop codon UGA and synthesized on its tRNA. The Sec-tRNA incorporates Sec into selenoproteins such as GPX4 (PMID: 33268902).
We showed that in MDA-MB-231 there is a very low amount of FSP1, therefore CoQ_{10} cannot promptly exert its antioxidant function (Fig. I).
We then consider that MDA-MB-231 and possibly other TNBC use the mevalonate pathway (MVA) and its intermediates such IPP and DMAPP to support selenoproteins and, thus, GPX4 synthesis (PMID: 33358111, PMID: 31105042) rather than using CoQ_{10}/FSP1 to protect cells from ferroptosis. Accordingly, we can therefore hypothesize that when UBIAD1 is low (TNBC), MVA intermediates are used to support the DMAPP-tRNASelCys-GPX4 branch while when UBIAD1 is high MVA intermediates are needed to support CoQ_{10} synthesis reducing GPX4 levels: this in turn leads to higher sensibility to ferroptosis since there is a reduced level of the selenoprotein GPX4 and an increased amount of oxidised CoQ10 due to the lack of FSP1.
To support this claim, we treated MDA-MB-231 cells with CoQ_{10} (see Fig. N) and we do not see a decrease of GPX4 levels suggesting that in these conditions we are not altering GPX4 synthesis. Accordingly, since FSP1 is still low expressed in MDA-MB-231, the oxidized CoQ_{10} loaded could be pro-ferroptotic rather than anti-ferroptotic, explaining the results showed in Fig. 6h.

[Redacted]

Figure N: *Western blot analysis of GPx4 expression in MDA-MB-231 treated for 24h with 0.45uM RSL3, 20μM CoQ_{10} or the combination. ACTIN was used as loading control. Uncropped blots at the end of the rebuttal letter.*

As suggested by the Reviewer we have now revised our model and prepare a new Fig. 6 (now Fig. 7) including the effect of UBIAD1/CoQ_{10} on GPX4 levels.

Minor issues:
#3.6- Supplementary Fig 2e. The figure indicates -/-. I guess it must be +/- because CoQ deficiency is not very severe. In that case, which are the CoQ9 levels in the -/- mice?

R#3.6. We thank the Reviewer, we apologize for the mistake and have change the figure accordingly.

Uncropped Blots for the RL:

Fig. H.b

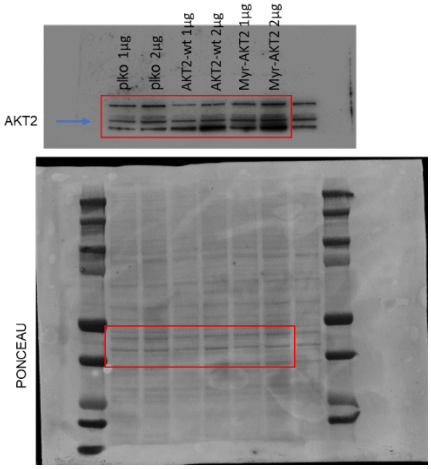


Fig. I.a

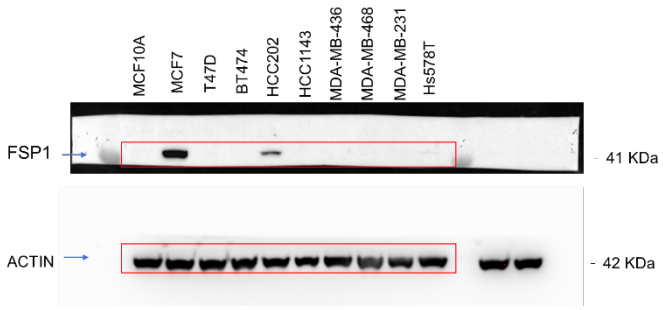


Fig. J.b

[Redacted]

Fig. J.c

[Redacted]

Fig. N

[Redacted]

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The authors addressed my concerns with the new additions to the revised manuscript. The transparency in discussing their results in the rebuttal and the manuscript is commendable; so is their effort to discuss the complexity of the study, particularly with respect to the dual role of CoQ10 (as a lipid antioxidant and as regulator of the membrane mechanical properties) in the context of breast cancer progression.

One remaining comment is to quantify the changes in cortical actin and focal adhesion in the immunofluorescence staining images of UBIAD-OE and CoQ10-loaded cells vs control. The observed increase in membrane blebbing in these cells aligns with cytoskeletal characteristics of high cortical contractility and low adhesion (consistent with low p-FAK and low attachment on laminin). This is a central point in the paper, and quantitative analyses will help determine the extent to which these cytoskeletal changes are due to CoQ10.

Additionally, it would be beneficial to examine cytoskeletal changes in cells that are treated with ROCK and/or MLCK inhibitors. It is very intriguing that inhibitor-treated cells retain their high cellular rigidity when concomitantly treated with CoQ10. Do the effects of CoQ10 on membrane fluidity independently alter invasion (and cellular stiffness?) regardless of cortical contractility? Answering this question (quantitatively) would reveal to what extent CoQ10 effects are mediated through adhesion and cortical contractility.

