nature portfolio

Peer Review File

Hypoxia-induced downregulation of PGK1 crotonylation promotes tumorigenesis by coordinating glycolysis and the TCA cycle

Editorial Note: Parts of this Peer Review File have been redacted as indicated to remove third-party material where no permission to publish could be obtained.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this manuscript, Guo et al described hypoxia induced decreased lysine crotonylation facilitate breast tumor progression. The results presented in the manuscript suggested that ECHS1 interacts with PGK1, leading to downregulation of PGK1 Kcr under hypoxia and thus promoting glycolysis. Overall, this manuscript provides a PTM guided metabolic reprogramming through PGK1 crotonylation in breast cancer progression. However, several key mechanistic experiments are missing to support their hypothesis. In addition, Impact of glycolysis in breast cancer promotion has been well established and the finding in this work could be incremental for the overall framework. The detail review is outlined below:

Major Points:
1. Glycolysis in breast cancer promotion has been well established. Most breast cancer cells have already rely heavily on glycolysis. To support the significance of PGK1 crotonylation in regulating cancer cells mitochondrial metabolic reprogramming, it will be important to validate the relevance by using a suitable spontaneous model.
2. Hypoxia has been previously subjected to regulation of tumor mitochondrial metabolism rewiring. It is not clear to distinguish the contribution between the emphasized PGK1 crotonylation from other well documented hypoxic factors.
3. Multiple elements could regulate the level of Acetyl-CoA, how to distinguish the contribution from metabolic pathways needs to be addressed.
4. The authors claim that ECHS1 can decrease intracellular crotonyl-CoA levels. However, considering that ECHS1 is a mitochondrial-localized protein, authors need to address if ECHS1 can influence crotonyl-CoA levels in the cytosol?
5. It is essential for the authors to assess hypoxic crotonyl-CoA levels in both the cytosol and mitochondria to understand how Hypoxia affects these levels.
6. Where exactly does PGK1 crotonylation occur? If crotonylation happens in the cytosol and PGK1 is subsequently transported to the mitochondria for de-crotonylation by ECHS1, the authors need to provide the data to support this.
7. Similarly, in which cellular compartment does PGK1 de-crotonylation predominantly occur - the cytosol or the mitochondria since author mentioned that ECHS1 could affect the intracellular crotonyl-CoA levels.
8. If PGK1 is crotonylated in the cytosol, is it then shuttled into the mitochondria specifically under Normoxia or Hypoxia?
9. The mechanism by which ECHS1 reduces PGK1 crotonylation is not clear, especially considering that ECHS1 can directly break down crotonyl-CoA, thus reducing its intracellular concentration. Is an interaction between ECHS1 and PGK1 necessary for crotonyl-CoA removal?
10. Figure 5 doesn't adequately elucidate how PGK1 induces P-PDHK1 and p-PDH1 under hypoxic conditions.
11. The impact of the PGK1-pPDHK1-pPDH1 axis on tumor progression isn't clearly described. Most experiments seem to rely solely on sgPGK1 or PGK1-3KR, leaving unanswered questions about how the PGK1-pPDHK1-pPDH1 pathway influences tumor growth both in vitro and in vivo. More cancer biology experiments are needed here to justify authors claim.

Minor Points:
1. Most of the experimental results from a single experiment with n=3. In most cases, data points should be at least n=6.
2. The authors should repeat the results presented in figure 5e. The whole cell lysate shows that Flag-PGK1 overexpression leads to a decrease in ECHS1, which contrasts with the findings in Fig 3b.

Reviewer #2 (Remarks to the Author):

This manuscript by Guo et al. reports the decrease of PGK1 crotonylation under hypoxia and its links to the altered glycolysis and TCA cycle in cancer cells. Lysine crotonylation (Kcr) was originally identified in histones and has recently been detected in a variety of other proteins. In this study, the authors performed quantitative proteomic analysis of the crotonylome in a breast cancer cell line MDA-MB-231 and found that the crotonylation levels of three lysine residues, K131, K156, and K220, of PGK1 were reduced under hypoxia compared with normoxia. The authors demonstrated that the PGK1 Kcr was regulated by ECSH1 and PCAF. Specifically, PCAF served as a crotonyltransferase using crotonyl-CoA as the crotonyl donor, while ECSH1 catalyzed the hydration of crotonyl-CoA, lowering the crotonyl-CoA level and thereby downregulating PGK1 Kcr. Mechanistically, hypoxia induced the mitochondrial localization of PGK1. The decreased PGK1 Kcr level promoted its interaction with PDHK1, strengthening the PGK1-PDHK1-PDH phosphorylation cascade, which eventually led to PDH inhibition and enhanced aerobic glycolysis as well as lactate production. The authors further showed that the reduced PGK1 Kcr level favored the proliferation and tumorigenic activity of MDA-MB-231 cells, and lower PGK1 Kcr levels in clinical samples were associated with higher malignancy and poorer prognosis.
Overall, the findings discussed in the manuscript are likely to provide new insights into the roles played by non-histone Kcr under pathogenic conditions and be of potential interest to the readers of Nature Communications. However, I am not fully convinced by the mechanistic studies that tried to illustrate the roles played by PGK1 Kcr in coordinating mitochondrial pyruvate metabolism and glycolysis and in regulating breast cancer cell proliferation and tumorigenesis, largely due to the oversimplified mimic of non-crotonylated PGK1 by the 3KR mutant. The authors are suggested to address the concerns/comments listed below before I can support the publication of this manuscript.

Major:
1. Lysine residues can be modified by different PTMs. The K to R mutation not just blocks Kcr but abolishes all the possible lysine PTMs. Although this study started from global crotonylome analysis, when it narrowed down to the K131, K156, and K220 residues of PGK1, other types of lysine PTMs at these three sites should be monitored. Specifically, IP of the endogenous PGK1 should be performed. After protease digestion, the peptide mixture should be analyzed by LC-MS/MS. The level changes of other PTMs at K131, K156, and K220 between hypoxia and normoxia should be quantified. And the stoichiometries of Kcr and other PTMs under both conditions should be determined. These experiments should be done at least for acetylation and lactylation, as lysine acetylation is known to be an abundant and widely distributed PTM that participates in the regulation of diverse cellular processes. More importantly, K220 acetylation of PGK1 has been detected and shown to regulate the PGK1 activity (Wang et al., PLoS Biol, 2015, 13, e1002243). And lysine lactylation is known to be upregulated in conditions with higher cellular lactate levels, for example, hypoxia (e.g.,

Zhang et al., Nature, 2019, 574, 575; Yang et al., Nat Metab, 2023, 5, 61). If the acetylation and lactylation levels of PGK1 K131, K156, and K220 are significantly changed under hypoxia comparing with normoxia, the conclusions of this study will be very much different from its current state.
2. The resolution of Supplementary Figure 1d is too low. In addition, since the entire study has been focusing on PGK1 K131cr, K156cr, and K220cr, the existence of these three Kcr sites should be validated and characterized more rigorously. Standard Kcr peptides with the same sequences should be synthesized. Co-elution of the synthetic peptides with the sample derived from MDA-MB-231 cells should be performed to show the identical retention time in liquid chromatography and fragmentation pattern in tandem mass spectrometry.
3. The authors demonstrated that crotonate treatment and ECHS1 manipulation could vary the levels of PGK1 Kcr. To make the results more Kcr-relevant, I suggest the authors add crotonate treated and/or ECHS1 knockdown samples for the mechanistic studies listed in Figure 5d,f,g,h,i,j, and 6b,d,e,g. The results should be compared to those of the PGK1 WT and 3KR mutant. Discussions of the results obtained from different conditions should be provided.
4. The PGK1 K131cr antibody is a key material for this study. The current characterization in terms of its specificity has not been efficient. In addition to the unmodified PGK1 K131, the site and modification type specificity of the antibody should be monitored in a broader scope using dot blot against different peptides, including PGK1 K131 acetylation, lactylation, and crotonylation of PGK1 K156 and K220. The antibody specificity should also be tested using whole cell lysate, and the full membrane should be provided but not just the PGK1 region. Besides, the procedure for antibody generation and purification should be provided in the Methods.
5. For almost all the immunoblotting results, the label of protein markers seemed to be random and casual, which seriously damages the reliability of the data. For example, the protein bands of endogenous ECHS1 in different membranes could be higher or lower than or right at the position of the 26 kD marker. The authors should check this issue and the raw data of all the immunoblotting in full membranes should be provided as supplementary figure(s).

Minor:
1. The raw mass spectrometry data and the files generated by Maxquant after data analysis were not provided.
2. Figure 1c, meanings of the color code and the dot size should be specified.
3. Page 3 Line 8, the Ref. 9 was wrong.
4. Page 4 Line 26, the sentence “Previous studies have shown that the concentration of crotonyl-CoA affects histone Kcr non-enzymatically” was not right. The cited reference (Sabari et al., Mol Cell, 2015, 58, 203) shows that an increased cellular crotonyl-CoA level can be sensed by the crotonyltransferase p300, which uses the crotonyl-CoA as a cofactor to elevate histone Kcr levels, but not crotonyl-CoA itself modified lysine residues non-enzymatically.
5. To follow up, the authors showed that crotonyl-CoA treatment could increase PGK1 Kcr at single protein level non-enzymatically in vitro (e.g., Fig. 1h and Fig. 2i) and they also showed that PCAF could catalyze the addition of PGK1 Kcr both in vitro and in live cells (e.g., Fig. 4). It is not clear to me that why the authors included the non-enzymatic assay, whose results, though, were convincing. Do the authors think and believe that, in live cells, PGK1 Kcr are added both non-enzymatically and enzymatically? If yes, more evidence should be provided and discussed. Alternatively, I suggest the authors remove the non-enzymatic data to avoid possible confusion. Such an action will not affect the main conclusion of this study.
6. Figure 2d, species names should be in italic.

