nature portfolio

Peer Review File

Citrullination modulation stabilizes HIF-1α to promote tumour progression

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Review of "Citrullination modulation stabilizes HIF-1α to promote tumour progression" by Chen et al.
This is an interesting paper describing a new phenomenon of Padi4 binding to HIF-1α and its subsequent citrullination, required for its stability, in hepatocellular carcinoma cells. The key findings of this manuscript are as follows:
1) PADI4 directly binds and citrullinates HIF-1α.
2) HIF-1αcitrullination stabilizes HIF-1α protein by disrupting the interaction of HIF-1α and VHL.
3) DHE is an antagonist of PADI4 and represses HIF-1α expression.
4) PADI4-mediated HIF-1α citrullination promotes HCC progression in vivo.
The manuscript is of potential interest. It is not completely novel, since it was previously found that inhibition of HIF1α decreases hypoxia-induced peptidylarginine deiminase 2, and that hypoxia increases the production of PAD2 and citrullinated proteins. However, it provides many mechanistic insights and has a potential clinical relevance.
Critiques of methodology/approach:
1) An IP-MS analysis is performed together with IgG control, as IgG can bind many proteins non-specifically. Did the authors find PADI4 bound to IgG control in the experiment in Figure 1A? Also, it would be a good positive control to check whether highly significant hits from Figure 1A are know binding partners of HIF-1. Are there any?
2) Using IF, the authors observe a colocalization of HIF-1α and PADI4 in Hep3B cells. However, the staining looks like strong background/autofluorescence, as 100% of cell areas are highly positive for both HIF-1α and PADI4. Liver cells (including hepatomas) frequently exhibit strong autofluorescence due to high amounts of metabolites. I would not show this data as it does not look very convincing. Again, Figure 2H staining in red and green channels looks completely non-specific. If the authors want to keep these data, negative and positive controls (Secondary antibody only and primary antibody only staining must be shown. Otherwise, you show 100% colocalization in the entire cell.
3) Staining area for citrullinated and non-citrullinated HIF-1a are almost identical. Does it mean that nearly 100% of HIF-1a is citrullinated at R698? If yes, why is your MS analysis identified <20 of R698 citrullinated peptides?
4) Achieving high specificity for a single amino acid residue or a post-translational modification (PTM) on a particular amino acid with polyclonal antibodies is challenging. The authors claim that their polyclonal antibody specifically recognizes R698-citrullinated HIF-1α. It is unclear for this reviewer how a polyclonal antibody can generate such high specificity.
5) The authors showed that ectopic expression of PADI4 led to greater accumulation of HIF-1α proteins (Figure 3A). However, the endogenous PADI4 expression is not taken into account. The authors should show the baseline expression of PADI4 in Hep3B and Hep2G and further modify their results based on the findings.
6) In Figure 3E, the authors showed that MG132 profoundly blocked PADI4 knockdown-mediated HIF-1α degradation, suggesting that PADI4 promotes HIF-1α stability by interfering with proteasomal-dependent degradation. But MG132 prevents degradation of any protein, including HIF-1α, which is known to be a subject to degradation. This experiment does not prove any involvement of PADI4 in stability of HIF-1α.
7) For Figure 3G, It is unclear for this reviewer what is the cause of the molecular weight difference between endo and exo Padi4? FLAG-tag weight is 1012 Da. Also, the endogenous Padi4 expression did not seem to be affected much by the shRNA knockdown. In this case, it is impossible to generate cells with 100% enzymatically inactivated Padi4 expression. It is recommended to transfect Padi4 KO cells with PADI4D473A and PADI4C645A mutant vectors to prove the author's point.
8) Did enzymatically inactivated Padi4 reduce/abrogate HIF-1α citrullination at R698?
9) The authors claimed that compared with normoxic conditions, the binding between HIF-1α and PADI4 was increased accompanied by increased citrullinated HIF-1αR698 levels under hypoxic conditions, suggesting that oxygen-mediated hydroxylation of HIF-1α may disrupt HIF-1α-PADI4 binding as well as PADI4-mediated HIF-1α citrullination. But this also could be because hypoxia increased Padi4 levels in the cell. Most of your experiments show that hypoxia elevates Padi4. Please clarify.
10) It is unclear why the normal liver cell numbers have not been affected by hypoxia at 48 h (Figure 5B).
11) Western blot results showed that the HIF-1α protein levels were decreased after DHE treatment in a dose dependent manner, while the PADI4 protein levels were unaffected. Does it

mean that DHE binding prevents HIF-1α WB antibody binding (resulting in weaker WB signal) or does it mean that DHE reduces mRNA expression of HIF-1α? At many instances, it is stated that DHE reduces HIF-1α expression. If so, the mechanism should be explored.
Suggestions on how to improve the manuscript:
1) Hypoxia induced PADI4 protein levels (Figure 3B). This is interesting and should be discussed.
2) In this study Dihydroergotamine is proposed as a drug preventing/inhibiting HIF-1α and PADI4 interaction. However, this drug does not inhibit free unbound PADI4 or HIF-1α or those bound to other proteins. This limitation should be clearly discussed. Some statements in the manuscript text should be corrected such as Figure 5 label: dihydroergotamine is an antagonist of PADI4 and represses HIF-1α expression.
3) More discussion should be provided on how these new data fit into current knowledge in the field. Citrullination of the HCC is not well reported, but there are studies documenting citrullination of colorectal cancer liver metastasis. Also, a thorough discussion of this highly relevant paper should be performed PMID: 29484680.
Minor Critiques:
1) In the sentence "To test these possibilities, ectopically expressed Flag-tagged HIF-1α was immunoprecipitated and detected with an anti-pancitrullination antibody in HEK293T and Hep3B cells" please change detected for probed.
2) In Figure 1G legend, the authors claim that they performed "probing with an anti pancitrulline antibody". But the methods section states that they used The Anti-Citrulline (Modified) Detection Kit which is not an anti pancitrulline antibody. Please clarify.
3) Most experiments in this study were performed in mouse cell lines. Therefore, mouse protein and gene nomenclature should be used throughout.
4) All bar graphs should display individual data points, and it should be indicated in the figure legends whether each dot means a technical replicate or a culture dish.

Reviewer #2 (Remarks to the Author):

The authors describe a novel mechanism for the regulation of HIF-1a. by PADI4-mediated citrullination of R698. This novel modification interferes with the binding of VHL, thus contribution to the stabilization of HIF-1a under hypoxic conditions. This finding will be of moderate significance to the field and builds on an already large body of work describing the regulation of HIF-1a by hypoxia and VHL. This was a well-written paper, and the data as a whole largely support the conclusions drawn.

To increase the rigor and applicability of the findings to the field, some additional data are required:

2. The authors elegantly outline a novel mechanism of regulation of HIF-1a during hypoxia. Since HIF-2a is regulated by similar mechanisms (PHDs and VHL) and also plays an important pro-tumorigenic role in HCC and other cancer types, the authors should demonstrate whether PADI4 also stabilize HIF-2a and whether citrullination plays a similar role in regulating the stability of HIF-2a.

3. The author suggest that citrullination only occurs when the HIF-1a hydroxylation by the PHDS are inhibited, either by hypoxia, or by expression of a HIF-1a mutant that cannot by hydroxylated. The authors should supplement this finding using shPADI4 in combination with other inhibitors of the PHDs (eg using iron chelators such as DFO or CoCL2) to verify that this effect is PHD, rather than hypoxia dependent.

1. In Fig. 1C, the authors show co-localization of HIF-1a and PADI4 in hypoxic HEP3B cells. It is surprising that HIF-1a is appears to be expressed throughout the cell (both cytoplasmic and nuclear) under these hypoxic conditions, when it is expected that HIF-1a should be largely nuclear. Similarly, no staining validations controls are shown for PADI4. Thus, the authors should provide validation of the HIF-1a and PADI4 staining conditions used for used for ICC (for example using normoxia vs hypoxia or siRNA knockdown) to validate that the signals seen are indeed specific for the proteins indicated.

Additional minor comments

1. Line 139 Page 6: The authors state that PADI4 significantly increased the citrullination levels of HIF-1α in a dose-dependent manner (Fig. 1g). Authors should provide statistical analysis to justify

this statement or modify statement accordingly

2. Line 225 page 10 :"The authors state that PADI4 significantly blocked VHL binding to HIF-1α under both normoxic and hypoxic conditions (Fig. 4c) Authors should provide statistical analysis to justify this statement or modify statement accordingly.

3. Page 16 Line 379: The authors state that "In addition to the long-held view that HIF-1α is stable owing to the decreased activities of PHDs under hypoxic conditions, we provide evidence based on epigenetic modification findings that PADI4-mediated citrullination of HIF-1 is equally critical for HIF-1 stabilization and activity under hypoxic conditions". This statement is not justified by the data. No comparison was made between the contribution of PHDs versus citrullination for the stabilization of HIF-1a during hypoxia. In addition, the authors suggest that citrullination may only occur in non-hydroxylated HIF suggesting that citrullination requires inhibition of the PHDs (by hypoxia).