Reviewer #2 (Remarks to the Author):

After its revision, the ferroptosis-related findings (Fig. 6) continue to represent a notable weakness in this study. These issues include:

At the conclusion of Fig. 6, the authors stated that “Overall, we conclude that treatment with CoQ10 or UBIAD1 expression sensitizes breast cancer cells to ferroptosis (and consequently reduces ferroptosis resistance) by potentially altering PM mechanical properties and stiffness, ECM/AKT2 survival signaling, and the GPX4 antioxidant system.” However, the authors failed to present any data that definitively establish a causal link between any of the proposed mechanisms and the observed ferroptosis phenotype in UBIAD1 overexpressing or knockdown cells. Despite exploring some rescue approaches (such as overexpression of constitutively active AKT2 and treatment with ROCK and MLCK inhibitors) in previous figures, no direct causal connection to the observed ferroptosis outcome was demonstrated by using these rescue approaches.

The effect of UBAD1 overexpression on GPX4 was weak and only revealed under a ferroptosis inducer treatment. The mechanisms behind this remain unknown.

The authors propose that CoQ enhances breast cancer cell sensitivity to ferroptosis inducers. However, this counterintuitive observation was only demonstrated in MDA-MB231 cells, and the ferroptosis-promoting effect by CoQ shown in Fig. 6h appears notably weak.

To substantiate this surprising conclusion, further testing across various breast cancer cell lines is necessary. In these studies, they should present dose curve data across different doses of a ferroptosis inducer.

The author posited that the ferroptosis-promoting effect caused by UBAD1 or CoQ might be due to the absence or low expression of FSP1 in particular breast cancer cells. To strengthen their assertion about the general role of UBAD1 or CoQ in promoting ferroptosis within breast cancer cells, exploring public RNAseq analysis to compare FSP1 expression across a wide spectrum of breast cancer cells against its expression in normal mammary epithelial cells or cancer cells from other cancer types would be helpful. This approach could clarify whether FSP1 is broadly downregulated in breast cancer cells or just downregulated in the specific cell lines chosen by the authors.

The description in Y axis of Fig. 1h, i was cut out.

Reviewer #3 (Remarks to the Author):

The authors have offered reasoned explanations addressing my previous concerns, reinforcing these explanations with supplementary experiments.

REVIEWER COMMENTS

Reviewer #1:

#1.1. One remaining comment is to quantify the changes in cortical actin and focal adhesion in the immunofluorescence staining images of UBIAD-OE and CoQ10-loaded cells vs control. The observed increase in membrane blebbing in these cells aligns with cytoskeletal characteristics of high cortical contractility and low adhesion (consistent with low p-FAK and low attachment on laminin). This is a central point in the paper, and quantitative analyses will help determine the extent to which these cytoskeletal changes are due to CoQ10.

R 1.1: We thank the Referee for his/her comments. We have now quantified the changes in cortical actin and focal adhesion in the immunofluorescence staining images of CoQ10-loaded and UBIAD-OE cells vs control as suggested. These data have been now included in Suppl Fig. 1g,h and Suppl Fig. 5j,k, respectively. These quantifications are statistically significant and confirm that the increase in membrane blebbing aligns with cytoskeletal characteristics of high cortical contractility and low adhesion.

#1.2. Additionally, it would be beneficial to examine cytoskeletal changes in cells that are treated with ROCK and/or MLCK inhibitors. It is very intriguing that inhibitor-treated cells retain their high cellular rigidity when concomitantly treated with CoQ10. Do the effects of CoQ10 on membrane fluidity independently alter invasion (and cellular stiffness?) regardless of cortical contractility? Answering this question (quantitatively) would reveal to what extent CoQ10 effects are mediated through adhesion and cortical contractility.

R 1.2: We thank the reviewer for this question. We have indeed repeated the invasion experiments (see new Fig. 1i) and immunofluorescence analyses (new Suppl. Fig. 1i-k and Suppl. Fig. 5l-n) to examine cytoskeletal changes in cells that are treated with ROCK and/or MLCK inhibitors.
We indeed showed that while ROCK and/or MLCK inhibitors increase invasion and lamellipodia/FA formation, such effect is counteracted by CoQ10 suggesting unequivocally that CoQ10 acts by altering cortical contractility.

Reviewer #2:

#2.1. At the conclusion of Fig. 6, the authors stated that “Overall, we conclude that treatment with CoQ10 or UBIAD1 expression sensitizes breast cancer cells to ferroptosis (and consequently reduces ferroptosis resistance) by potentially altering PM mechanical properties and stiffness, ECM/AKT2 survival signaling, and the GPX4 antioxidant system.” However, the authors failed to present any data that definitively establish a causal link between any of the proposed mechanisms and the observed ferroptosis phenotype in UBIAD1 overexpressing or knockdown cells. Despite exploring some rescue approaches (such as overexpression of constitutively active AKT2 and treatment with ROCK and MLCK inhibitors) in previous figures, no direct causal connection to the observed ferroptosis outcome was demonstrated by using these rescue approaches.

R 2.1: These experiments, which include rescue experiments, were conducted to address concerns regarding the role of UBIAD1/CoQ10 in regulating cell stiffness/softness (Ref 1’s request). Therefore, the primary aim of these experiments was not to establish a causal link between the proposed mechanisms and the observed *ferroptosis phenotype* in UBIAD1-overexpressing cells.