7. The sentence starting from Page 4 Line 43 to Page 5 Line 1 is ambiguous and should be revised.
8. Page 5 Line 5, K156r.
9. Data quality of Figure 4A is not up to the standard and should be repeated.
10. Data shown in Supplementary Figure 5b,c had nothing to do with the manuscript, and the current 5d should be 5b.
11. Page 7 Line 18, the word “essential” is an overstatement.
12. Figure 5h, the color bar should be di-colored.

Reviewer #3 (Remarks to the Author):

In this manuscript, Guo et al. reported that hypoxia-induced downregulation of PGK1 crotonylation, which further alters glycolysis and TCA through the non-enzymatic function of PGK1. Using the molecular biology and biochemistry approaches, the authors primarily focus on how PGK1 crotonylation is regulated by writers, erasers, and subtracts. There are also functional studies of how PGK1 controls metabolism and contributes to tumor development. The finding of PGK1 crotonylation is new and interesting. However, the lack of evidence for the regulation and function of endogenous PGK1 lowers the interest of this study.
Major concerns:
MDA-MB-231 and 293T cells are the only cells used in this study. The essential findings of the endogenous PGK1 and its regulators should be tested in additional cancer cell lines.
Does ECHS1 bind to K-to-R mutated PGK1?
IF staining (3i, 4h) shows PGK1 expression over the whole cells, which will overlap will any other protein. Is it true that PGK1 is expressed over the whole cell, including nuclear?
Will crotonyl-CoA treatment increase crotonyl-CoA levels in hypoxia-cultured 231 cells?
Rescue PKG1-Kcr?
In vitro, does ECHS1 reduce crotonyl-CoA in the tubes?
PGK1 crotonylation occurs with or without enzymes, such as PCAF. What is their relative contribution?
Does hypoxia alter PGK1 expression?

Point-by-point responses to comments from reviewers

Dear all reviewers,

We appreciate you so much for your constructive suggestions and helpful comments to improve our manuscript. We have conducted all the experiments and provided detailed explanations to address the concerns raised by the reviewers. For detailed information, please see the "point-by-point responses" provided below.

Reviewer #1 (Remarks to the Author):

In this manuscript, Guo et al described hypoxia induced decreased lysine crotonylation facilitate breast tumor progression. The results presented in the manuscript suggested that ECHS1 interacts with PGK1, leading to downregulation of PGK1 Kcr under hypoxia and thus promoting glycolysis. Overall, this manuscript provides a PTM guided metabolic reprogramming through PGK1 crotonylation in breast cancer progression. However, several key mechanistic experiments are missing to support their hypothesis. In addition, Impact of glycolysis in breast cancer promotion has been well established and the finding in this work could be incremental for the overall framework. The detail review is outlined below:

Major Points:

1. Glycolysis in breast cancer promotion has been well established. Most breast cancer cells have already rely heavily on glycolysis. To support the significance of PGK1 crotonylation in regulating cancer cells mitochondrial metabolic reprogramming, it will be important to validate the relevance by using a suitable spontaneous model.

Response: Thank you so much for your suggestions.

As you suggested, we now evaluated the significance of PGK1 crotonylation (Kcr) on tumor mitochondrial metabolic reprogramming by using the normal breast tissues from wild-type (FVB) mice and tumor tissues from the MMTV-PyMT mice with early (week 7) or late (week 18) stages of spontaneous breast cancer. We found that the expression of PGK1 K131cr was suppressed in breast cancer tissues from MMTV-PyMT mice when compared to normal mouse breast tissues, and late-stage breast cancers exhibited lower levels of PGK1 K131cr in comparison to early-stage cancers (Fig.7a,b in the current version, new data). Moreover, the levels of PGK1, ECHS1, phosphorylated PDHK1, PDHK1, and HIF1α showed a gradient increase during this spontaneous mouse breast cancer progression (Fig.7a,c-g in the current version, new data), indicative of a gradual dependence of mitochondrial metabolic reprogramming in breast cancer progression. These observations are consistent with our findings in human breast cancer tissues (Fig. 7h-j,m and Supplementary Fig. 9b-e,g,h in the current version).

Moreover, we provided additional evidence supporting the regulation of PGK1 crotonylation in mitochondrial pyruvate metabolism by manipulating PGK1 Kcr levels through ECHS1 knockdown and mutation of the PGK1 Kcr sites to arginine. The results demonstrated that promoting PGK1 Kcr by ECHS1 depletion led to decreased levels of phosphorylated

PDHK1 and PDH, and increased levels of OCR, TCA cycle metabolites, acetyl-CoA and ROS (Fig. 5d,h,j-l in the current version, new data). In contrast, abolishing PGK1 Kcr by mutating lysine sites to arginine resulted in elevated levels of phosphorylated PDHK1 and PDH, and reduced levels of OCR, TCA cycle metabolites, acetyl-CoA and ROS in the presence or absence of ECHS1 (Fig. 5d,h,j-l in the current version, new data). Furthermore, the enhanced PGK1-PDHK1 interaction and phosphorylated PDHK1 under hypoxia were abrogated by the expression of shRNA targeting ECHS1 (Fig. 5f,g and Supplementary Supplementary Fig. 7d,f in the current version, new data). Taken together, these data indicate that PGK1 crotonylation could regulate PDHK1-PDH phosphorylation cascade and mitochondrial metabolic reprogramming during breast cancer progression.

2. Hypoxia has been previously subjected to regulation of tumor mitochondrial metabolism rewiring. It is not clear to distinguish the contribution between the emphasized PGK1 crotonylation from other well documented hypoxic factors.

Response: Thank you so much for pointing out these issues.

Indeed, it has been shown that hypoxia exerts significant impacts on tumor metabolic reprogramming. Hypoxia-inducible factor (HIF), a sensor of oxygen, is considered as a key regulator of hypoxic response (PMID: 28602395; PMID: 28842498). HIF-1α has been shown to upregulate pyruvate dehydrogenase kinase 1 (PDHK1 or PDK1) via transcription. PDHK1 phosphorylates the E1α subunit of pyruvate dehydrogenase (PDH), which converts pyruvate to acetyl-coenzyme A (acetyl-CoA), thus inhibiting its activity and limiting the entry of acetyl-CoA into the tricarboxylic acid (TCA) cycle and subsequent mitochondrial respiration (PMID: 16517405; PMID: 16517406). Moreover, hypoxia induces the phosphorylation of phosphoglycerate kinase 1 (PGK1) at Ser-203 and subsequently the mitochondrial translocation of PGK1, which acts as a protein kinase to phosphate PDHK1, thereby affecting mitochondrial pyruvate metabolism (PMID: 26942675).

In our study, we found that hypoxia-induced upregulation of phosphorylated PDHK1 and downregulation of PGK1 crotonylation, but not the upregulation of PDHK1 expression, were reversed by the expression of shRNA targeting ECHS1 (Fig. 5g and Supplementary Supplementary Fig. 7f in the current version, new data). Consistently, PGK1 S203D increased the phosphorylation of PDHK1 depending on the existence of ECHS1 (Supplementary Fig. 7e in the current version). Additionally, blocking PGK1 Kcr led to increased levels of phosphorylated PDHK1 and PDH, regardless of ECHS1 presence (Fig. 5d in the current version, new data). These findings suggest that the downregulation of PGK1 Kcr is crucial for PDHK1 phosphorylation. Furthermore, the expression of ECHS1 is upregulated by HIF-1α, which is required for the reduction of PGK1 Kcr under hypoxia (Fig.3j-n,q and Supplementary Fig. 5f-j in the current version). Collectively, our results indicate that PGK1 crotonylation functions downstream of HIF-1α and upstream of PDHK1 phosphorylation. PGK1 crotonylation, along with other hypoxic factors, might play important roles at different stages in regulating the tumor mitochondrial metabolism rewiring.

3. Multiple elements could regulate the level of Acetyl-CoA, how to distinguish the contribution from metabolic pathways needs to be addressed.

Response: Thank you for your valuable suggestions.

Our results showed that abrogation of PGK1 Kcr was required for the phosphorylation of PDHK1 and PDH (Fig. 5d in the current version, new data), suggesting that PGK1 Kcr might affect the conversion of pyruvate to acetyl-CoA catalyzed by PDH. To specifically evaluate the impact of PGK1 Kcr on this pathway, we directly detected the level of $^{13}C_3$-pyruvate-derived acetyl-CoA. This approach allowed us to exclude the influence of other metabolic pathways leading to acetyl-CoA. We carried out the experiments of $^{13}C_3$- pyruvate tracing experiments in PGK1-deleted MDA-MB231 cells expressing sgRNA-resistant PGK1 (rPGK1) WT or 3KR, with or without ECHS1 knockdown. The results demonstrated that promoting PGK1 Kcr by ECHS1 depletion was associated with enhanced synthesis of acetyl-CoA derived from pyruvate while blocking PGK1 Kcr inhibited the synthesis of acetyl-CoA derived from pyruvate (Fig. 5k in the current version, new data). Moreover, hypoxia also led to the decreased conversion of $^{13}C_3$- pyruvate to acetyl-CoA, which was restored by the knockdown of ECHS1 (Supplementary Fig. 7g in the current version, new data). These findings indicate that downregulation of PGK1 Kcr restricts the conversion of pyruvate to acetyl-CoA. We already added the related descriptions in the part of "PGK1 crotonylation is indispensable for coordinating mitochondrial pyruvate metabolism and glycolysis" in the Results section.

4. The authors claim that ECHS1 can decrease intracellular crotonyl-CoA levels. However, considering that ECHS1 is a mitochondrial-localized protein, authors need to address if ECHS1 can influence crotonyl-CoA levels in the cytosol?

Response: Thank you so much for your concerns.