Reviewer #3 (Remarks to the Author):

In this study, the researchers investigated the role of peptidyl arginine deiminase 4 (PADI4) in regulating tumor progression and its potential therapeutic significance. Through a series of in vitro and in vivo experiments, the researchers demonstrated that the enzymatic activity of PADI4 contributes to tumor growth. Mechanistically, PADI4-mediated citrullination of HIF-1α blocks VHL binding, thereby preventing VHL-mediated HIF-1α ubiquitination and subsequent proteasome degradation. Overall, the results of the study reveal citrullination as a previously unrecognized posttranslational modification related to HIF-1α stability and suggest that targeting PADI4-mediated HIF-1α citrullination could be a promising therapeutic strategy for cancers with elevated HIF-1α expression.

Question 1: According to the literature, the PAD4 protein has a nuclear localization signal and is expressed in the nucleus. However, in the authors' immunofluorescence images, PAD4 is distributed throughout the entire cytoplasm. Considering that the authors already have the Flag-PAD4 vector, this reviewer suggest the authors use a Flag antibody for immunofluorescence to test the localization of PAD4 in normal and hypoxia conditions. In Fig. 1c and Fig. 2h, please perform control experiments to stain the cells with HIF-1α and PAD4 antibodies separately.

Question 2: Please provide the molecular weight of PAD4 in the Western blot? In Figure 3-g, please provide the molecular weights of endogenous PAD4 and exogenous PAD4?

Question 3: In reference 39, it was showed that the expression levels of PAD4 and HIF-1α increase under hypoxic conditions. In Figure 5f, the expression level of PAD4 under hypoxic conditions is largely consistent with that under normal conditions; however, in Figure S3c-d, the expression level of PAD4 is significantly increased under hypoxia conditions. There seems to be a contradiction in the results of these two figures. Please clarify these data points.

Question 4: The oral bioavailability of DHE is low, please test the serum and tumor DHE concentrations in the mouse model. Please offer rationale for choosing oral administration instead of intravenous administration.

Question 5: PAD4 plays a role in maintaining the stability of HIF-1α by citrullination. In animal experiments, DHE is also utilized to inhibit the citrullination of HIF-1α by PAD4. Please test if HIF-1α citrullination and histone citrullination is altered after DHE administration in the mouse model?

Point-by-point response to the comments of the Reviewers

We appreciate the editor for your kind decision and the opportunity to provide our point-by-point responses based on the positive comments of the three reviewers. We are grateful to our reviewers for their constructive comments and suggestions that have greatly improved the quality of our study. In the past three months, we have performed additional experiments and addressed all the concerns raised by the reviewers. Here we submitted a substantially improved manuscript along with our point-by-point response. For the reviewers' convenience, we have appended all the revised figures in this file, which we labeled as **Figure R1** to **Figure R22**.

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Responses: We appreciate the reviewer for the positive comments and summary of our study. Meanwhile, we thank the reviewer for the valuable suggestions which helped us strengthen this study.

Critiques of methodology/approach:

1) An IP-MS analysis is performed together with IgG control, as IgG can bind many proteins non-specifically. Did the authors find PADI4 bound to IgG control in the experiment in Figure 1A? Also, it would be a good positive control to check whether highly significant hits from Figure 1A are known binding partners of HIF-1. Are there any?

Response: We appreciate the reviewer for the concern. For immunoprecipitation-mass spectrometry (IP-MS) assays, Hep3B-Flag-HIF-1α was set up as the experimental group while Hep3B-Flag-EV was used as the control group, and both Flag-HIF-1α and Flag-EV were immunoprecipitated with the anti-Flag antibody. Theoretically, the control Flag-EV group could largely exclude non-specific binding proteins. Furthermore, to determine whether IgG binds to PADI4 non-specifically, we have performed Co-IP assays with IgG as the control group including **Figure 1b**, **Figure S1b** and **S1c** in the original manuscript, these results showed no interactions between IgG antibody and PADI4 protein.

Meanwhile, we re-examined the top 10 highly significant candidates in **Figure 1a** in the original manuscript and found that apart from the SPECC1L, the left candidates were all included in the interaction network of HIF-1α, and six of these 10 proteins including PIK3C2A, DAB2, PICALM, IGF2BP3, PADI4 and HSPA8 were predicted to bind to HIF-1α based on STRING database (**Figure R1**). Moreover, the molecular chaperone HSPA8 is a known binding partner of HIF-1α, promoting its lysosomal-dependent protein degradation[1]. Collectively, these results indicated that our IP-MS results are reasonable.

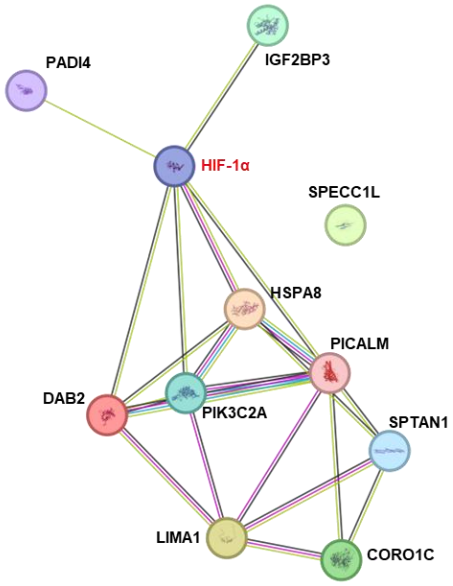


Figure R1. The protein-protein interaction network showed potential interaction between HIF-1α and indicated proteins. The relation between HIF-1α and the top 10 candidates in our IP-MS result was

analyzed by the STRING database. Network nodes represent proteins and different colors of the line mean types of interactions (Purple: experimentally determined interactions; Green: text-mining predicted interactions; Black: co-expressions; Cyan: known interactions from curated databases).

2) Using IF, the authors observe a colocalization of HIF-1α and PADI4 in Hep3B cells. However, the staining looks like strong background/autofluorescence, as 100% of cell areas are highly positive for both HIF-1α and PADI4. Liver cells (including hepatomas) frequently exhibit strong autofluorescence due to high amounts of metabolites. I would not show this data as it does not look very convincing. Again, Figure 2H staining in red and green channels looks completely non-specific. If the authors want to keep these data, negative and positive controls (Secondary antibody only and primary antibody only staining must be shown. Otherwise, you show 100% colocalization in the entire cell.

Response: We appreciate the reviewer for pointing this out and Referee #2&3 raised the similar concerns. According to the reviewer's kind and thoughtful suggestions, immunofluorescence (IF) assays were reperformed to exclude the potential interferences of strong autofluorescence of liver cells. We stained the cells either with secondary antibodies (anti-Mouse-488/594, anti-Rabbit-488/594) or primary antibodies (anti-HIF-1α, -PADI4, -HIF-1$\alpha^{R698Cit}$) alone, and no autofluorescence was observed during confocal imaging (**Figure R2a, b**). To avoid strong autofluorescence interference, we further optimized the dilution ratio of primary antibodies and found obvious nuclear localization of HIF-1α under hypoxic conditions (**Figure R2c**). PADI4 antibody was also validated in Hep3B-shPADI4 cells. Being consistent with previous reports[2,3], both nuclear and cytoplasmic localization of PADI4 was observed in NTC control cells (**Figure R2d, upper panel**), while almost no signal was detected upon PADI4 knockdown (**Figure R2d, lower panel**).

By employing these negative and positive controls suggested by our reviewer, experimental conditions of **Figure 1c** and **Figure 2h** in the original manuscript were further reperformed. There is a clear colocalization between HIF-1α, PADI4 and HIF-1$\alpha^{R698Cit}$ with high quality (**Figure R3a, b**), which is consistent with our original conclusion. We have included **Figure R3a, R3b** as **Fig. 1c** and **Fig. 2h** in the revised manuscript, respectively.