To address this additional request from Ref.2, we have performed novel rescue experiments specifically for the ferroptosis sensitivity using ROCK and MLCK inhibitors, active-AKT2 to prove the causal connection. While there is no causative role between AKT2 activation and UBIAD1/CoQ10 levels in ferroptosis sensitivity (see Figure A), we found a strong inverse correlation between UBIAD1 expression and ROCK and MLCK inhibitors (see new Fig. 6c). In details while ROCK and MLCK inhibitors treatment make MDA-MB-231 cells less sensitive to RSL3 treatment, such effect is less pronounced in UBIAD1-expressing cells suggesting that UBIAD1/CoQ10 act at the level of PM cortical actin to influence cell sensitivity to ferroptosis.

Figure A: *Cytofluorimetric analysis of cellular lipid peroxidation levels using BODIPY C11 staining in MDA-MB-231 cells transfected with Scramble (Scr), UBIAD1^{OE}, and UBIAD1^{OE} with Myr-AKT2 (constitutively active form of AKT2) overexpression plasmid, and treated with either Vehicle (DMSO) or RSL3 (0.45μM) for 12 hours. The fluorescent intensity signal was quantified as the ratio between oxidized and reduced BODIPY C11. Data are expressed as fold change over DMSO-treated MDA-MB-231 cells. Each dot represents a biological replicate. Mean ± SEM. Adjusted p-value was calculated using one-way Anova followed by Tukey's multiple comparisons test. ns, not significant.*

Please see also replies 2.3 and 2.4.

#2.2. The effect of UBAD1 overexpression on GPX4 was weak and only revealed under a ferroptosis inducer treatment. The mechanisms behind this remain unknown.

R 2.2: We gently, by firmly, disagree with the referee's comments. The effect on GPX4 was shown both in ex vivo tumor tissue PyMT (Fig. 6h) and MDA-MB-231 cell line (Suppl Fig. 6d) and achieved by using two different ferroptosis inducers, Erastin and RLS3. The effect of such ferroptosis inducers was tested on MDA-231, MDA-MB-436, Hs578T, and PyMT cells (Fig. 6a-g and suppl Fig. 6b-c). Acsl4 was used as an independent marker for ferroptosis.
Overall, we believe that the effect of UBIAD1 overexpression on ferroptosis is quite significant. Despite ongoing debates regarding the identification of ferroptosis markers, our RNA-seq analysis reveals that more than 27 of the 203 differentially expressed genes (including ALDH2, ALDOA, C3, CELF2, CEMIP, COL12A1, CREB3L1, CRIM1, CST1, CTGF, CYP1A1, EGR1, EPO, FKBP1A, FOSL2, HMGB2, IGFBP6, IGFBP7, MIR4435-2HG, NQO1, OIP5, RAB13, S100A10, SCD, SQSTM1, SRC, and THBS1) in UBIAD1^{OE} cells have been previously identified as associated with ferroptosis in multiple studies.
It is well known that ferroptosis is not a physiological basal condition found in cell lines or tissues and researchers working on ferroptosis need to engage such process *in vitro* and *in vivo* using ferroptosis inducers. Also, established markers for ferroptosis are still undefined and treatment with ferroptosis inducers is the only way to study ferroptosis specifically in *in vitro* settings.

Please see new data Fig. 6

#2.3. The authors propose that CoQ enhances breast cancer cell sensitivity to ferroptosis inducers. However, this counterintuitive observation was only demonstrated in MDA-MB-231 cells, and the ferroptosis-promoting effect by CoQ shown in Fig. 6h appears notably weak. To substantiate this surprising conclusion, further testing across various breast cancer cell lines is necessary. In these studies, they should present dose curve data across different doses of a ferroptosis inducer.

R 2.3: We did not implement Fig. 6h since in the first round of revision of this manuscript Ref 2 did not ask for specific questions to demonstrate such an effect also on different BC and/or different doses.
We would like to remind the Referee that in Fig. 6d-g and Suppl Fig. 6b-c, the effect of UBIAD1 expression on ferroptosis sensitivity has been already shown using different doses and on multiple models such as MDA-MB-231, MDA-MB-436, Hs578T and tumor-derived PyMT cultures.
In addition, we have now implemented these data by using BC with FSP1^{High} compared to FSP1^{Low}. We tested then MCF7 (FSP1^{High}) and MDA-231 (FSP1^{Low}) regarding sensitivity to the ferroptosis inducers RLS3 for lipid peroxidation (new Suppl Fig. 6i) and its correlation with CoQ10 treatments for cell survival (new Suppl Fig. 6l). Dose curve data across different doses of a ferroptosis inducer across various breast cancer cell lines is shown.
These data suggest that when BC cells express low levels of FSP1 they are more sensitive to CoQ10 treatments since they cannot convert CoQ10 in the reduced form efficiently.

#2.4 The author posited that the ferroptosis-promoting effect caused by UBAD1 or CoQ might be due to the absence or low expression of FSP1 in particular breast cancer cells. To strengthen their assertion about the general role of UBAD1 or CoQ in promoting ferroptosis within breast cancer cells, exploring public RNAseq analysis to compare FSP1 expression across a wide spectrum of breast cancer cells against its expression in normal mammary epithelial cells or cancer cells from other cancer types would be helpful. This approach could clarify whether FSP1 is broadly downregulated in breast cancer cells or just downregulated in the specific cell lines chosen by the authors.