To examine whether ECHS1 influences crotonyl-CoA levels in the cytosol, we isolated both the cytosolic and mitochondrial fractions from MDA-MB231 cells and measured the levels of crotonyl-CoA in these different cellular compartments. Our findings revealed that the level of crotonyl-CoA decreased in both the cytosolic and mitochondrial fractions under hypoxic conditions, while the knockdown of ECHS1 abolished this phenomenon (Fig. 3o in the current version, new data). Additionally, we observed a more pronounced decline in crotonyl-CoA levels within the mitochondria compared to the cytosol under hypoxia (Fig. 3o in the current version, new data). Previous studies indicated that acyl-CoAs can be exported from the mitochondria through acylcarnitine shuttling. Nonetheless, the underlying mechanism governing this process requires further elucidation (PMID: 37134161; PMID: 26239049). Crotonyl-carnitine was also been identified by the LC-MS/MS (PMID: 26239049). Therefore, the alterations observed in cytosolic crotonyl-CoA levels may be attributed to changes occurring within the mitochondria.

5. It is essential for the authors to assess hypoxic crotonyl-CoA levels in both the cytosol and mitochondria to understand how Hypoxia affects these levels.

Response: Thank you so much for the constructive suggestions.

As mentioned above, our results demonstrated a decrease in the level of crotonyl-CoA in both the cytosolic and mitochondrial fractions under hypoxic conditions. This decrease can be reversed upon the knockdown of ECHS1 (Fig. 3o in the current version, new data). These results suggest that ECHS1 is responsible for the reduction of crotonyl-CoA in both the cytosol and mitochondria under hypoxia. Additionally, we found that HIF-1α bound to the ECHS1 promoter, which led to the upregulation of ECHS1 under hypoxic conditions (Fig. 3j-n and

Supplementary Fig. 5f-j in the current version). Taken together, we conclude that HIF-1α upregulates ECHS1 expression upon hypoxia, which consequently leads to a decrease in crotonyl-CoA levels in both the cytosol and mitochondria.

6. Where exactly does PGK1 crotonylation occur? If crotonylation happens in the cytosol and PGK1 is subsequently transported to the mitochondria for de-crotonylation by ECHS1, the authors need to provide the data to support this.

Response: Grateful to you for the constructive suggestions.

PGK1 has been reported to be a cytosol-localized glycolytic enzyme under normoxia and translocates from the cytosol to the mitochondria under hypoxic conditions (PMID: 26942675). They demonstrated that hypoxia induces about 12% of cytosolic PGK1 translocation to the mitochondria (please see the attached figure from the indicated reference). Our immunofluorescence assays and cell fractionation assays confirmed this phenomenon (Fig. 3p and 5b in the current version). Moreover, we identified PCAF as a crotonyltransferase responsible for PGK1 Kcr, which localized in cytosol mainly and mitochondria to a lesser extent (Fig. 4i in the current version, new data). Furthermore, hypoxic-induced upregulation of ECHS1 resulted in a more decreased crotonyl-CoA level in mitochondria than that in the cytosol (Fig. 3o in the current version, new data), and barely PGK1 Kcr was detected in mitochondria (Fig. 3p in the current version, new data), indicating that PGK1 Kcr in mitochondria is strictly constrained. Therefore, PGK1 Kcr occurs in the cytosol under normoxia because of PGK1's cytosolic localization and happens mainly in the cytosol under hypoxia owing to the limited crotonyl-CoA availability in mitochondria. Our cell fractionation assays verified this and showed that PGK1 was crotonylated mainly in the cytosol (Fig. 3p in the current version, new data).

[Redacted]

(PMID: 26942675) Figure S1. Mitochondrial Translocation of PGK1 Is Mediated by ERK1/2-Dependent Phosphorylation. B, Total cell lysate, cytosolic, and mitochondrial fractions were prepared from U87 and U251 cells stimulated with or without hypoxia for 6 h. Cellular fractions from equal number of cells were analyzed using immunoblotting analyses with the indicated antibodies. Hypoxia-induced HIF1α expression (left panel) was a control for hypoxic stimulation. Mitochondrial COX IV and cytosolic tubulin were used as controls. WCL: whole-cell lysate; Cyto: cytosol; Mito: mitochondria. WB, Western blot. The images were quantified by scanning densitometry.

In detail, as depicted in Fig.5b, hypoxia promoted the mitochondrial localization of Flag-PGK1 and Flag-PGK1 3KR, indicating that crotonylation did not affect the mitochondrial translocation of PGK1 induced by hypoxia. Thus, both the crotonylated or uncrotonylated

forms of PGK1 entered the mitochondria and interacted with ECHS1 upon hypoxia, based on the findings that crotonylation did not influence the interaction between PGK1 and ECHS1 (Fig. 3e in the current version, new data). Furthermore, we found the loss of interaction between ECHS1 and PGK1 promoted PGK1 Kcr (Fig. 3f in the current version, new data), suggesting that the interaction between ECHS1 and PGK1 is indispensable for PGK1 Kcr suppression in mitochondria and this may result from the clearance of crotonyl-CoA surrounding PGK1 by PGK1-interacting ECHS1 and the restricted PGK1's utilization efficiency of crotonyl-CoA.

Based on these findings, we conclude that PGK1 crotonylation primarily occurs in the cytosol. In response to hypoxia, the upregulation of ECHS1 leads to a decrease in cytosolic crotonyl-CoA levels, consequently impairing PGK1 Kcr. Moreover, PGK1 translocates from the cytosol to the mitochondria and interacts with ECHS1 under hypoxic conditions, further limiting the availability of crotonyl-CoA for PGK1 and resulting in the downregulation of PGK1 crotonylation.

7. Similarly, in which cellular compartment does PGK1 de-crotonylation predominantly occur - the cytosol or the mitochondria since author mentioned that ECHS1 could affect the intracellular crotonyl-CoA levels.

Response: Thank you so much for pointing out these issues.

As we have explained above from Issue#6, hypoxia leads to a decrease in PGK1 crotonylation in the cytosol due to a reduced crotonyl-CoA level caused by ECHS1 upregulation. ECHS1 interacted with mitochondrial PGK1 translocating from the cytosol and abrogated PGK1 Kcr by degrading crotonyl-CoA surrounding PGK1. These findings indicate the downregulation of PGK1 Kcr occurs in both the cytosol and mitochondria. However, mitochondrial PGK1 Kcr exhibited a more significant suppression than the cytosolic PGK1 Kcr (Fig. 3p in the current version, new data).

8. If PGK1 is crotonylated in the cytosol, is it then shuttled into the mitochondria specifically under Normoxia or Hypoxia?

Response: Greatly grateful to you for mentioning this issue.

Indeed, PGK1 is mainly crotonylated in the cytosol and its crotonylated state does not affect its shuttle into the mitochondria (Fig. 3p and 5b in the current version). Li. et al. have demonstrated that hypoxia induces ERK1/2-dependent phosphorylation of PGK1 at S203, which is required for the translocation from the cytosol to the mitochondria of PGK1. In the mitochondria, PGK1 acts as a protein kinase to phosphorylate PDHK1. Subsequently, PDHK1 phosphorylates PDH, leading to the suppression of mitochondrial pyruvate metabolism (PMID: 26942675). We found that the elimination of PGK1 Kcr leads to an increase in phosphorylated PDHK1 and PDH, regardless of the presence or absence of ECHS1 (Fig.5d in the current version, new data). Meanwhile, we expressed Flag-tagged PGK1 S203D, a phospho-mimicking mutant (Ser-to-Asp), or PGK1 wild-type with or without ECHS1 knockdown in MDA-MB231 and found that PGK1 S203D increased the phosphorylation of PDHK1 depending on the existence of ECHS1 (Supplementary Fig. 7e in the current version). Overall, these findings indicate that while PGK1 Kcr does not affect its translocation into the mitochondria under hypoxic conditions, it is critical for the phosphorylation and activation of PDHK1 by PGK1 within the mitochondria.

9. The mechanism by which ECHS1 reduces PGK1 crotonylation is not clear, especially considering that ECHS1 can directly break down crotonyl-CoA, thus reducing its intracellular concentration. Is an interaction between ECHS1 and PGK1 necessary for crotonyl-CoA removal?

Response: Grateful to you for pointing out this issue.

We have shown that the downregulation of PGK1 Kcr is induced by the decreased levels of crotonyl-CoA induced by ECHS1 and the interaction between ECHS1 and PGK1 (Fig. 3 and Supplementary Fig. 5). To investigate whether the interaction between ECHS1 and PGK1 is essential for this phenomenon, we utilized AlphaFold2 to predict the interaction interface between these two proteins. The result indicated that Lys133, Asn138, Lys139, and Lys144 of PGK1 are responsible for its association with ECHS1. We then generated a Flag-tagged PGK1 mutant with amino acid substitutions K133D, N138A, K139D and K144D, and compared its interaction with ECHS1 to wild-type Flag-tagged PGK1. As shown in Fig. 3f, mutations in these four amino acids of PGK1 abolished its interaction with ECHS1, while simultaneously enhancing PGK1 Kcr, indicating that the association between ECHS1 and PGK1 is necessary for the downregulation of PGK1 Kcr.

In the responses to Issue#6, we previously explained that ECHS1 reduced the intracellular crotonyl-CoA levels to decrease PGK1 Kcr under hypoxic conditions, and the interaction between ECHS1 and PCK1 was crucial for maintaining the low level of PGK1 Kcr in the mitochondria. In our global crotonylome data, we observed a total of 128 Kcr sites in 101 proteins changed in response to hypoxia, among which PGK1 Kcr showed significant changes (Fig. 1d,g in the current version). We hypothesized that the association between ECHS1 and PGK1 might be responsible for these significant changes in PGK1 Kcr.

10. Figure 5 doesn't adequately elucidate how PGK1 induces P-PDHK1 and p-PDH1 under hypoxic conditions.

Response: Thank you so much for your concerns.