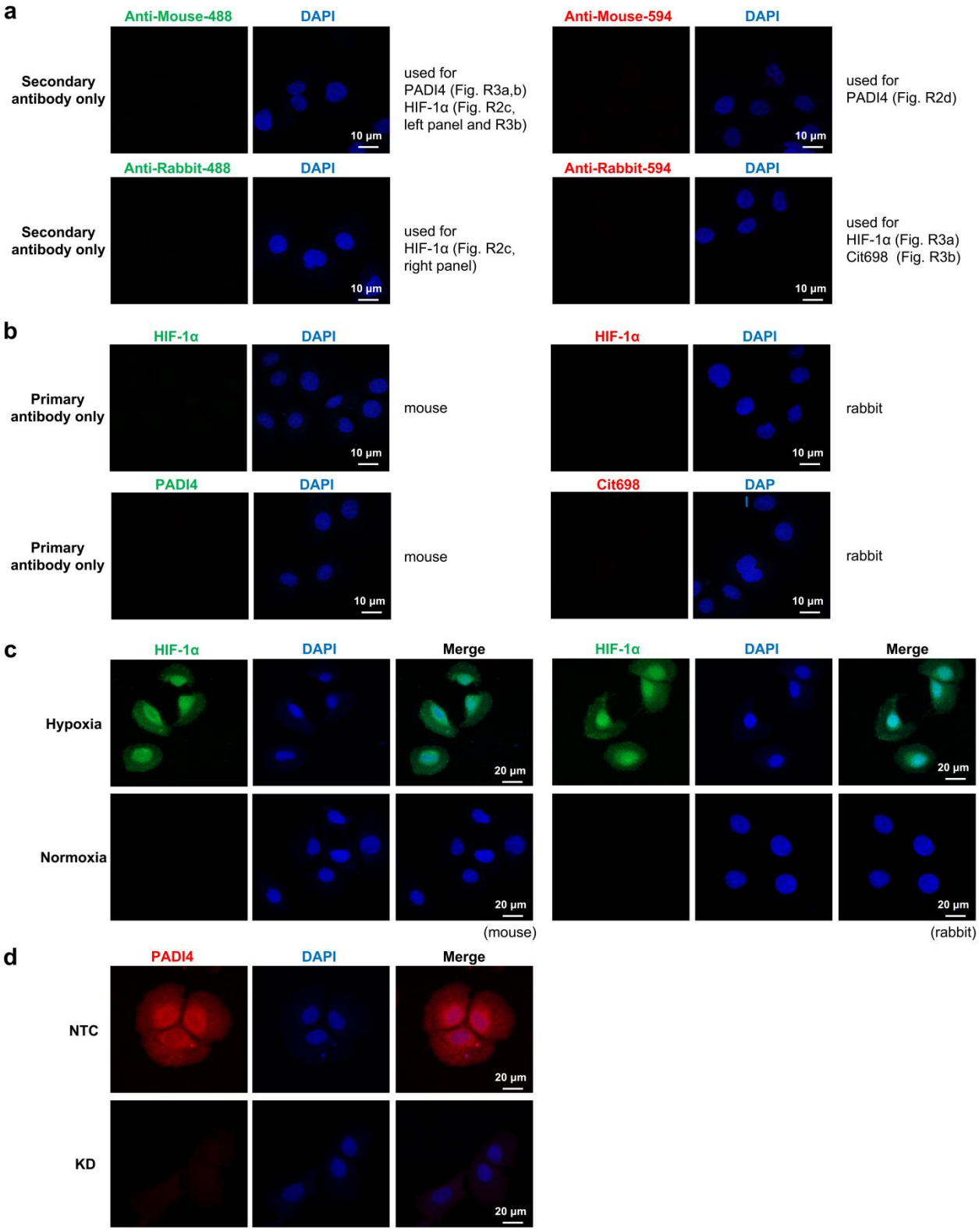


Figure R2. Verification of the staining specificity of HIF-1α and PADI4 antibodies for immunofluorescence (IF) assays. (**a-b**) Hep3B cells cultured under hypoxic conditions for 6 h were separately stained with secondary antibodies alone (CoraLite488/594-conjugated Anti-Mouse IgG, CoraLite488/594-conjugated Anti-Rabbit IgG) (**a**) or primary antibodies alone (HIF-1α, PADI4, HIF-1α^{R1698Cit}) (**b**). Scare bar: 10 μm. (**c-d**) Specificity verification of HIF-1α and PADI4 staining. IF staining of endogenous HIF-1α in Hep3B cells

cultured under hypoxic or normoxic conditions for 6 h (**c**). IF staining of endogenous PADI4 in Hep3B cells expressing NTC or shPADI4 (**d**). Scare bar: 20 μm. Images were acquired by a confocal microscope system (Zeiss LSM 800).

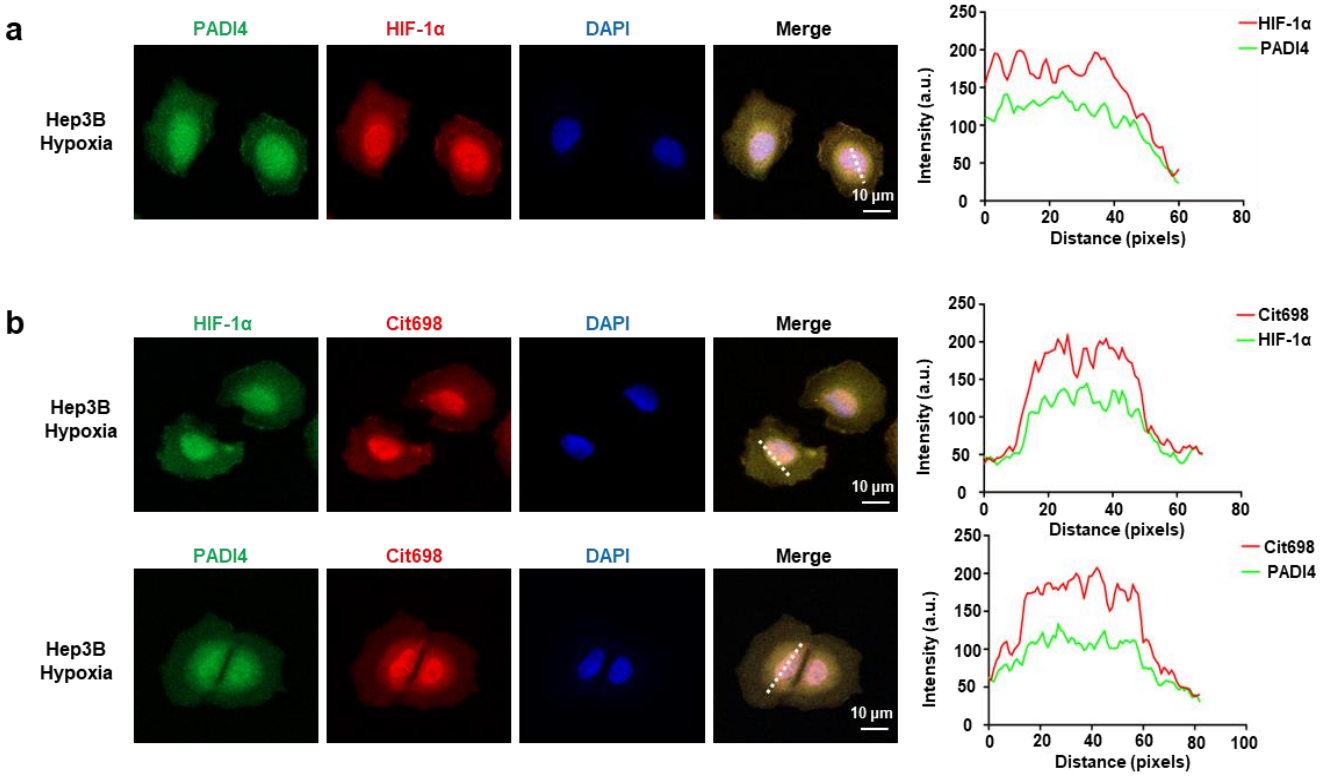


Figure R3. Analysis of IF staining of PADI4, HIF-1α and HIF-1α^{R698Cit} in Hep3B cells. (**a-b**) IF analysis of the colocalization of PADI4 and HIF-1α (**a**), citrullinated HIF-1α^{R698} and HIF-1α or PADI4 (**b**) in Hep3B cells cultured under hypoxic conditions for 6 h (left panel). Scale bars: 10 μm. Intensity profiles of each line were quantified by ImageJ software and drawn with GraphPad Prism 7.0 (right panel). We have included **Figure R3a, R3b** as **Fig. 1c** and **Fig. 2h** in the revised manuscript, respectively.

3) Staining area for citrullinated and non-citrullinated HIF-1a are almost identical. Does it mean that nearly 100% of HIF-1a is citrullinated at R698? If yes, why is your MS analysis identified <20 of R698 citrullinated peptides?

Response: We appreciate the reviewer's concern. We consider that it's difficult to say the almost identical staining region means that 100% of HIF-1α is citrullinated at R698, as the IF detection may not be accurate enough for precise quantitative analysis. In addition, The strength of antibody signals and fluorescence exposure time will affect the visual effect of IF staining.