R 2.4: We have recovered such publicly available information, and we are here reporting several independent works showing that normal breasts expressed high levels of FSP1/AIFM2 compared to other tissues. Using The Protein Atlas database and looking at RNA tissue expression for AIFM2 is evident that normal breast is the tissue with the highest expression of FSP1 after adipose tissue.
https://www.proteinatlas.org/ENSG00000042286-AIFM2/tissue#rna_expression

RNA EXPRESSION OVERVIEW[i]

Consensus dataset[i]

RNA tissue specificity: Low tissue specificity

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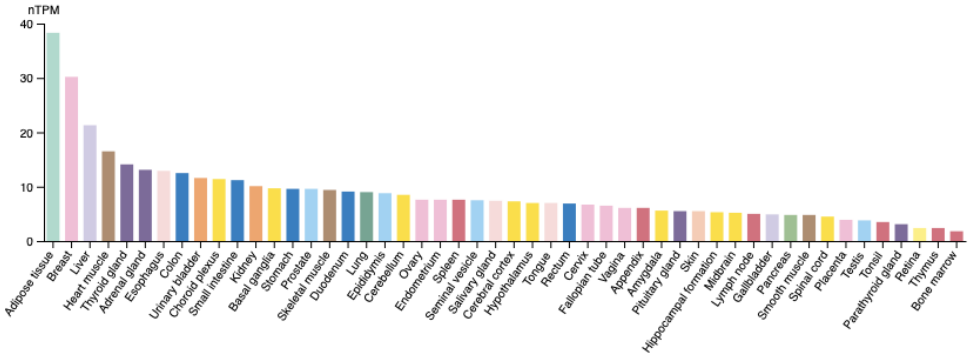

HPA dataset[i]

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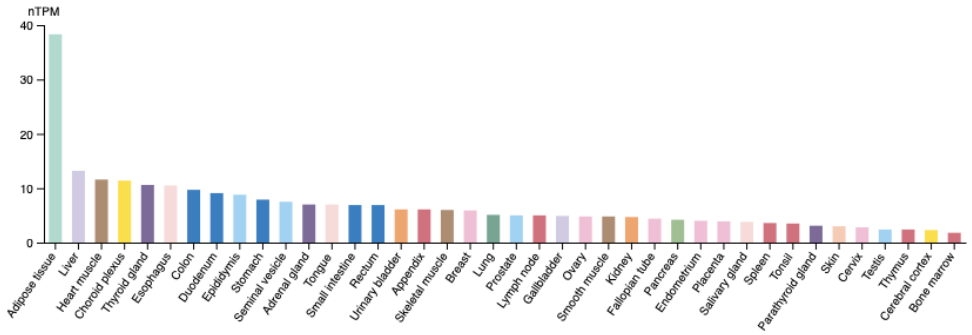

GTEx dataset[i]

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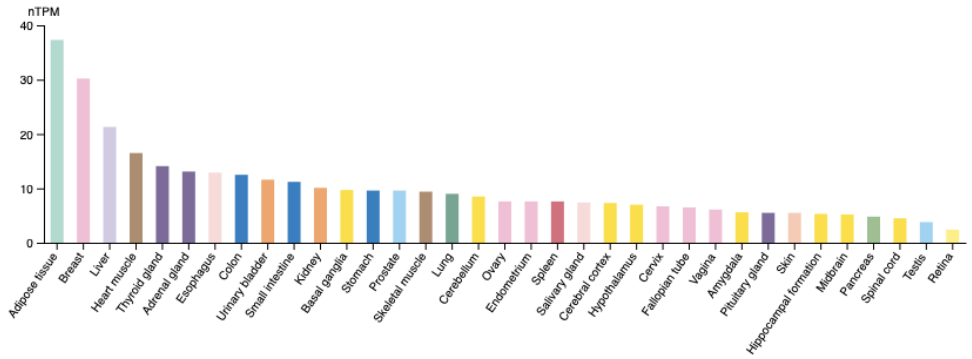

FANTOM EXPRESSION OVERVIEW

FANTOM5 dataset[i]