It has been reported that hypoxia triggers the translocation of PGK1 to mitochondria, where it functions as a protein kinase to phosphate PDHK1, thereby enhancing its activity. PDHK1 then phosphorylates PDH, leading to the suppression of mitochondrial pyruvate metabolism (PMID: 26942675). In line with these findings, we observed that PGK1 S203 phosphorylation increased PDH phosphorylation in the presence of PDHK1 (Supplementary Fig. 7a in the current version, new data). Therefore, we focused on investigating the role of PGK1 Kcr in modulating the phosphorylation of PDHK1 and PDH, as well as its relationship with PGK1 phosphorylation. Our results showed that the downregulation of PGK1 Kcr promoted the interaction between PGK1 and PDHK1, resulting in elevated levels of phosphorylated PDHK1 and PDH (Fig. 5d,f in the current version, new data). We then also utilized MDA-MB231 cells expressing Flag-PGK1 or Flag-PGK1 3KR, with or without shRNA targeting PDHK1, to assess the phosphorylation of PDH. We found that downregulation of PGK1 Kcr induced PDH phosphorylation in a PDHK1-dependent manner (Fig. 5e in the current version, new data). Moreover, the knockdown of ECHS1 disrupted the hypoxia-induced interaction between PGK1 and PDHK1, as well as the phosphorylation of PDHK1 (Fig. 5f,g in the current version, new data). Additionally, we found PGK1 S203D

increased the phosphorylation of PDHK1 depending on the existence of ECHS1 (Supplementary Fig. 7e in the current version). These results support the essential role of downregulating PGK1 Kcr in facilitating PGK1-mediated phosphorylation and activation of PDHK1, which subsequently phosphorylates PDH.

11. The impact of the PGK1-pPDHK1-pPDH1 axis on tumor progression isn't clearly described. Most experiments seem to rely solely on sgPGK1 or PGK1-3KR, leaving unanswered questions about how the PGK1-pPDHK1-pPDH1 pathway influences tumor growth both in vitro and in vivo. More cancer biology experiments are needed here to justify authors claim.

Response: Thank you so much for your constructive suggestions.

As you suggested, we used PGK1-deleted MDA-MB231 cells expressing sgRNA-resistant PGK1 (rPGK1) WT or 3KR, with or without shRNA targeting PDHK1, to explore the effect of PGK1 Kcr-pPDHK1-pPDH1 pathway on tumor growth both *in vitro* and *in vivo*. The results revealed that the expression of rPGK1 3KR mutant resulted in enhanced tumor cell growth, which was reversed by the expression of shRNA targeting PDHK1 (Fig. 6k,l in the current version, new data). In addition, we observed tumors of MDA-MB231 cells expressing rPGK1 3KR mutant were larger than those of MDA-MB231 cells expressing rPGK1 wild-type. Notably, tumor growth was suppressed in MDA-MB231 cells expressing rPGK1 wild-type and the 3KR mutant upon knockdown of PDHK1 in xenograft models of nude mice (Fig. 6m-o in the current version, new data). Taken together, these results suggest the regulation of PGK1 Kcr on tumor growth is dependent on PDHK1. We added the related descriptions in the Parts of "Downregulation of PGK1 crotonylation promotes cell proliferation and tumorigenesis" in the Results section.

Minor Points:

1. Most of the experimental results from a single experiment with n=3. In most cases, data points should be at least n=6.

Response: Thank you so much for pointing out these issues.

The data presented in the study were verified in at least two independent experiments with similar results. Following your suggestions, the results of animal studies, immunofluorescence assays, immunohistochemistry assays and some cancer biology experiments are presented with at least 6 data points. The accurate number of biological independent samples was noted in the figure legend.

2. The authors should repeat the results presented in figure 5e. The whole cell lysate shows that Flag-PGK1 overexpression leads to a decrease in ECHS1, which contrasts with the findings in Fig 3b.

Response: Thank you for your valuable suggestions.

As you suggested, we repeated the results presented in Figure 5e in the last version. The results showed that overexpression of PGK1 did not affect the expression of ECHS1 (Fig.5f in the current version, new data).

Reviewer #2 (Remarks to the Author):

This manuscript by Guo et al. reports the decrease of PGK1 crotonylation under hypoxia and its links to the altered glycolysis and TCA cycle in cancer cells. Lysine crotonylation (Kcr) was originally identified in histones and has recently been detected in a variety of other proteins. In this study, the authors performed quantitative proteomic analysis of the crotonylome in a breast cancer cell line MDA-MB-231 and found that the crotonylation levels of three lysine residues, K131, K156, and K220, of PGK1 were reduced under hypoxia compared with normoxia. The authors demonstrated that the PGK1 Kcr was regulated by ECSH1 and PCAF. Specifically, PCAF served as a crotonyltransferase using crotonyl-CoA as the crotonyl donor, while ECSH1 catalyzed the hydration of crotonyl-CoA, lowering the crotonyl-CoA level and thereby downregulating PGK1 Kcr. Mechanistically, hypoxia induced the mitochondrial localization of PGK1. The decreased PGK1 Kcr level promoted its interaction with PDHK1, strengthening the PGK1-PDHK1-PDH phosphorylation cascade, which eventually led to PDH inhibition and enhanced aerobic glycolysis as well as lactate production. The authors further showed that the reduced PGK1 Kcr level favored the proliferation and tumorigenic activity of MDA-MB-231 cells, and lower PGK1 Kcr levels in clinical samples were associated with higher malignancy and poorer prognosis.

Overall, the findings discussed in the manuscript are likely to provide new insights into the roles played by non-histone Kcr under pathogenic conditions and be of potential interest to the readers of Nature Communications. However, I am not fully convinced by the mechanistic studies that tried to illustrate the roles played by PGK1 Kcr in coordinating mitochondrial pyruvate metabolism and glycolysis and in regulating breast cancer cell proliferation and tumorigenesis, largely due to the oversimplified mimic of non-crotonylated PGK1 by the 3KR mutant. The authors are suggested to address the concerns/comments listed below before I can support the publication of this manuscript.

Major:

1. Lysine residues can be modified by different PTMs. The K to R mutation not just blocks Kcr but abolishes all the possible lysine PTMs. Although this study started from global crotonylome analysis, when it narrowed down to the K131, K156, and K220 residues of PGK1, other types of lysine PTMs at these three sites should be monitored. Specifically, IP of the endogenous PGK1 should be performed. After protease digestion, the peptide mixture should be analyzed by LC-MS/MS. The level changes of other PTMs at K131, K156, and K220 between hypoxia and normoxia should be quantified . And the stoichiometries of Kcr and other PTMs under both conditions should be determined. These experiments should be done at least for acetylation and lactylation, as lysine acetylation is known to be an abundant and widely distributed PTM that participates in the regulation of diverse cellular processes. More importantly, K220 acetylation of PGK1 has been detected and shown to regulate the PGK1 activity (Wang et al., PLoS Biol, 2015, 13, e1002243). And lysine lactylation is known to be upregulated in conditions with higher cellular lactate levels, for example, hypoxia (e.g., Zhang et al., Nature, 2019, 574, 575; Yang et al., Nat Metab, 2023, 5, 61). If the acetylation and lactylation levels of PGK1 K131, K156, and K220 are significantly changed under hypoxia comparing with normoxia, the

conclusions of this study will be very much different from its current state.

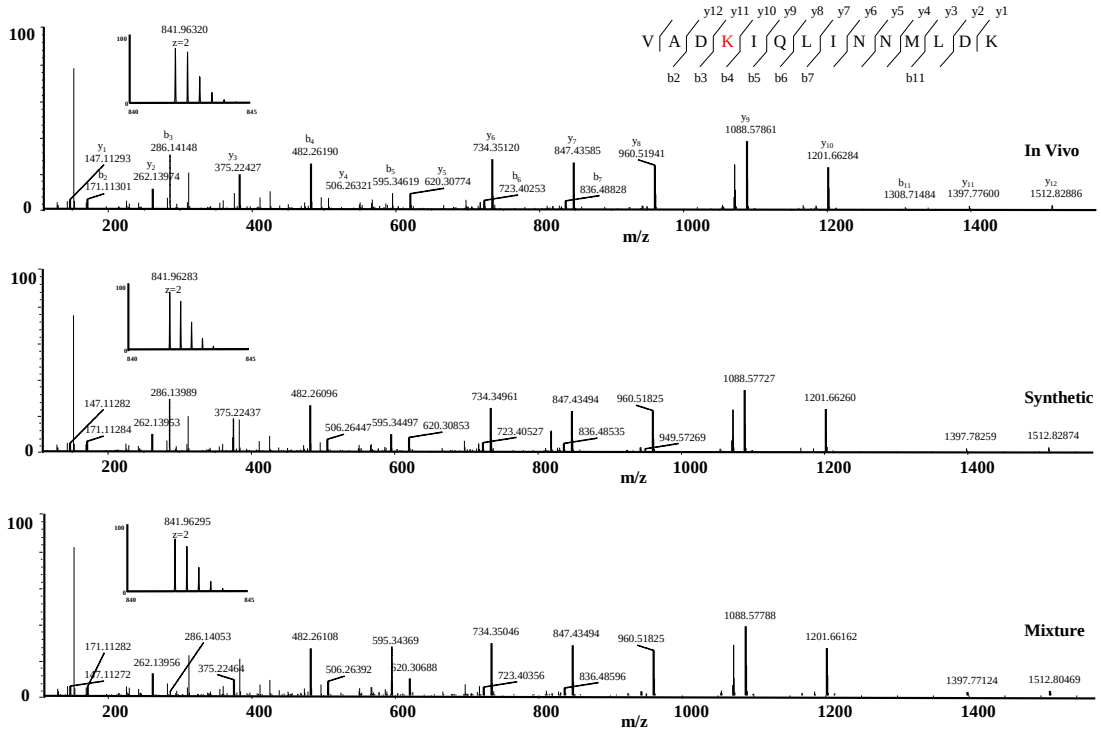
Response: Thank you so much for your concerns and constructive suggestions.