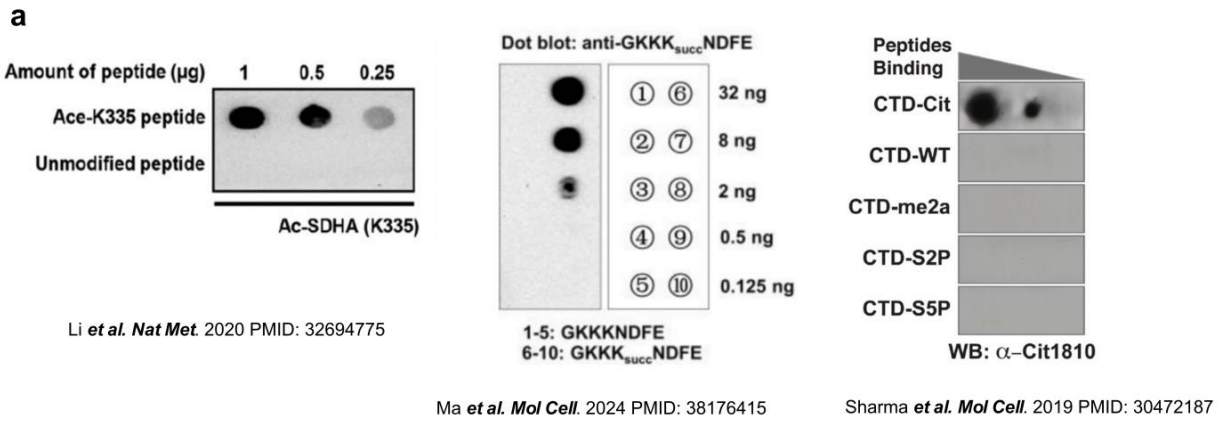
As for the mass spectrometry (MS) result of R698 citrullinated peptides < 20, there are three reasons: 1) HIF-1α proteins were digested into dozens of peptides by trypsin for the MS analysis and some

peptide information would be lost during the experiment. 2) to identify the citrullinated residues on HIF-1α, we carried out the *in vitro* citrullination assay and the total HIF-1α may not be 100% modified by PADI4 in the catalytic system. 3) the identification of protein post-translational modification by mass spectrometry is a qualitative method, the number of citrullinated peptides identified by MS analysis could not reflect the real proportion of citrullinated HIF-1α. Therefore, the high peptide-spectrum match (PSM) score of R698Cit only gave us a clue that the R698 is most likely to be the citrullinated site of HIF-1α (**Figure 2b** in the original manuscrip). Our further molecular biology experiments showed that the R698 is the truly citrullinated site of HIF-1α (**Figure 2f, g** and **Figure S2c** in the original manuscript).

4) Achieving high specificity for a single amino acid residue or a post-translational modification (PTM) on a particular amino acid with polyclonal antibodies is challenging. The authors claim that their polyclonal antibody specifically recognizes R698-citrullinated HIF-1α. It is unclear for this reviewer how a polyclonal antibody can generate such high specificity.

Response: This is a good point. It is undeniable that the specificity of monoclonal antibodies is better than that of polyclonal antibodies. With the improvement of antibody production technology, polyclonal antibodies against point mutation of single amino aicd residue including SDHA-K335Ac, LACTB-K284succ and RNAP2-R1810cit have been used in our previous published papers in *Nature Metabolism*[4] and *Molecular cell*[5], and other protein citrullination research[6] (**Figure R4a**). In addition, considering that the preparation of monoclonal antibodies can incur considerable time and money costs, we selected polyclonal antibodies targeting citrullination at R698 site for further study.

HIF-1α^{R698Cit} antibody in this study was customized by Abclonal company. Briefly, a KLH (KeyholeLimpetHemocyanin)-coupled peptide sequence (CALSQ-cit-TTVP) derived from HIF-1α was synthesized and injected into 3 rabbits. Rabbits were then sacrificed after 5 injections and the serum was collected for further purification. Affinity purification was performed first on columns containing the citrullinated peptide, followed by elution of non-citrullinated specific antibodies on columns containing the non-citrullinated peptide to obtain antibodies that specifically recognize HIF-1α^{R698} citrullination. Dot blot assays were further performed to screen out the antibody with the best specificity, and we select antibody-2 for our follow-up experiments in the original manuscript (**Figure R4b**).



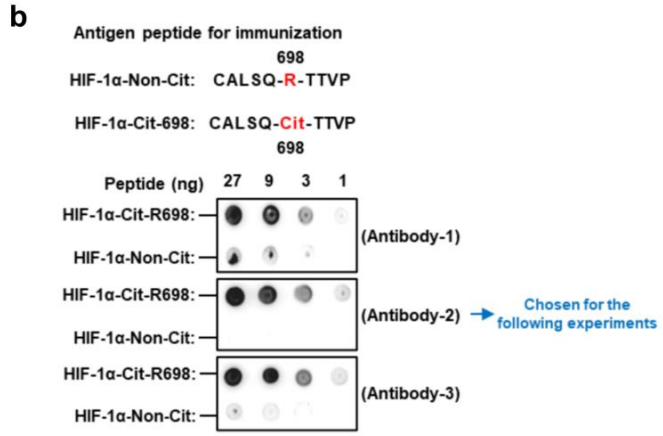


Figure R4. The specificity analysis of HIF-1α^{R698Cit} antibody by dot blot. (**a**) The specificity of SDHA-K335Ac (PMID: 32694775), LACTB-K284succ (PMID: 38176415) and RNAP2-R1810cit (PMID: 30472187) polyclonal antibodies was verified by dot blot using a nitrocellulose membrane. (**b**) The specificity of citrullinated HIF-1α^{R698} antibody was verified by dot blot using a nitrocellulose membrane spotted with 1, 3, 9 and 27 ng of citrullinated HIF-1α peptide with the R698 residue (CALSQ-Cit-TTVP) or the corresponding unmodified peptide (CALSQ-R-TTVP). Antibodies 1-3 represent 3 antibodies purified from 3 rabbits, respectively.

5) The authors showed that ectopic expression of PADI4 led to greater accumulation of HIF-1α proteins (Figure 3A). However, the endogenous PADI4 expression is not taken into account. The authors should show the baseline expression of PADI4 in Hep3B and Hep2G and further modify their results based on the findings.

Response: Following our reviewer's suggestion, we repeated the same experiment and western blotting experiments were conducted using endogenous PADI4 antibody to show the baseline and ectopic expression of PADI4. Consistent with our previous conclusion, overexpression of PADI4 led to obvious accumulation of HIF-1α proteins (**Figure R5**). We have included **Figure R5** as **Fig. 3a** in the revised manuscript.

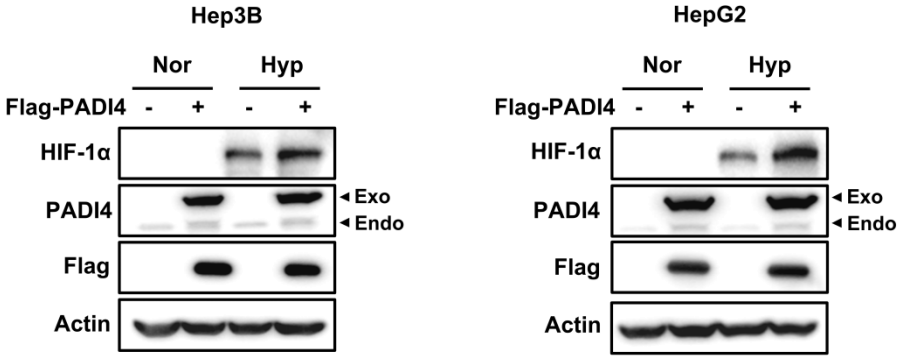


Figure R5. PADI4 overexpression promotes the accumulation of HIF-1α protein levels under hypoxic conditions. Hep3B (left panel) and HepG2 cells (right panel) expressing Flag-EV or Flag-PADI4 were cultured under normoxic or hypoxic conditions for 6 h, cell lysates were harvested and protein levels of HIF-1α were measured by western blotting. Actin served as the loading control. We have included **Figure R5** as **Fig. 3a** in the revised manuscript.

6) In Figure 3E, the authors showed that MG132 profoundly blocked PADI4 knockdown-mediated HIF-1α degradation, suggesting that PADI4 promotes HIF-1α stability by interfering with proteasomal-dependent degradation. But MG132 prevents degradation of any protein, including HIF-1α, which is known to be a subject to degradation. This experiment does not prove any involvement of PADI4 in stability of HIF-1α.

Response: This is a good point. As indicated by the reviewer, HIF-1α is recognized by E3 ubiquitin ligase, then ubiquitinated and degraded by 26S proteasome-mediated degradation, which can be blocked by MG132, a proteasome inhibitor[7-9]. To further demonstrate the involvement of PADI4 in HIF-1α stability, we detected the ubiquitination levels of HIF-1α in 293T-PADI4 cells. Results showed that overexpression of PADI4 could obviously reduce HIF-1α ubiquitination levels (**Figure R6a**), suggesting that PADI4 prevents HIF-1α degradation from ubiquitination. In addition, we investigated the impact of PADI4 knockdown on HIF-1α protein half-life by cycloheximide (CHX) chase experiments. The results showed that the degradation rate of HIF-1α became faster when PADI4 was knocked down (**Figure R6b**). Collectively, **Figure R6** and **Figure 3e** in the original manuscript concluded that PADI4 promotes HIF-1α protein stability by blocking proteasomal-dependent degradation. We have included **Figure R6a, R6b** as **Fig. S3d, S3e** in the revised manuscript, respectively.