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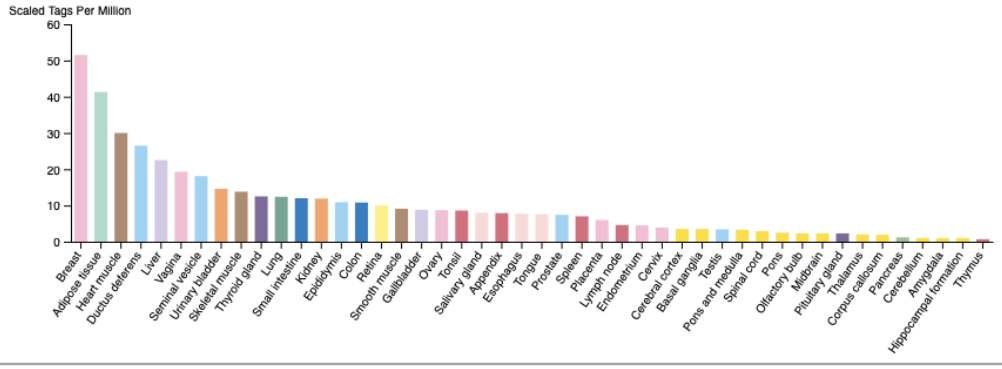
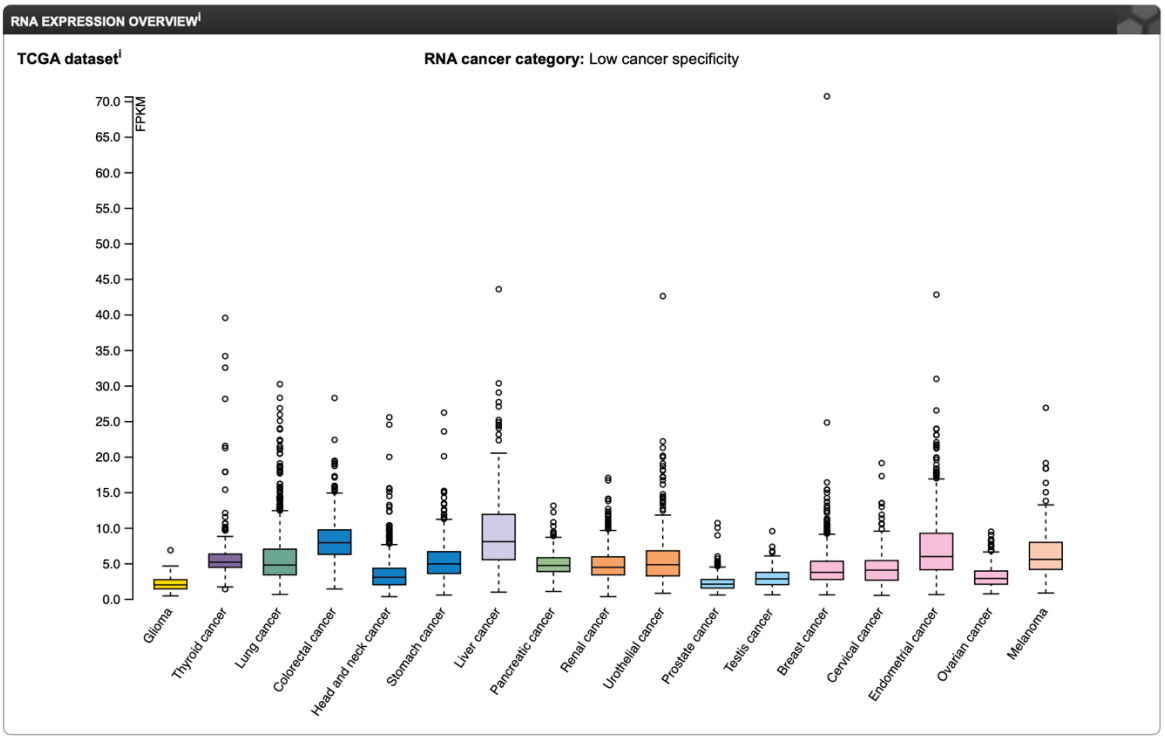
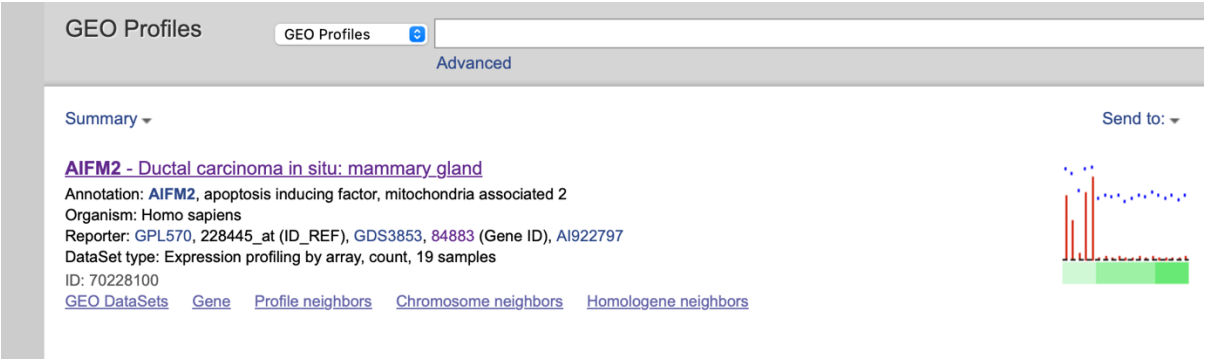


On the contrary, when utilizing the TCGA RNA expression dataset to assess FSP1 expression across various cancer types, it is evident that breast cancer exhibits not an elevated, but rather a medium/low expression in comparison to other cancers. This observation indicates a notable reduction in FSP1 expression during the transition from a healthy mammary gland to breast cancer.



We also discover two independent studies showing that breast cancer initiation leads to a loss/decrease of FSP1/AIFM2 expression: PMID: 21314937 (https://pubmed.ncbi.nlm.nih.gov/21314937/ and https://www.ncbi.nlm.nih.gov/geo/tools/profileGraph.cgi?ID=GDS3853:228445_at) and PMID: 34722530 (https://www.frontiersin.org/articles/10.3389/fcell.2021.748925/).