You are right in pointing out the possibility of other lysine PTMs occurring at K131, K156, and K220 of PGK1. To address this, we followed your suggestions and conducted IP assays using MDA-MB231 cells cultured under normoxia or hypoxia for 12 hours. The enriched PGK1 proteins were subjected to trypsin digestion, and the resulting peptides were collected and processed for LC-MS/MS analysis. We only detected crotonylation of PGK1 at K220, which showed a decrease under hypoxia (please refer to the attached Panel a). Additionally, we observed that the *in vivo* derived PGK K220cr peptide, the synthetic standard PGK K220cr peptide, and the mixture of the two peptides exhibited nearly identical retention time in liquid chromatography and fragmentation patterns in tandem mass spectrometry (please see the attached Panel b and c). However, we were unable to detect PGK1 K131cr and K156cr, as well as the acetylation and lactylation of K131, K156, and K220. This limitation could be attributed to the low efficiency of PGK1 enrichment due to insufficient antibody affinity or the low abundance of these specific protein modifications.

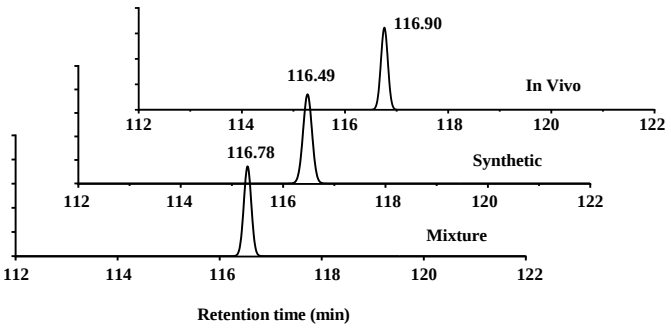
a

Gene	Position	Modification sites	Intensity N	Intensity H	Ratio H/N
PGK1	K220	VADK(Cr)IQLINNMLDK	8.21E+07	4.44E+07	0.54

b



c



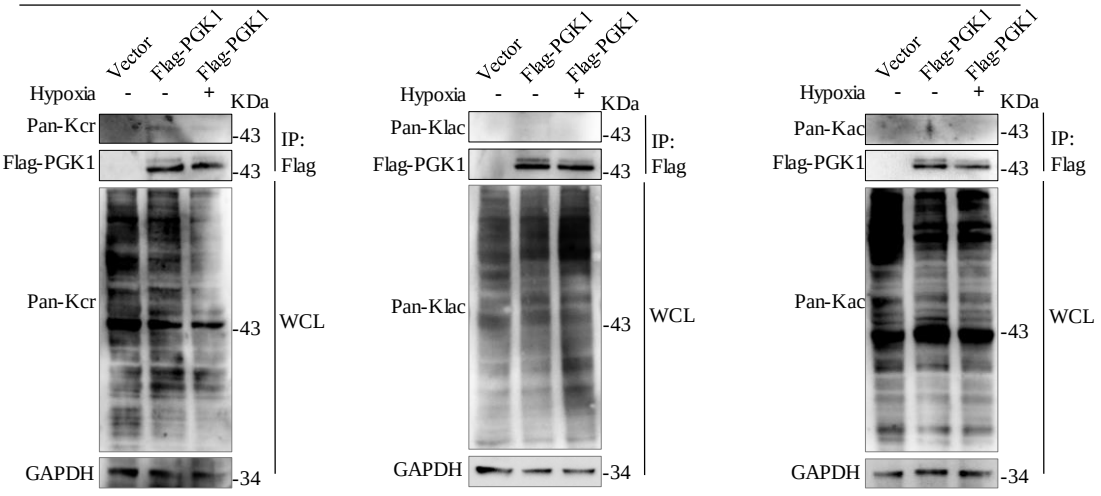
Legends: a, MDA-MB231 cells cultured under normoxia or hypoxia for 12 hours and subjected to IP assays, followed by protein digestion and LC-MS/MS analysis. *The level changes of PGK1 K220cr between hypoxia and normoxia were quantified as indicated. b, MS/MS spectrum of in vivo derived PGK K220cr peptide, the synthetic standard PGK K220cr peptide, and the mixture of the two peptides. c, Extracted ion chromatograms from LC-MS/MS analysis of in vivo derived PGK K220cr peptide, the synthetic standard PGK K220cr peptide, and the mixture of the two peptides.*

Next, we attempted to enrich crotonylated, lactylated, and acetylated proteins using pan anti-Kcr, pan anti-Kla, or pan anti-Kac antibody-conjugated agarose beads. We utilized the same whole-cell lysates obtained from MDA-MB231 cells cultured under normoxia or hypoxia for 12 hours. The enriched proteins were subjected to trypsin digestion, and the resulting peptides were collected and processed for LC-MS/MS analysis. The results revealed a reduction in crotonylation at K131, K156, and K220 of PGK1, which is consistent with the crotonylome data (Supplementary Fig. 4a in the current version, new data; please also see the attached Panel a). Moreover, lactylation was observed at K131 of PGK1, which remained almost unaffected by hypoxic treatment (Supplementary Fig. 4a in the current version, new data; please also see the attached Panel a). However, we did not identify the acetylation of PGK1 at the K131, K156 and K220 sites. Subsequently, we performed IP assays to investigate other lysine PTMs on PGK1. The results demonstrated the presence of crotonylation on PGK1, while lactylation and acetylation were not detected (Supplementary Fig. 4b in the current version, new data; please also see the attached Panel b). Notably, although hypoxia led to an increase in protein modification by lactylation, we did not observe lactylation on PGK1, possibly due to the low intensity of PGK1 lactylation at K131 (Supplementary Fig. 4b in the current version, new data; please also see the attached Panel b).

a

Gene	Position	Score	Modification sites	Normalized intensity N	Normalized intensity H	Ratio H/N
PGK1	K131	157.96	FHVEEEGK(La)GK	5.34E+06	5.66E+06	1.06
PGK1	K156	224.89	ASLSK(Cr)LGDVYVNDAFGTAHR	1.35E+08	1.70E+07	0.13
PGK1	K131	158.76	FHVEEEGK(Cr)GK	1.46E+07	8.71E+06	0.60
PGK1	K220	212.59	VADK(Cr)IQLINNMLDK	2.18E+08	7.57E+07	0.35

b



Legends: a, MDA-MB231 cells cultured under normoxia or hypoxia for 12 hours and subjected to IP assays using pan anti-Kcr or pan anti-Kla antibody-conjugated agarose beads*, followed by protein digestion and* LC-MS/MS analysis*. The level changes of PGK1 modifications between hypoxia and normoxia were quantified as indicated. b,* IP assays were performed with anti-Flag M2 beads using HEK293T cells expressing the Flag-tagged PGK1 cultured under normoxia or hypoxia for 12 h, followed by immunoblotting with the indicated antibodies.

Although previous studies demonstrated that PGK1 is acetylated at K220 and this acetylation of PGK1 regulates its activity, we did not detect the acetylation of PGK1 K220 in our LC-MS/MS analysis. This observation could be attributed to the low abundance of this modification in specific cellular contexts. It appears that the crotonylation at K131, K156, and K220 of PGK1 might be relatively abundant and responsive to hypoxia. However, due to technical limitations, we cannot entirely exclude the possibility of other lysine PTMs occurring at these three sites. Additionally, in response to the reviewer's suggestions in Issue#3, we incorporated ECHS1 knockdown samples, as well as PGK1 WT and 3KR mutant overexpression samples, in our mechanistic studies to make the results more Kcr-relevant.

2. The resolution of Supplementary Figure 1d is too low. In addition, since the entire study has been focusing on PGK1 K131cr, K156cr, and K220cr, the existence of these three Kcr sites should be validated and characterized more rigorously. Standard Kcr peptides with the same sequences should be synthesized. Co-elution of the synthetic peptides with the sample derived from MDA-MB-231 cells should be performed to show the identical retention time in liquid chromatography and fragmentation pattern in tandem mass spectrometry.

Response: Thank you for your valuable suggestions.

We sincerely apologize for the low resolution of Supplementary Figure 1d in the previous version. Now, we synthesized standard Kcr peptides with the same peptide sequence as PGK1 K131cr, K156cr and K220cr, and compared their MS/MS spectra with those derived from the MDA-MB231 cells. The results showed that the *in vivo* derived PGK1 K131cr peptide, the synthetic standard PGK1 K131cr peptide and the mixture of the two peptides had almost identical retention times in liquid chromatography and fragmentation patterns in tandem mass spectrometry (Fig. 2a,b in the current version, new data). Similar results were obtained for PGK1 K156cr and K220cr (Supplementary Fig. 2a,b and 3a,b in the current version, new data). Taken together, these findings suggest the presence of PGK1 K131cr, K156cr, and K220cr in breast cancer cells. In the revised manuscript, we have replaced the original Supplementary Figure 1d with Fig. 2a, Supplementary Fig. 2a, and Supplementary Fig. 3a to provide clearer and more rigorous data.

3. The authors demonstrated that crotonate treatment and ECHS1 manipulation could vary the levels of PGK1 Kcr. To make the results more Kcr-relevant, I suggest the authors add crotonate treated and/or ECHS1 knockdown samples for the mechanistic studies listed in Figure 5d,f,g,h,i,j, and 6b,d,e,g. The results should be compared to those of the PGK1 WT and 3KR mutant. Discussions of the results obtained from different conditions should be provided.

Response: Grateful to you for your suggestions.