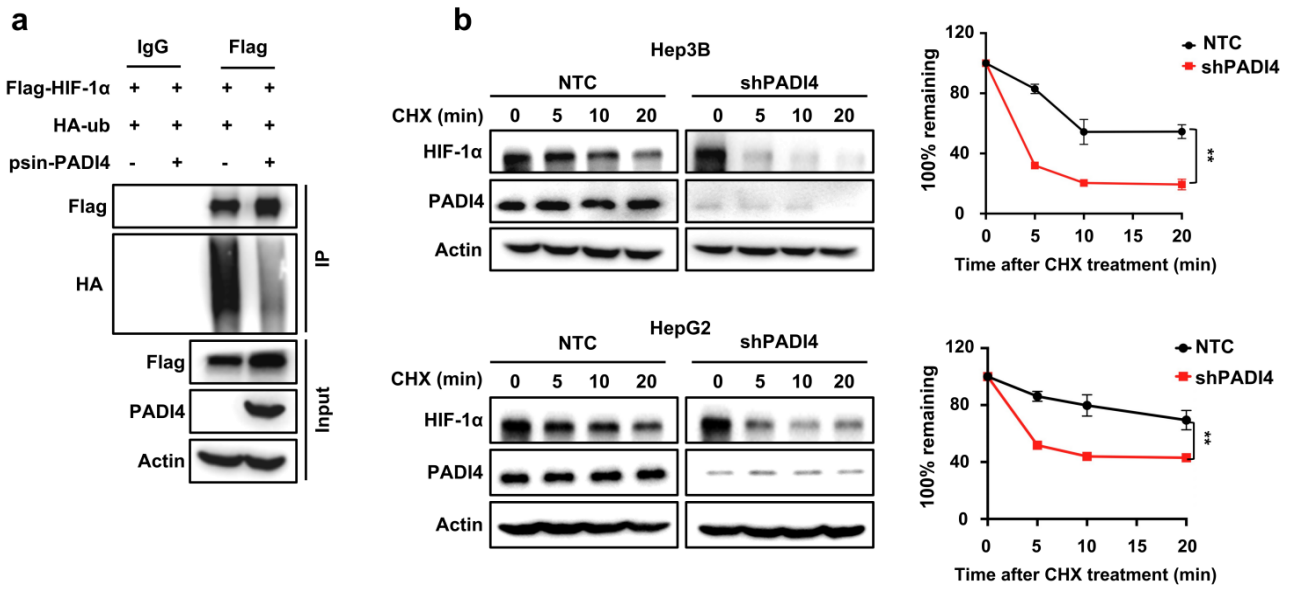


Figure R6. PADI4 suppresses HIF-1α ubiquitination and maintains HIF-1α stability. (**a**) HEK293T cells were transfected with EV or PADI4 plasmids together with Flag-HIF-1α and HA-Ub plasmids. After 48 h transfection, cells were treated by 10 μM MG132 for another 6 h and cell lysates were harvested for immunoprecipitation with either IgG or anti-Flag antibodies and analyzed by western blotting. (**b**) Western blot analysis (left panel) of HIF-1α protein levels in Hep3B and HepG2 cells expressing NTC or PADI4 shRNA treated with 40 μg/ml CHX for the indicated times. Actin served as the loading control. Quantification of HIF-1α protein levels relative to Actin (right panel). Data are presented as the mean ± SEM of 3 independent experiments (*n*=3 biological replicates).We have included **Figure R6a, R6b** as **Fig. S3d, S3e** in the revised manuscript, respectively.

7) For Figure 3G, it is unclear for this reviewer what is the cause of the molecular weight difference between endo and exo Padi4? FLAG-tag weight is 1012 Da. Also, the endogenous Padi4 expression did not seem to be affected much by the shRNA knockdown. In this case, it is impossible to generate cells with 100% enzymatically inactivated Padi4 expression. It is recommended to transfect Padi4 KO cells with PADI4D473A and PADI4C645A mutant vectors to prove the author's point.

Response: We apologize for this confusion. Actually, we used the 3×FLAG tag vector to construct the exogenous PADI4, which could lead to a 3~4 kDa molecular weight shift. In western blotting, the lower concentration of resolving gel, the more obvious the shift of proteins with higher molecular weight (exogenous PADI4 about 70kDa). To make sure that the exogenous PADI4 was overexpressed and the endogenous PADI4 was knocked down, 7% SDS-PAGE gel was employed in this study to better separate the exogenous and endogenous PADI4.

Following the reviewer's suggestion, PADI4 KO cells were established by CRISPR-Cas9 and further overexpressed PADI4^{WT}, PADI4^{D473A} and PADI4^{C645A} mutants. Western blot results are consistent with our previous results in **Figure 3g** in the original manuscript. Namely, the ectopic

expression of PADI4^{WT}, but not that of the inactivated mutants, rescued PADI4 knockout-induced decrease in HIF-1α protein levels (**Figure R7a, b, upper panel**). The original data of exogenous and endogenous PADI4 was shown with the protein ladder (**Figure R7a, b, lower panel**). As for the efficiency of shRNA, we have reperformed these assays and observed similar phenomenon (**Figure R7c, d**), therefore, we have included **Figure R7c** and **R7d** as **Figure 3g** and **S3g** in the revised manuscript, respectively.

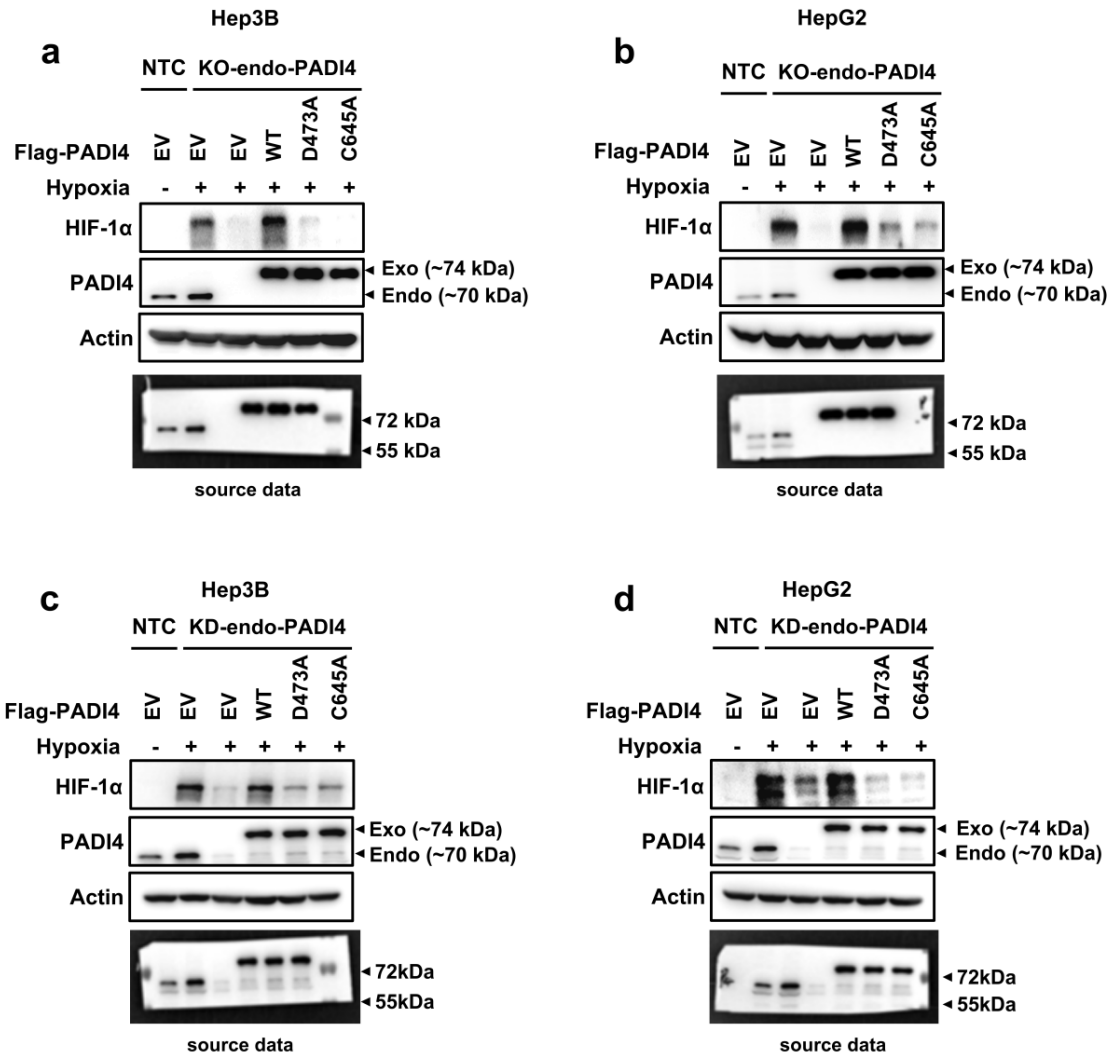


Figure R7. PADI4 enzymatic activity is essential for maintaining HIF-1α protein levels. (**a-b**) Endogenous PADI4-knockout Hep3B cells (**a**) and HepG2 cells (**b**) were infected with viruses expressing Flag-EV, Flag-PADI4^{WT}, Flag-PADI4^{D473A}, or Flag-PADI4^{C645A} and further cultured under normoxic or hypoxic conditions for 6 h. Cell lysates were harvested, and the protein levels of HIF-1α were analyzed by western blotting. Source data of the exogenous and endogenous PADI4 with the protein ladder are shown. Actin served as the loading control. (**c-d**) Endogenous PADI4-knockdown Hep3B cells (**c**) and HepG2 cells (**d**) were infected with viruses expressing Flag-EV, Flag-PADI4^{WT}, Flag-PADI4^{D473A}, or Flag-PADI4^{C645A} and further cultured under normoxic or hypoxic conditions for 6 h. Cell lysates were harvested, and the protein levels of HIF-1α were analyzed by western blotting. Source data of