Also mRNA levels of FSP1/ AIFM2 using Oncomime database showed that AIFM2 is under-expressed in breast cancer https://www.frontiersin.org/articles/10.3389/fcell.2021.748925/full).

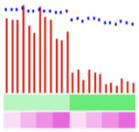
Moreover, Tamoxifen-resistant cells exhibit a decreased FSP1 expression, further substantiating the evidence that TNBC cells potentially express lower levels of FSP1 compared to cells reliant on estrogen-receptor signaling (https://www.ncbi.nlm.nih.gov/geo/tools/profileGraph.cgi?ID=GDS4051:224461_s_at).

Summary

Send to:

AIFM2 - Tamoxifen-resistant **breast cancer** cell line MCF7 subclones

Annotation: **AIFM2**, apoptosis inducing factor, mitochondria associated 2
Organism: Homo sapiens
Reporter: GPL570, 224461_s_at (ID_REF), GDS4051, 84883 (Gene ID), BC006121
DataSet type: Expression profiling by array, count, 24 samples
ID: 77287223
GEO DataSets Gene Profile neighbors Chromosome neighbors Homologene neighbors



As asked by the reviewer, such information has been recovered by public RNAseq analyses by comparing FSP1 expression across a wide spectrum of normal and neoplastic breast tissues. The FSP1 analyses showed in our paper was on breast cancer cell lines coherent with the rest of the paper and were not chosen *ad hoc*.

To further confirm the involvement of FSP1 in CoQ10-mediated sensitivity to ferroptosis we have analyzed FSP1 levels in CoQ10-treated cells after ferroptosis inductions (see new Fig. 6j). It is statistically clear that CoQ10 treatment makes FSP1 protein less stable after RLS3 explaining the increased sensitivity of the CoQ10-treated cells for ferroptosis inducers (Fig. 6h). We strongly believe that the stability/levels of the NADH-dependent oxidoreductases FSP1 drive different response on ferroptosis.

Interestingly, it has been recently shown that the myristoylation of the N-terminal of FSP1 is the key reason for its membrane localization and ferroptosis inhibition: when mutated at the myristoylation site (G2A) FSP1 is not localized properly at the plasma membrane domain (and possibly to lipid rafts) and had impaired ferroptosis inhibition activity (see https://doi.org/10.1038/s41467-023-36446-8 and 10.1038/s41586-019-1705-2). Therefore, UBIAD1/CoQ10 by reducing the number of lipids rafts and altering cortical contractility could act by reducing the stability of FSP1 and then affecting sensitivity to ferroptosis.

#2.5. *The description in the Y axis of Fig. 1h, I was cut out.*

R 2.5: We apologize for that due to a format issue during the upload of the original file to the Nature submission website.

Reviewer #3:

Ref 3 by complimenting with us about reinforcing with new data our findings, do not ask for further questions.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors addressed all my concerns.

Reviewer 2 raises very important points. I think that these points could be addressed by rewording of the results and adding discussion points highlighting the shortcomings of the findings. In my opinion, this does not take away from their strength of the conclusions.

Here are some suggestions:

1. The first two points raised by reviewer 2 are totally valid in that the effects of CoQ10/UBIAD1oe are only partial. This should be made clear when interpreting the results (ie, not using words like “blunted” and “lost”). Nonetheless, this is not unexpected in such experiments since the combination of Y2736 and ML7 is so powerful in decreasing cortical contractility that I find it impressive that CoQ10 or UBIAD1oe had any significant effects at all. The authors did not say that CoQ10 is directly interacting with ROCK and MLCK. The inhibitors were used as a tool to diminish cortical contractility. Having said that, the authors need to include a more detailed discussion about how CoQ10 alters cortical contractility independently of these two central myosin activators. In my opinion, it would be unreasonable to ask them to mechanistically investigate the regulation of cortical actin since this would entail a lot of work and would significantly delay publishing the study (and as is, they have enough to make their case about changes in cellular rigidity).

2. Importantly, I would like to add that the authors should refrain from talking about “focal adhesion”, such as on page 24, since all their quantification is of membrane protrusion and blebbing (gross cellular morphology). Again, just changing the wording would not diminish their conclusions.

3. Finally, regarding reviewer 2’s point about GPX4 being regulated only under ferroptosis induction, I would like to add that there are differences between PyMT and MDA231 cells: in Fig 6h, GPX4 was not significantly induced in response to RSL3 in control PyMT cells, whereas in Fig S6d, RSL3 increased GPX4 levels in control MDA231 cells. It is important to discuss these points and certainly to re-write the results section to reflect these differences.

Reviewer #2 (Remarks to the Author):

This reviewer appreciates the efforts made by the authors to address review comments, but feels obligated to point out that several statements made by the authors are not consistent with their corresponding data. If the authors do not agree with the reviewer, I suggest that the editor send the manuscript to another reviewer to specifically comment on the following points:

Page 24 “On the other hand, in the presence of the ROCK inhibitor Y27632 and the MLCK inhibitor ML7, cell invasiveness increases, but such effect is blunted when CoQ10 is present (Fig. 1i).” (blue text added during the second revision”) In this statement, “such effect” refers to the cell invasiveness increases caused by Y27632 + ML7 treatment. The wording

“blunted” suggests that the combination of Y27632 + ML7 with CoQ10 (the fourth bar) should result in a minimal change in invasiveness compared to the Y27632 + ML7 treatment (the second bar). However, Fig. 1i does not support this interpretation. The figure shows that while Y27632 + ML7 increases invasiveness and CoQ10 decreases invasiveness, the combination of Y27632 + ML7 + CoQ10 results in an intermediate level of invasiveness. This indicates that the effects of Y27632 + ML7 treatment and CoQ10 treatment may be independent rather than interactive, contradicting the authors' statement.