We deleted endogenous PGK1 in MDA-MB231 cells using CRISPR/Cas9 technology, then rescued PGK1 expression with sgRNA-resistant PGK1 (rPGK1) WT or 3KR. These

rPGK1 WT and 3KR cells were further depleted with ECHS1 and subjected to the mechanistic studies listed in Figure 5d,f,g,h,i,j, and 6b,d,e,g in the previous version of our manuscript. The results demonstrated that expression of the rPGK1 3KR mutant resulted in upregulated levels of phosphorylated PDHK1 and PDH compared to rPGK1 WT in the presence or absence of ECHS1 (updated as Fig. 5d in the current version). rPGK1 WT cells with ECHS1 depletion exhibited reduced levels of phosphorylated PDHK1 and PDH compared to rPGK1 WT cells (updated as Fig. 5d in the current version). The original Figure 5f,g,h,i,j were changed to Figure 5h,i,j,k,l in the revised manuscript. We added these results and descriptions in the part of "PGK1 crotonylation is indispensable for coordinating mitochondrial pyruvate metabolism and glycolysis": "We then determined whether PGK1 Kcr regulated mitochondrial pyruvate metabolism and glycolysis. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) assays were performed using rPGK1 WT and 3KR cells with or without ECHS1 depletion. Our results showed that rPGK1 WT cells with ECHS1 depletion displayed an increase in OCR and a reduction in ECAR in comparison to rPGK1 WT cells. Conversely, rPGK1 3KR cells exhibited decreased OCR and elevated ECAR compared to rPGK1 WT cells, regardless of the presence or absence of ECHS1(Fig. 5h,i in the current version, new data). Additionally, LC-MS analysis of metabolites in rPGK1 WT- or 3KR-rescued MDA-MB231 cells with or without ECHS1 depletion indicated that promoting PGK1 Kcr by ECHS1 depletion was associated with reduced pyruvate and lactate levels, and elevated levels of TCA cycle metabolites and acetyl-CoA. Conversely, abolishing PGK1 Kcr by mutating lysine sites to arginine resulted in the inverse effects (Fig. 5j,k in the current version, new data). Hypoxia also led to the decreased conversion of pyruvate to lactate, which was restored by the knockdown of ECHS1 (Supplementary Fig. 7g in the current version, new data). We also found that rPGK1 3KR expression inhibited ROS production while ECHS1 depletion promoted ROS production (Fig. 5l in the current version, new data). Through the manipulation of PGK1 Kcr by ECHS1 knockdown and the introduction of a non-crotonylated PGK1 mimic, our findings indicate that abrogation of PGK1 crotonylation attenuates mitochondrial pyruvate metabolism and promotes lactate production."

Moreover, in response to the reviewer's suggestions for Figure 6b,d,e,g in the previous version of our manuscript, we conducted colony formation assays, cell proliferation assays, and migration assays by using rPGK1 WT and 3KR cells with or without ECHS1 depletion. The results demonstrated that rPGK1 3KR displayed increased colony formation and enhanced cell proliferation compared to rPGK1 WT cells, irrespective of ECHS1 status (Supplementary Fig. 8a-c in the current version, new data). Additionally, the depletion of ECHS1 in rPGK1 WT cells significantly inhibited colony formation and cell proliferation when compared to their parental cells (Supplementary Fig. 8a-c in the current version, new data). Moreover, transwell and wound healing migration assays showed that rPGK1 3KR expression promoted tumor cell migration more effectively than rPGK1 WT, regardless of the presence or absence of ECHS1 (Supplementary Fig. 8d-g in the current version, new data). Notably, the promotion of PGK1 Kcr through ECHS1 depletion led to impaired transwell migration and wound healing activity (Supplementary Fig. 8d-g). We organized these results as Supplementary Fig. 8a-g in the revised manuscript.

Taken together, these results suggest that crotonylation of PGK1 plays a pivotal role in orchestrating mitochondrial pyruvate metabolism and glycolysis and regulating breast cancer

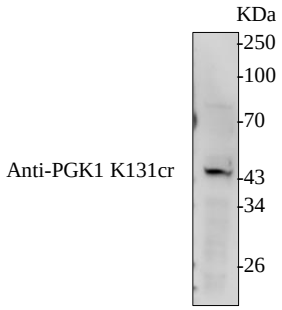
cell proliferation and migration. We also discussed it in the second paragraph of the discussion section: "Although our study indicates the crotonylation of PGK1 at K131, K156 and K220 sites influences hypoxia-induced metabolic reprogramming, we cannot rule out the possibility that other lysine PTMs occurring at these three sites could also contribute to this process.”

4. The PGK1 K131cr antibody is a key material for this study. The current characterization in terms of its specificity has not been efficient. In addition to the unmodified PGK1 K131, the site and modification type specificity of the antibody should be monitored in a broader scope using dot blot against different peptides, including PGK1 K131 acetylation, lactylation, and crotonylation of PGK1 K156 and K220 (1). The antibody specificity should also be tested using whole cell lysate, and the full membrane should be provided but not just the PGK1 region (2). Besides, the procedure for antibody generation and purification should be provided in the Methods (3).

Response: Thank you so much for your constructive suggestions.

(1) According to your suggestions, we performed dot blots using synthesized peptides of PGK1 K131 acetylation, lactylation, and crotonylation, as well as PGK1 K156, and K220 crotonylation to further test the specificity of PGK1 K131cr antibody. The results showed that the PGK1 K131cr antibody specifically recognized crotonylation of PGK1 at K131, but not acetylation, lactylation, or crotonylation at K156 and K220 (Fig. 2f and Supplementary Fig. 4e in the current version, new data). These results indicate the site and modification type specificity of the PGK1 K131cr antibody.

(2) The whole-cell lysate of MDA-MB231 cells was subjected to immunoblotting using the PGK1 K131cr antibody. The results showed specific recognition of a band around 43 kDa, corresponding to crotonylated PGK1 (please see the attached figure below).



Legends: MDA-MB231 cells were collected and lysed. The whole-cell lysate was subjected to immunoblotting using the PGK1 K131cr antibody.

(3) Now we provided the detailed procedure for antibody generation and purification in the Parts of “Generation and purification of the PGK1 K131cr/156cr antibody” in the Methods section: “Based on the sequence information, two modified and one unmodified polypeptides corresponding to each site were synthesized and conjugated with KLH. After being diluted with physiological saline and mixed 1:1 with the corresponding adjuvant, the immunogen was injected into six New Zealand rabbits four times in 5 weeks. On the 45th day, 30 mL of whole blood was collected and centrifuged to obtain the supernatant for serum screening. Subsequent collections of 20 mL of whole blood were performed on the 50th, 65th, and 70th days. Positive serum samples were purified using a protein A column and eluted with 150 mM glycine buffer. The crude IgG obtained was loaded onto an equilibrated immunogen affinity chromatography

column to specifically enrich the target antibody. Further purification was carried out using an unmodified affinity chromatography column to remove non-specific antibody components. The resulting effluent was directly collected, and 10% glycerol was added to the purified antibody before it was aliquoted and stored at -20℃."

5. For almost all the immunoblotting results, the label of protein markers seemed to be random and casual, which seriously damages the reliability of the data. For example, the protein bands of endogenous ECHS1 in different membranes could be higher or lower than or right at the position of the 26 kD marker. The authors should check this issue and the raw data of all the immunoblotting in full membranes should be provided as supplementary figure(s).

Response: Grateful to you for pointing out these issues.

We appreciate your feedback and apologize for any inaccuracies. We have thoroughly reviewed and corrected the labeling of protein markers to ensure their accuracy in our research. In addition, we have also provided the raw data of all immunoblotting in full membranes as Source data.

Minor:

1. The raw mass spectrometry data and the files generated by Maxquant after data analysis were not provided.

Response: Thank you so much for your concerns.

The raw mass spectrometry data for the global crotonylome and the files generated by Maxquant after data analysis have been deposited to the ProteomeXchange Consortium (https://proteomecentral.proteomexchange.org) via the iProX partner repository with the dataset identifier PXD051809.

2. Figure 1c, meanings of the color code and the dot size should be specified.

Response: Thank you so much for your suggestions.

The original Figure 1c was changed to Figure 1f in the revised manuscript. We added the related descriptions in the Parts of "Fig. 1 The global crotonylome reveals downregulation of PGK1 crotonylation under hypoxia" in the Figure legend section: "The dot size and color represent protein number and the p value of the corresponding pathway, respectively."

3. Page 3 Line 8, the Ref. 9 was wrong.

Response: Thank you so much for pointing out this issue.

We sincerely apologize for the error in citing the reference. We have now corrected the citation to ensure accurate referencing in our revised manuscript.

4. Page 4 Line 26, the sentence "Previous studies have shown that the concentration of crotonyl-CoA affects histone Kcr non-enzymatically" was not right. The cited reference (Sabari et al., Mol Cell, 2015, 58, 203) shows that an increased cellular crotonyl-CoA level can be sensed by the crotonyltransferase p300, which uses the crotonyl-CoA as a cofactor to elevate histone Kcr levels, but not crotonyl-CoA itself modified lysine residues non-enzymatically.

Response: Thank you for pointing out this problem.

You are correct. The cited reference demonstrates that histone Kcr is regulated

enzymatically by the crotonyltransferase p300, utilizing crotonyl-CoA as a cofactor. We apologize for the confusion caused by the sentence, and we have now removed it from our revised manuscript.

5. To follow up, the authors showed that crotonyl-CoA treatment could increase PGK1 Kcr at single protein level non-enzymatically in vitro (e.g., Fig. 1h and Fig. 2i) and they also showed that PCAF could catalyze the addition of PGK1 Kcr both in vitro and in live cells (e.g., Fig. 4). It is not clear to me that why the authors included the non-enzymatic assay, whose results, though, were convincing. Do the authors think and believe that, in live cells, PGK1 Kcr are added both non-enzymatically and enzymatically? If yes, more evidence should be provided and discussed. Alternatively, I suggest the authors remove the non-enzymatic data to avoid possible confusion. Such an action will not affect the main conclusion of this study.

Response: Grateful to you for pointing out these issues.