the exogenous and endogenous PADI4 with the protein ladder are shown. Actin served as the loading control. We have included **Figure R7c** and **R7d** as **Figure 3g** and **S3g** in the revised manuscript, respectively.

8) Did enzymatically inactivated Padi4 reduce/abrogate HIF-1α citrullination at R698?

Response: The reviewer's point is well-taken. To determine whether enzyme activity is essential for PADI4-enhanced HIF-1α^{R698} citrullination, His-PADI4-WT, His-PADI4^{D473A}, His-PADI4^{C645A} proteins and GST-HIF-1α proteins were purified from the *Escherichia coli* strain BL21 (DE3) for the *in vitro* citrullination assay. The results showed that, unlike WT-PADI4, the inactivated PADI4 mutants failed to catalyze the citrullination of HIF-1α at R698 (**Figure R8**). We have included **Figure R8** as **Fig. S3f** in the revised manuscript.

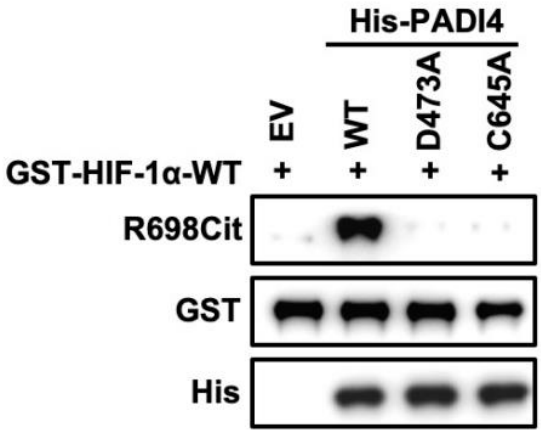


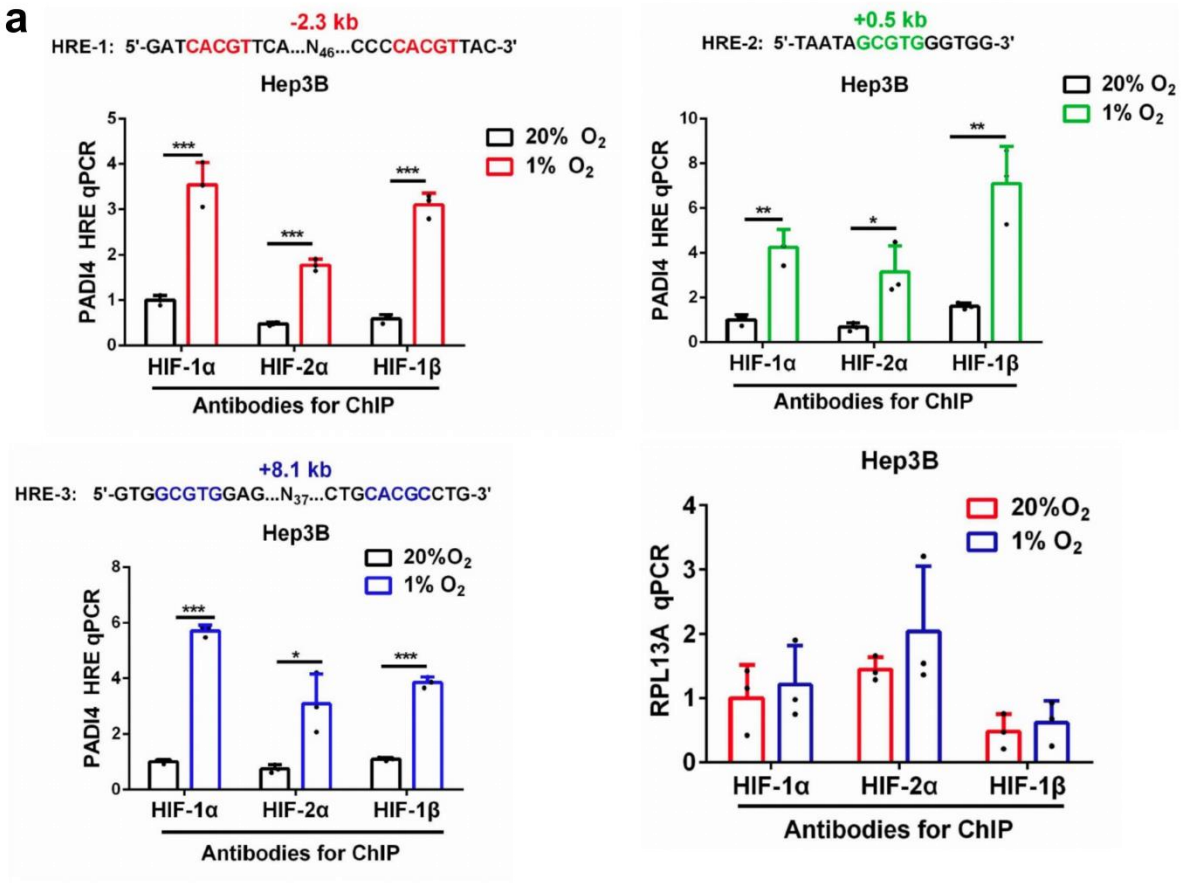
Figure R8. *In vitro* citrullination of HIF-1α by WT or enzymatically inactivated PADI4. An *in vitro* citrullination assay was performed by incubating purified GST-HIF-1α with WT His-PADI4 or enzymatically inactivated His-PADI4^{D473A} or His-PADI4^{C645A} proteins at 37 °C for 1 h in the catalytic buffer and probing with the anti-HIF-1α^{R698Cit} antibody. We have included **Figure R8** as **Fig. S3f** in the revised manuscript.

9) The authors claimed that compared with normoxic conditions, the binding between HIF-1α and PADI4 was increased accompanied by increased citrullinated HIF-1αR698 levels under hypoxic conditions, suggesting that oxygen-mediated hydroxylation of HIF-1α may disrupt HIF-1α-PADI4 binding as well as PADI4-mediated HIF-1α citrullination. But this also could be because hypoxia increased Padi4 levels in the cell. Most of your experiments show that hypoxia elevates Padi4. Please clarify.

Response: We truly appreciate the reviewer for the insightful comments. PADI4 has been reported to be upregulated by HIF-1α[10], hypoxia-induced binding of HIFs to consensus hypoxia-response element (HRE) located in the intergenic region (2.3 kb 5' to the transcription start site (TSS)) and intron (0.5 and 8.1 kb 3' to the TSS), but not coding sequence (CDS) of PADI4 gene in Hep3B cells as shown by

Wang *et al.*[10] (**Figure R9a, b**). Our western blot results also showed that hypoxia treatment induced the upregulation of endogenous PADI4 (**Figure R9c**). However, CDS without HRE was cloned into the vectors to overexpress PADI4, so exogenous PADI4 is not regulated by hypoxia (**Figure R9d**).

To further verify hydroxylation of HIF-1α disrupts HIF-1α-PADI4 binding and HIF-1α citrullination, we examined the interaction of HIF-1α and PADI4, as well as HIF-1α citrullination under normoxic conditions with PHDs inhibitor (Deferoxamine mesylate, DFO). Results showed that DFO could efficiently repress HIF-1α hydroxylation, accompanied by enhanced interaction of HIF-1α and PADI4 as well as increased HIF-1α^{R698} citrullination levels (**Figure R10**). These results were consistent with the enhanced interaction of PADI4 with HIF-1α^{DM} (the P402/564A double mutant lacks residues that can be hydroxylated) compared to wild type HIF-1α, as shown in **Figure 4b** in the original manuscript. Together, these data suggested that HIF-1α hydroxylation disrupts HIF-1α-PADI4 interaction as well as PADI4-mediated HIF-1α citrullination. We have included **Figure R10** as **Fig. 4c** in the revised manuscript.