Likewise, for the statement “we found that while treatment with the ROCK inhibitor Y27632 and the MLCK inhibitor ML7 tends to protect cells from RSL3, such effects are lost in MDA-MB-231 UBIAD1OE cells, suggesting that changes in PM cortical properties might influence ferroptosis sensitivities (Fig. 6c).”, the data in Fig. 6c shows that Y27632 + ML7 still decreases BODIPY C11 staining in UBIAD1-OE cells (compare the 4th and 5th bars), similar to its effect in control cells (compare 2nd and 3rd bars). This again suggests that the effects of UBIAD1 OE and Y27632 + ML7 treatment are independent.

For my previous comment “The effect of UBAD1 overexpression on GPX4 was weak and only revealed under a ferroptosis inducer treatment. The mechanisms behind this remain unknown.” I specifically refer to the minimal changes of GPX4 levels between PyMT and MyMT-UBIAD1 samples under vehicle treatment in Fig. 6h (the first 6 lanes). In fig. S6d, the authors did not compare GPX4 levels between control and UBIAD1-OE cells under vehicle condition. I also commented why UBIAD1 affects GPX4 levels only under a ferroptosis inducer treatment condition, but not under normal condition, seems strange and the underlying mechanism remains unclear.

We thanks ref.1 for his/her comments and suggestions. Please find below our reply.

1. The first two points raised by reviewer 2 are totally valid in that the effects of CoQ10/UBIAD1oe are only partial. This should be made clear when interpreting the results (ie, not using words like “blunted” and “lost”). Nonetheless, this is not unexpected in such experiments since the combination of Y2736 and ML7 is so powerful in decreasing cortical contractility that I find it impressive that CoQ10 or UBIAD1oe had any significant effects at all. The authors did not say that CoQ10 is directly interacting with ROCK and MLCK. The inhibitors were used as a tool to diminish cortical contractility. Having said that, the authors need to include a more detailed discussion about how CoQ10 alters cortical contractility independently of these two central myosin activators. In my opinion, it would be unreasonable to ask them to mechanistically investigate the regulation of cortical actin since this would entail a lot of work and would significantly delay publishing the study (and as is, they have enough to make their case about changes in cellular rigidity).
We completely agree with the reviewer’s points, and we were also really astonished by the fact that CoQ10 can alter ROCK and MLCK inhibitors’ effects in our cell culture model. These data led us to enthusiastically talk about “lost” and “blunted” but we also understand that without a mechanism behind that explaining it, such statements would sound too prevailing and powerful for a reader.
We have now removed the words “blunted” or “lost” and tone down our conclusions throughout the manuscript. We used track changes to easily follow our edits.

2. Importantly, I would like to add that the authors should refrain from talking about “focal adhesion”, such as on page 24, since all their quantification is of membrane protrusion and blebbing (gross cellular morphology). Again, just changing the wording would not diminish their conclusions.
We have been following these suggestions and the “focal adhesion” term has been removed. We have rephrased the paragraph on pag 24 and the discussion section.

3. Finally, regarding reviewer 2’s point about GPX4 being regulated only under ferroptosis induction, I would like to add that there are differences between PyMT and MDA231 cells: in Fig 6h, GPX4 was not significantly induced in response to RSL3 in control PyMT cells, whereas in Fig S6d, RSL3 increased GPX4 levels in control MDA231 cells. It is important to discuss these points and certainly to re-write the results section to reflect these differences.
We agree indeed that there are differences on GPX4 levels when PyMT or MDA-MB-231 cells were treated with RSL3. However, we need to consider that we are comparing two different models: from one side mouse tumor-derived explants (PyMT) and from the other side human breast cancer cell lines (MDA-MB-231). It is therefore not surprising that the two different cellular models react differently to RSL3 treatment. Also, RSL3 has been shown to have different outcomes on different cell lines and models regarding GPX4 induction (e.g. increase or decrease GPX4 levels depending on its concentration, timing of treatment, and different commercial GPX4 antibodies used). The conclusion we want to highlight here is that the expression of UBIAD1 itself can reduce the GPX4 levels compared to scramble upon different ferroptosis stimuli and this happens in both models analysed.
We have also now rephrase the discussion section making this point clearer.