Previous studies have reported that protein lysine acylation, including acetylation, succinylation, and crotonylation, can occur through nonenzymatic processes (PMID: 30131192; PMID: 26382620; PMID: 28803779). The occurrence of nonenzymatic protein acylation is dependent on the local substrate concentrations (PMID: 30131192). However, the cellular concentrations of crotonyl-CoA are significantly lower compared to acetyl-CoA, which is the most abundant CoA species (PMID: 25818647). In our previous version, we observed nonenzymatic PGK1 Kcr *in vitro* using relatively high concentrations of crotonyl-CoA (Fig. 1h, 2i in the previous version). More importantly, PCAF could catalyze the addition of PGK1 Kcr both *in vitro* and in live cells (Fig.4 in the current version). Thus, we speculated that PGK1 Kcr primarily occurs through the enzymatic pathway catalyzed by the crotonyltransferase PCAF in cells. Therefore, we deleted all the non-enzymatic data as you suggested.

6. Figure 2d, species names should be in italic.

Response: Thank you so much for pointing out this issue.

As you suggested, the species names are now appropriately formatted in italic.

7. The sentence starting from Page 4 Line 43 to Page 5 Line 1 is ambiguous and should be revised.

Response: Thank you so much for this suggestion.

We have rewritten the sentence and added the related descriptions in the part of “PGK1 Kcr at the K131, K156 and K220 sites decreases under hypoxia” in the Results section: “To further validate the above results, we attempted to develop specific antibodies targeting PGK1 K131cr, K156cr, and K220cr. However, we successfully generated antibodies for K131cr and K156cr, but encountered difficulties in generating an antibody for K220cr due to challenges associated with the antigenicity, hydrophilicity, and spatial location of the amino acids surrounding K220.”

8. Page 5 Line 5, K156r.

Response: Grateful to you for pointing out this issue.

We sincerely apologize for the error. We have made the necessary correction in our revised manuscript in the part of “PGK1 Kcr at the K131, K156 and K220 sites decreases under

hypoxia": "The IP analysis of HEK293T cells transfected with Flag-tagged PGK1 WT, K131R, or K156R demonstrated that K131R abolished the recognition by PGK1 K131cr antibody, whereas K156R did not affect the recognition by the indicated site-specific antibody, suggesting that only PGK1 K131cr antibody was available and specific (Fig. 2g and Supplementary Fig. 4g in the current version). "

9. Data quality of Figure 4A is not up to the standard and should be repeated.
Response: Thank you so much for pointing out this issue.

According to your suggestions, we repeated the experiment to ensure the data quality. We have updated Figure 4A in the new version of our manuscript.

10. Data shown in Supplementary Figure 5b,c had nothing to do with the manuscript, and the current 5d should be 5b.
Response: Grateful to you for pointing out these issues.

You are right. We now deleted Supplementary Figure 5b,c in the previous version of our manuscript and corrected Supplementary Figure 5d in the previous version as Supplementary Figure 7e in the revised manuscript.

11. Page 7 Line 18, the word "essential" is an overstatement.
Response: Thank you so much for this suggestion.

The word "essential" has been replaced with "important" to ensure that the statement accurate and avoids any overstatement.

12. Figure 5h, the color bar should be di-colored.
Response: Thank you so much for pointing out this issue.

The original Figure 5h was changed to Figure 5j in the revised manuscript. The color bar has now been updated to be di-colored in Figure 5j in the current version.

Reviewer #3 (Remarks to the Author):

In this manuscript, Guo et al. reported that hypoxia-induced downregulation of PGK1 crotonylation, which further alters glycolysis and TCA through the non-enzymatic function of PGK1. Using the molecular biology and biochemistry approaches, the authors primarily focus on how PGK1 crotonylation is regulated by writers, erasers, and subtracts. There are also functional studies of how PGK1 controls metabolism and contributes to tumor development. The finding of PGK1 crotonylation is new and interesting. However, the lack of evidence for the regulation and function of endogenous PGK1 lowers the interest of this study.
Major concerns:
1. MDA-MB-231 and 293T cells are the only cells used in this study. The essential findings of the endogenous PGK1 and its regulators should be tested in additional cancer cell lines.
Response: Thank you so much for your concerns.

To address this, we conducted additional experiments using HCC1806, another triple-negative breast cancer cell line, to investigate the association of endogenous PGK1 and its regulators. Immunoprecipitation (IP) analysis performed using HCC1806 cells cultured under

normoxia or hypoxia demonstrated a decrease in PGK1 Kcr levels under hypoxia (Supplementary Fig. 1c,d in the current version, new data). Additionally, Co-IP analysis conducted on HCC1806 cells cultured under normoxia or hypoxia revealed an enhanced interaction between PGK1 and ECHS1 under hypoxic conditions (Supplementary Fig. 5d,e in the current version, new data). Furthermore, we found that crotonyltransferase PCAF interacts with PGK1 (Supplementary Fig. 6d in the current version, new data). Collectively, these data indicate that endogenous PGK1 crotonylation and its dynamic regulation are present in various cancer cell lines.

2. Does ECHS1 bind to K-to-R mutated PGK1?

Response: Thank you so much for pointing out this issue.

To answer this question, we performed Co-IP analysis using MDA-MB231 cells expressing Flag-tagged PGK1 wild-type or PGK1 3KR mutant. The result revealed that ECHS1 bound to both PGK1 and PGK1 3KR, suggesting that crotonylation did not affect the interaction between ECHS1 and PGK1 (Fig. 3e in the current version, new data). Analysis of the interface between PGK1 and ECHS1 in the AlphaFold2-predicted structure identified four putative ECHS1-binding sites in PGK1, including Lys133, Asn138, Lys139, and Lys144. We generated a Flag-tagged PGK1 mutant with four amino acid substitutions (K133D, N138A, K139D, K144D) and compared its interaction with ECHS1 to wild-type Flag-tagged PGK1. As depicted in updated Fig. 3f (new data), mutations in these amino acids of PGK1 abolished its interaction with ECHS1, while simultaneously enhancing PGK1 Kcr.

3. IF staining (3i, 4h) shows PGK1 expression over the whole cells, which will overlap will any other protein. Is it true that PGK1 is expressed over the whole cell, including nuclear?

Response: Thank you for pointing out this problem.

It is well-established that PGK1 is primarily a cytosolic glycolytic enzyme that catalyzes the conversion of 1,3-diphosphoglycerate and ADP to 3-phosphoglycerate and ATP. However, under certain conditions such as hypoxia, EGFR activation, and expression of mutated oncogenic genes, a small portion of cytosolic PGK1 can translocate to the mitochondria. In the mitochondria, PGK1 functions as a protein kinase to phosphate PDHK1, thereby affecting mitochondrial pyruvate metabolism (PMID: 26942675; PMID: 31911580). We have also confirmed in our study that hypoxia promotes the mitochondrial localization of PGK1 (Fig. 3i,p and 5b in the current version). Furthermore, it has been reported that EGFR activation promotes CK2α-dependent phosphorylation of nuclear PGK1 at Ser256 and interaction of PGK1 with CDC7. This interaction releases the inhibitory effect of ADP on CDC7, thereby promoting DNA replication (PMID: 32479953; PMID: 30392930). Hence, in addition to the cytosolic localization, PGK1 can also localize in the nuclear (please see the attached figure from the indicated reference).

[Redacted]

(PMID: 30392930) Figure S1. EGFR activation results in CK2α-dependent phosphorylation of PGK1 at Ser256 and subsequent PGK1/CDC7 complex formation. (D) U87/EGFR cells were treated with or without EGF (100 ng/ml) for 30 min. Immunofluorescent analyses were performed with anti-PGK1 and anti-CDC7 antibodies. DAPI (blue) was used to stain the cell nuclei. (E) Total cell lysates and cytosolic and nuclear fractions were prepared from U87/EGFR and T98G cells treated with or without EGF (100 ng/ml) for 3 h. Immunoblot analyses were performed with the indicated antibodies. The percentage of nuclear PGK1 protein expression was determined by quantifying the band intensity.

PCAF is also expressed in multiple cellular compartments (PMID: 31908141; PMID: 21131905; PMID: 37244254). Therefore, to enhance the clarity of our findings, we substituted the IF staining results from Fig. 4h in the previous version with cell fractionation results depicting the localization of PCAF and PGK1 (Fig. 4i in the current version, new data). Our observations indicated that both PCAF and PGK1 exhibited localization in the cytosol and mitochondria, suggesting a spatial basis for their interaction. Additionally, we conducted Co-IP analysis and *in vitro* pull-down experiments to confirm the interaction between PGK1 and PCAF or ECHS1 (Fig. 3b-d, Fig. 4c-e, Supplementary Fig. 5b and Supplementary Fig. 6c,d in the current version). Based on all the results of these experiments, we have concluded that PGK1 interacts with PCAF and ECHS1.

4. Will crotonyl-CoA treatment increase crotonyl-CoA levels in hypoxia-cultured 231 cells? Rescue PKG1-Kcr?

Response: Grateful to you for pointing out these issues.

To explore whether crotonyl-CoA treatment increases crotonyl-CoA levels in MDA-MB231 cells under hypoxic conditions, we treated cells with 10 mM sodium crotonate (pH 7.4) and cultured under normoxic or hypoxic conditions for 12 h. Our results showed that the addition of crotonate significantly increased the level of crotonyl-CoA under normoxic conditions. However, the intracellular level of crotonyl-CoA decreased under hypoxia in the presence or absence of crotonate treatment (Supplementary Fig. 1e in the current version, new data). Consistently, the PGK1 Kcr was enhanced with the addition of crotonate under normoxic conditions while hypoxic treatment abolished this phenomenon (Fig. 1k in the current version, new data). We added the related descriptions in the Parts of “The global crotonylome demonstrates downregulation of PGK1 crotonylation under hypoxia” in the Results section.