Wang *et al.* ***Sci Adv***. 2021 PMID:34452909 (**Fig. S2**)

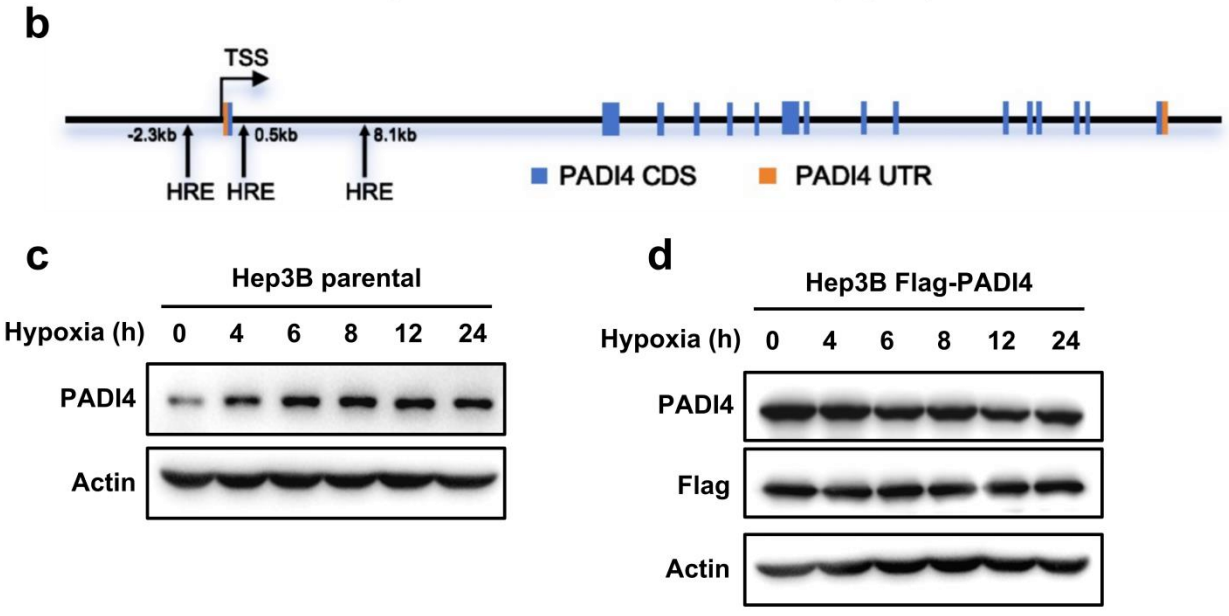


Figure R9. Hypoxia induces the upregulation of endogenous but not exogenous PADI4 proteins. (**a**) Hep3B cells were exposed to 20% or 1% O_2 for 16 h, and ChIP assays were performed using the indicated antibodies. Primers encompassing candidate HIF binding sites located 2.3 kb 5' (red), 0.5 kb 3' (green) and 8.1 kb 3' (blue) to the PADI4 transcription start site were used for qPCR and results were normalized to the first lane (mean ±

SD; $n = 3$). *$P < 0.05$, **$P < 0.01$, *** $P < 0.001$ vs. 20% O_2 (Student's t test). HIF binding to the RPL13A gene was assayed as a negative control. **Figure R9a** from the article of PMID 34452909. (**b**) Diagram showing the location of HREs in *PADI4* gene. (**c-d**) Parental Hep3B cells (**c**) and Hep3B cells expressing Flag-PADI4 (**d**) were cultured under normoxic or hypoxic conditions for indicated time, cell lysates were harvested and protein levels of PADI4 or Flag were measured by western blotting. Actin served as the loading control.

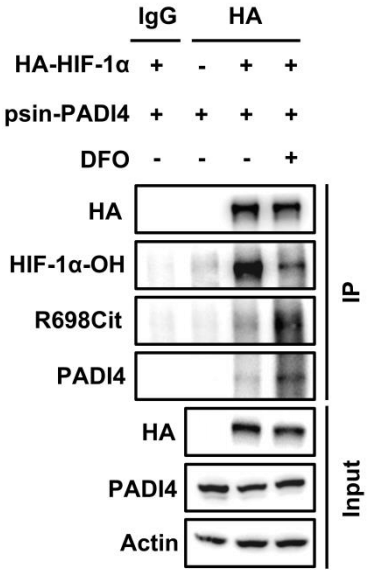


Figure R10. PHDs inhibitor DFO disrupts the interaction of HIF-1α and PADI4 as well as HIF-1α^{R698} citrullination levels under normoxic conditions. HEK293T cells were transfected with HA-EV or HA-HIF-1α plasmids together with psin-PADI4 plasmids with or without DFO in the presence of MG132 under normoxic condition for 6 h. Cell lysates were immunoprecipitated with IgG or anti-HA antibodies, followed by western blotting analysis. Actin served as the loading control. We have included **Figure R10** as **Fig. 4c** in the revised manuscript.

10) It is unclear why the normal liver cell numbers have not been affected by hypoxia at 48 h (Figure 5B).

Response: This is a good point. By detecting the expression of PADI4 in normal liver cell line (THLE3) and liver cancer cell lines (Hep3B and HepG2) under normoxic or hypoxic conditions for 6 h and 24 h, we found that compared with Hep3B and HepG2, the basal protein levels of PADI4 in THLE3 cells remained at a lower level and was not significant increase after hypoxia treatment (**Figure R11a**). Further cell proliferation assays also showed that knockdown of PADI4 obviously suppresses the growth of liver cancer cells, but had little inhibitory effect on THLE3 cells under hypoxic conditions for indicated times (**Figure R11b**). This study found that PADI4 is a potential target for DHE (**Figure 5a** in the original manuscript), that is why DHE treatment had little effect on the cell numbers of THLE3 cells with low PADI4 basal levels as shown in **Figure 5b** in the original manuscript.