Reviewer #2 (Remarks to the Author):

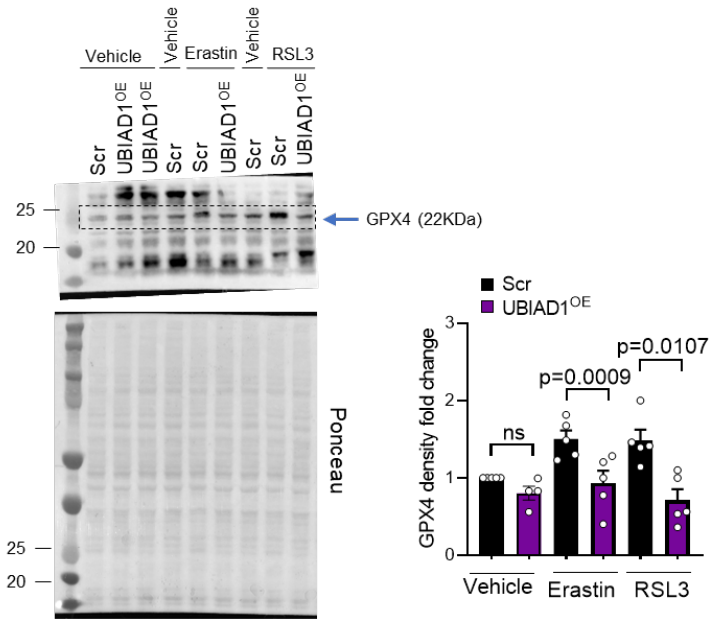
This reviewer appreciates the efforts made by the authors to address review comments, but feels obligated to point out that several statements made by the authors are not consistent with their corresponding data. If the authors do not agree with the reviewer, I suggest that the editor send the manuscript to another reviewer to specifically comment on the following points:

Page 24 “On the other hand, in the presence of the ROCK inhibitor Y27632 and the MLCK inhibitor ML7, cell invasiveness increases, but such effect is blunted when CoQ10 is present (Fig. 1i).” (blue text added during the second revision”) In this statement, “such effect” refers to the cell invasiveness increases caused by Y27632 + ML7 treatment. The wording “blunted” suggests that the combination of Y27632 + ML7 with CoQ10 (the fourth bar) should result in a minimal change in invasiveness compared to the Y27632 + ML7 treatment (the second bar). However, Fig. 1i does not support this interpretation. The figure shows that while Y27632 + ML7 increases invasiveness and CoQ10

decreases invasiveness, the combination of Y27632 + ML7 + CoQ10 results in an intermediate level of invasiveness. This indicates that the effects of Y27632 + ML7 treatment and CoQ10 treatment may be independent rather than interactive, contradicting the authors' statement.
We thank the Referee for these comments. We must say that depending on how you see these data, both an *independent* as well as an *interactive* effect of the two treatments can be proposed. Accordingly, and following also Ref 1 suggestions we have no rephrased and tone down the conclusions.

Likewise, for the statement “we found that while treatment with the ROCK inhibitor Y27632 and the MLCK inhibitor ML7 tends to protect cells from RSL3, such effects are lost in MDA-MB-231 UBIAD1OE cells, suggesting that changes in PM cortical properties might influence ferroptosis sensitivities (Fig. 6c).”, the data in Fig. 6c shows that Y27632 + ML7 still decreases BODIPY C11 staining in UBIAD1-OE cells (compare the 4th and 5th bars), similar to its effect in control cells (compare 2nd and 3rd bars). This again suggests that the effects of UBIAD1 OE and Y27632 + ML7 treatment are independent.
Respectfully, we do not agree on Referee’s comment since our data show that the two different treatments can influence each other evaluated as biological outputs. If the treatments were independent we should not have noticed an intermediate phenotype by IF analyses (Suppl Fig. 1i, 5n). Although we did not identify a specific mechanism to explain why CoQ10 interacts with actin cytoskeletal and/or stifness, we can score a clear intermediate phenotype regarding blebs and cell protrusions suggesting that the two conditions act in opposite ways (see Suppl Fig. 1i, 5n). Possibly, a titration of the different concentrations of both treatments at the same time would have helped to identify the right conditions to reinforce conclusions. We also believe that the cellular stiffness is then linked to changes in cell sensitivity to ferroptosis.

For my previous comment “The effect of UBAD1 overexpression on GPX4 was weak and only revealed under a ferroptosis inducer treatment. The mechanisms behind this remain unknown.” I specifically refer to the minimal changes of GPX4 levels between PyMT and MyMT-UBIAD1 samples under vehicle treatment in Fig. 6h (the first 6 lanes). In fig. S6d, the authors did not compare GPX4 levels between control and UBIAD1-OE cells under vehicle condition. I also commented why UBIAD1 affects GPX4 levels only under a ferroptosis inducer treatment condition, but not under normal condition, seems strange and the underlying mechanism remains unclear.
In Fig. S6d we indeed did not compare GPX4 levels between control and UBIAD1-OE cells under vehicle conditions since there was no difference between these two conditions. Such a conclusion can be also extrapolated from experiments shown in Fig. 6a, 6b, where in untreated conditions there are significant differences between scr and Ubiad1oe cells.
For transparency, we here report our results in untreated conditions. Please see below a representative blot with quantification (each dot is an independent experiment):



We indeed agree that it is unclear why UBIAD1 affects GPX4 levels only under a ferroptosis inducer treatment condition, but not under normal condition. Such observation has now been added in the discussion sections.
Overall, our point here is that nonetheless the models we used (PyMT and MDA-MB-231 cells) UBIAD1 expression impaired GPX4 levels under treatment with different ferroptosis inducers.