5. In vitro, does ECHS1 reduce crotonyl-CoA in the tubes?

Response: Thank you so much for this helpful comment.

To investigate whether ECHS1 reduces crotonyl-CoA *in vitro*, we incubated His-ECHS1 with crotonyl-CoA in the tubes. As ECHS1 catalyzes the conversion of crotonyl-CoA to 3-hydroxybutyryl-CoA, we measured the contents of both crotonyl-CoA and 3-hydroxybutyryl-CoA by performing the mass spectrometry analysis. Our results demonstrated a significant reduction in the level of crotonyl-CoA in the presence of ECHS1, accompanied by an increase in the level of 3-hydroxybutyryl-CoA (Supplementary Fig. 5a in the current version, new data).

6. PGK1 crotonylation occurs with or without enzymes, such as PCAF. What is their relative contribution?

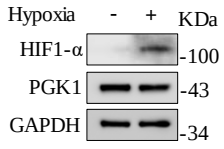
Response: Thank you so much for your concerns.

Regarding the minor Issue#5 raised by reviewer 2, we have explained that protein lysine acylation, including acetylation, succinylation, and crotonylation, has been reported to occur through nonenzymatic processes (PMID: 30131192; PMID: 26382620; PMID: 28803779). The occurrence of nonenzymatic protein acylation is dependent on the local substrate concentrations (PMID: 30131192). However, the cellular concentrations of crotonyl-CoA are significantly lower compared to acetyl-CoA, which is the most abundant CoA species (PMID: 25818647). In our previous version, we observed nonenzymatic PGK1 Kcr *in vitro* using relatively high concentrations of crotonyl-CoA (Fig. 1h, 2i in the previous version). More importantly, PCAF could catalyze the addition of PGK1 Kcr both *in vitro* and in live cells (Fig.4 in the current version). Thus, we conclude that PGK1 Kcr primarily occurs through the enzymatic pathway catalyzed by the crotonyltransferase PCAF in cells. Furthermore, as suggested by reviewer 2, we have removed the nonenzymatic data to avoid any potential confusion.

7. Does hypoxia alter PGK1 expression?

Response: Thank you so much for pointing out this issue.

As suggested by the reviewer, we detected the expression levels of PGK1 and HIF1α in cells cultured under normoxic or hypoxic conditions for 12 h. The results showed that hypoxia did not affect the expression of PGK1, but induced the expression of HIF1α (please see the attached figure below). We did not put it in the submitted manuscript as other figures also indicate hypoxia does not affect PGK1 expression (Fig. 5g in the current version, new data).



Legends: MDA-MB231 cells were collected and lysed. The whole-cell lysate was subjected to immunoblotting using the indicated antibodies.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

In the revised manuscript, Guo et al. have made significant efforts to enhance the quality of the work. The additional experimental data provided have addressed all my concerns. Therefore, I support the publication of this manuscript in Nature Communications.

Reviewer #2 (Remarks to the Author):

In the revised version, the authors provide new experimental data and related discussion, which address all of my comments. Therefore, I support the publication of this manuscript.

Reviewer #3 (Remarks to the Author):

The authors provide additional data to address most of my previous concerns. However, the new data also rinse some new issues, which should be further clarified.
Related to previous Number 2: PGK1 mutant with four amino acid substitutions abolished its interaction with ECHS1, but PGK1-3KR doesn’t. Together, these data suggest that N138A plays a major role in the interaction with ECHS1, which should be directed test with PGK1-N138A single mutant.
Related to previous Number 3: First, 4i didn't show PGK1 expression! Second, the Co-IP assays were performed in WCL, which subcellular component do these interactions occur in? Since the author cited previous studies showing PCAF and PGK1 could express in most subcellular locations, further evidence is required to show which subcellular location(s) their interaction has function.
Related to previous Number 5: It will be better to show the quantitative data, instead of the relative level of crotonyl-CoA to 3-hydroxybutyryl-CoA in Fig. S5A.

Point-by-point responses to comments from reviewers

Reviewer #1 (Remarks to the Author):

In the revised manuscript, Guo et al. have made significant efforts to enhance the quality of the work. The additional experimental data provided have addressed all my concerns. Therefore, I support the publication of this manuscript in Nature Communications.

Response: Greatly grateful to you for your recognition of our findings and your support for the publication of our manuscript in Nature Communications.

Reviewer #2 (Remarks to the Author):

In the revised version, the authors provide new experimental data and related discussion, which address all of my comments. Therefore, I support the publication of this manuscript.

Response: We greatly appreciate your recognition of our findings and your support for the publication of our manuscript.

Reviewer #3 (Remarks to the Author):

The authors provide additional data to address most of my previous concerns. However, the new data also rinse some new issues, which should be further clarified.

1. Related to previous Number 2: PGK1 mutant with four amino acid substitutions abolished its interaction with ECHS1, but PGK1-3KR doesn't. Together, these data suggest that N138A plays a major role in the interaction with ECHS1, which should be directed test with PGK1-N138A single mutant.

Response: Grateful to you for pointing out this issue.

We sincerely apologize for any confusion caused by our inaccurate description of the PGK1 mutant with four amino acid substitutions and the PGK1-3KR mutant. Our results showed that PGK1 mutant with four amino acid substitutions (K133D, N138A, K139D, K144D), but not PGK1 3KR mutant (K131/156/220R), abolished its interaction with ECHS1. Notably, the three lysine residues of PGK1 mutant with K133D, N138A, K139D and K144D are different from those of PGK1 3KR (K131/156/220R). Based on these data, we could not conclude that N138A plays a major role in the interaction with ECHS1, as these two PGK1 mutants have different lysine site mutations.

2. Related to previous Number 3: First, 4i didn't show PGK1 expression! Second, the Co-IP assays were performed in WCL, which subcellular component do these interactions occur in? Since the author cited previous studies showing PCAF and PGK1 could express in most subcellular locations, further evidence is required to show which subcellular location(s) their interaction has function.

Response: Thank you so much for pointing out this issue.

We apologize for the error in describing the results within our previous point-by-point response, and we also indeed forgot to put the blot of PGK1 in Fig. 4i. Now we added the blot

in Fig. 4i, please see the attached Figure. Actually, the localization of PGK1 was already blotted in Fig. 3p. The experiments in Fig. 3p and 4i were carried out under the same conditions, where cell fractionation samples were blotted. Our conclusions were based on the results presented in both Fig. 3p and 4i. Specifically, our results showed that PGK1 mainly localized in the cytosol and a small portion of cytosolic PGK1 can translocate to the mitochondria under hypoxic conditions whereas PCAF localized in both the cytosol and mitochondria under normoxic and hypoxic conditions, suggesting PCAF might interact with PGK1 in cytosol mainly and mitochondria to a lesser extent (Fig. 3p and 4i).

Fig. 4i

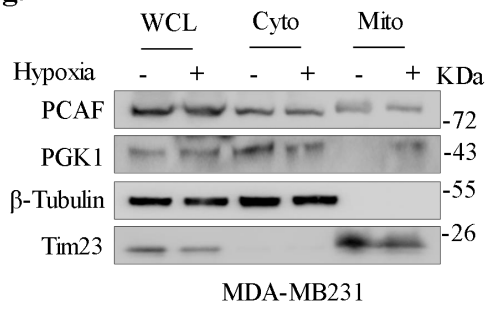
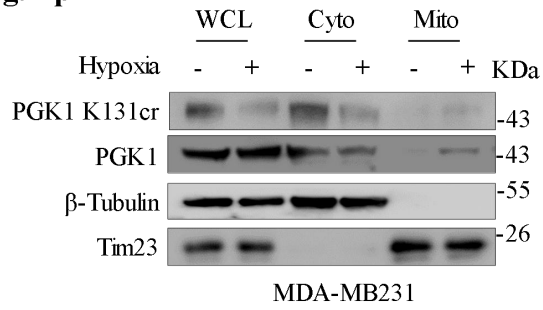


Fig. 3p



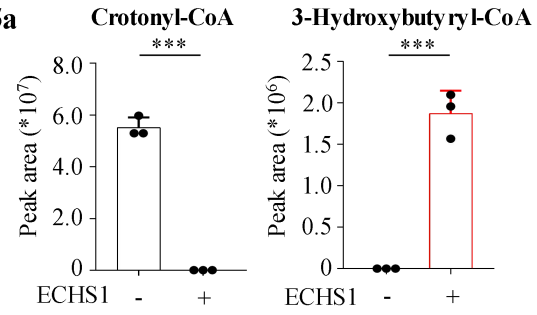
Legends: MDA-MB231 cells were cultured under normoxia or hypoxia for 12 h, followed by cell fractionation assays. Immunoblotting was performed to detect the indicated proteins.

3.Related to previous Number 5: It will be better to show the quantitative data, instead of the relative level of crotonyl-CoA to 3-hydroxybutyryl-CoA in Fig. S5A.

Response: Thank you so much for this suggestion.

We conducted an experiment to determine whether ECHS1 reduces crotonyl-CoA *in vitro*. We incubated 10 μM crotonyl-CoA with or without 2.5 μg ECHS1 recombinant protein in a 50 μL volume of reaction buffer at 30°C for 1 hour. Subsequently, we measured crotonyl-CoA and 3-hydroxybutyryl-CoA levels using LC-MS/MS. Following your suggestions, we have now provided the quantitative peak areas for crotonyl-CoA and 3-hydroxybutyryl-CoA in Fig. S5a.

Fig. S5a



*Legends: In vitro ECHS1 enzyme activity assays were performed by incubating crotonyl-CoA with or without ECHS1 recombinant protein. The levels of crotonyl-CoA and 3-hydroxybutyryl-CoA were detected by LC-MS/MS. Data are shown as the mean ±SD, n = 3 biologically independent samples. ***p < 0.001 (unpaired two-tailed Student's t-test).*