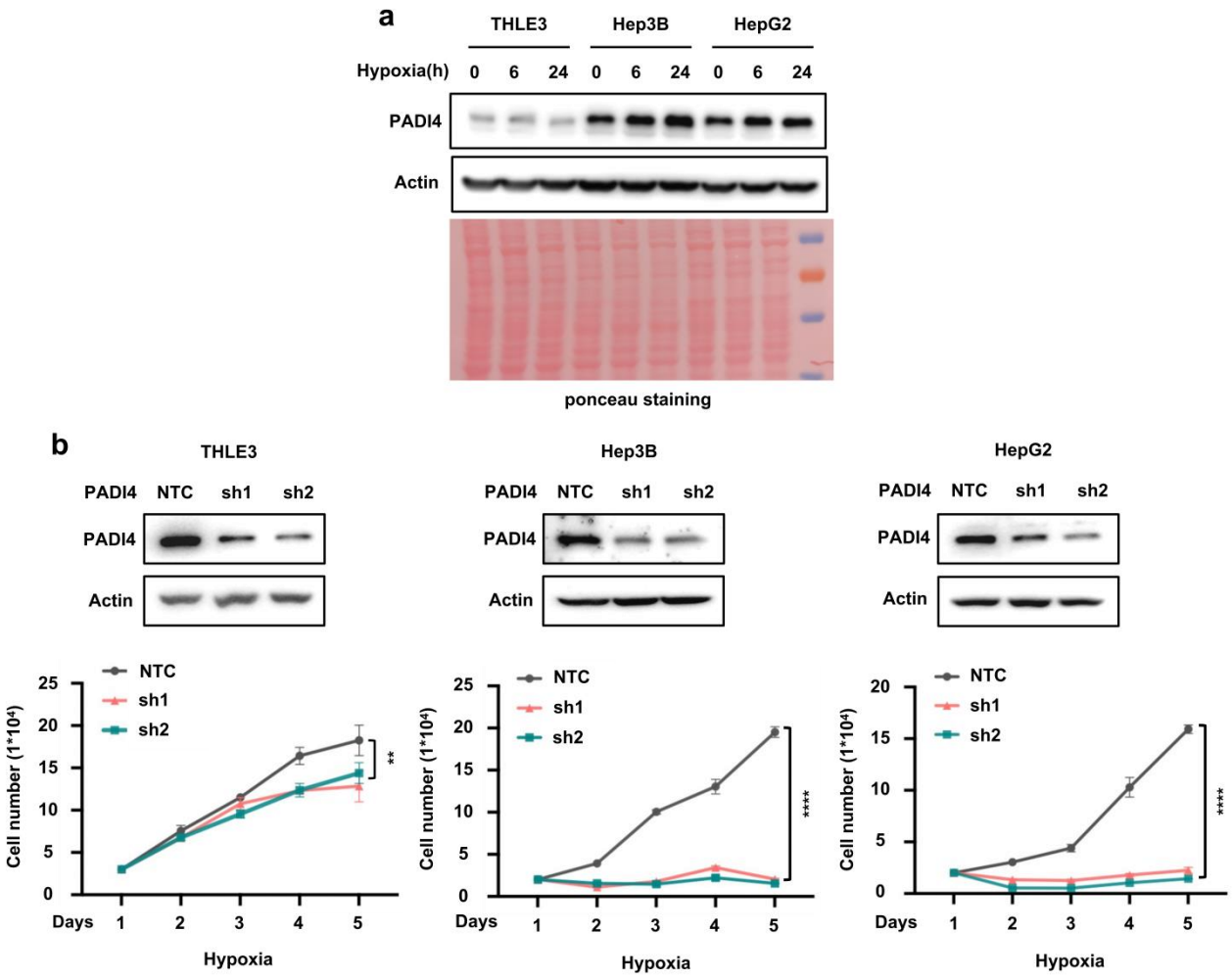


Figure R11. The expression of PADI4 is important for the proliferation of Hep3B and HepG2 cells, but not for THLE3 cells. (**a**) THLE3, Hep3B, and HepG2 cells were cultured under normoxic or hypoxic conditions for 6 h or 24 h. Cell lysates were harvested and protein levels of PADI4 were measured by western blotting. Actin and ponceau staining served as the loading control. (**b**) THLE3, Hep3B, and HepG2 cells expressing NTC or PADI4 shRNAs were harvested and the protein levels of PADI4 were measured by western blotting. Actin served as the loading control (upper panel). Growth curves of the cells were determined by trypan blue counting (lower panel). The data are presented as the mean ± SEM (*n*=3 independent assays).

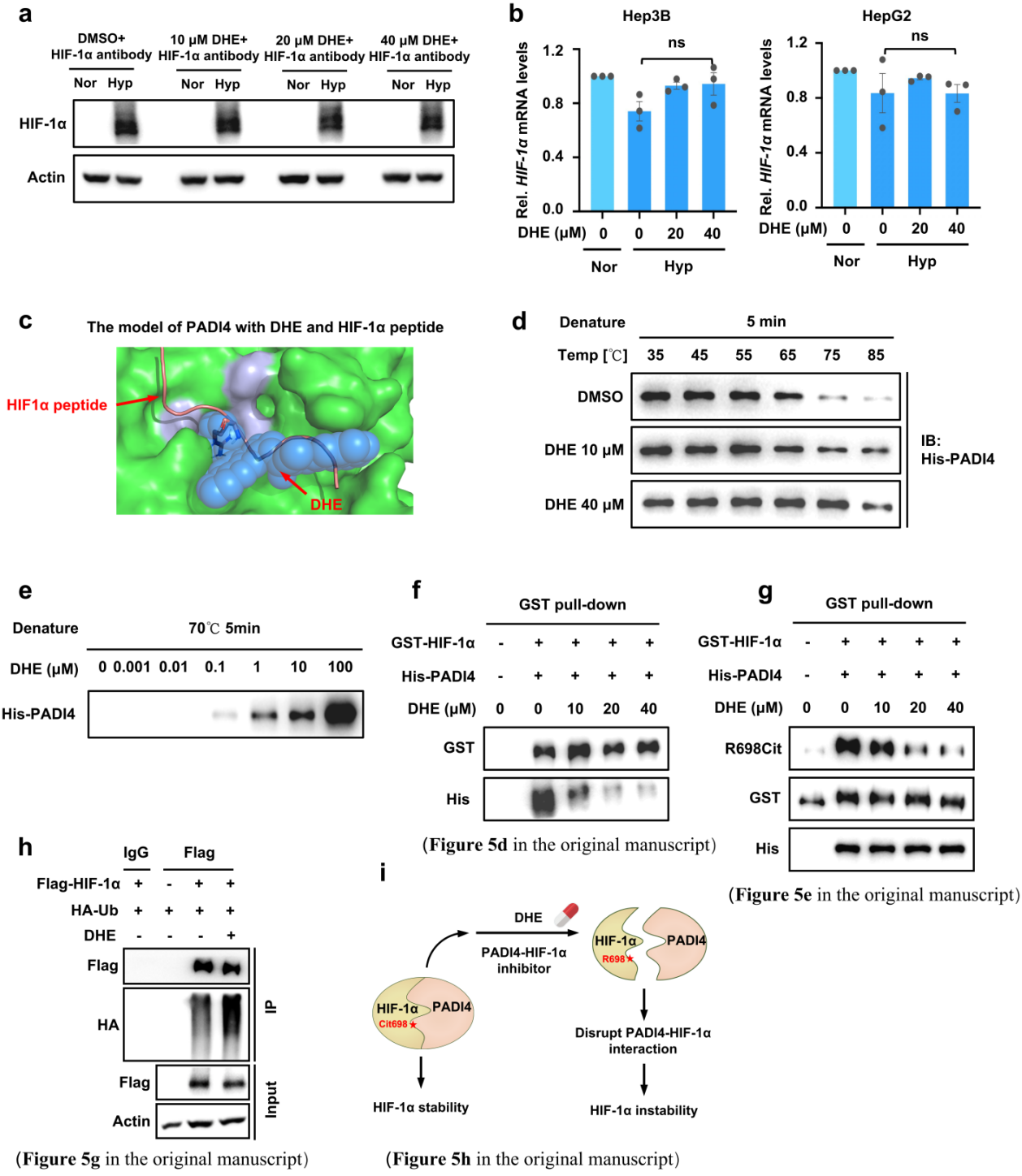
11) Western blot results showed that the HIF-1α protein levels were decreased after DHE treatment in a dose dependent manner, while the PADI4 protein levels were unaffected. Does it mean that DHE binding prevents HIF-1α WB antibody binding (resulting in weaker WB signal) or does it mean that DHE reduces mRNA expression of HIF-1α? At many instances, it is stated that DHE reduces HIF-1α expression. If so, the mechanism should be explored.

Response: In general, during the WB experiment, the SDS component of the protein lysis buffer completely disrupts the intact protein structure, and small molecules that are non-covalently bound to

the protein will fall off and lost during the subsequent SDS-PAGE electrophoresis. To further rule out the possibility raised by our reviewer, we added different concentrations of DHE during the antibody incubation to observe whether DHE prevents HIF-1α antibody from binding to HIF-1α protein. The conclusion is that DHE does not affect the binding efficiency of HIF-1α antibody to HIF-1α protein. (**Figure R12a**). qPCR results showed that DHE does not affect *HIF-1α* mRNA levels in Hep3B and HepG2 cells (**Figure R12b**).

Besides, based on our findings that PADI4-HIF-1α interaction is essential for HIF-1α stability, we screened out DHE that could bind to the pocket of PADI4, which may antagonize the binding of PADI4 and HIF-1α (**Figure R12c**). Accordingly, to confirm the direct binding of DHE to PADI4, the thermal shift assay (TSA) was performed based on the biophysical principle of ligand-induced thermal stabilization of its target protein[11,12]. The results showed that 10 μM or 40 μM DHE treatment substantially upregulated the thermal stability of purified His-PADI4 under different temperatures with 5 min denaturation (**Figure R12d**). Moreover, the thermal stability of PADI4 was also significantly enhanced by serial increase of DHE concentration (**Figure R12e**). These TSA results showed that DHE heat-stabilized PADI4 and indicated that DHE directly interacts with PADI4.

Furthermore, we have proved that DHE antagonizes the interaction between PADI4 and HIF-1α and subsequently suppresses HIF-1α citrullination, resulting in HIF-1α ubiquitination as shown in **Figure 5d**, **5e, 5g** in the original manuscript. (**Figure R12f-h**). Collectively, these data demonstrated that DHE reduces HIF-1α protein levels by directly interacting with PADI4, which disrupts the binding of PADI4 and HIF-1α and reduces the HIF-1α^{R698} citrullination levels, and eventually resulting in HIF-1α degradation in the proteasomal pathway (**Figure R12i**). We have included **Figure R12b, 12d,** and **12e** as **Fig. S4d, Fig. 5c,** and **5d** in the revised manuscript, respectively.



(**Figure 5d** in the original manuscript)

(**Figure 5e** in the original manuscript)

(**Figure 5g** in the original manuscript)

(**Figure 5h** in the original manuscript)

Figure R12. The mechanism of DHE represses HIF-1α protein levels. (**a**) Hep3B cells were cultured under nomoxic or hypoxic conditions for 6 h. Cell lysates were analyzed by western blotting using anti-HIF-1α antibody added with DMSO, 10 μM, 20 μM or 40 μM DHE, respectively. (**b**) Hep3B and HepG2 cells treated with DMSO, 20 μM or 40 μM DHE were cultured under normoxic or hypoxic conditions for 6 h. The mRNA levels of *HIF-1α* were analyzed by qPCR. Data are presented as the mean ± SEM of 3 independent experiments (n=3 biological replicates). Group differences are analyzed by ordinary one-way ANOVA test. (**c**) Molecular docking model of DHE and HIF-1α peptide in the enzymatic pocket of PADI4. (**d**) Purified His-PADI4 proteins were incubated with DMSO, 10 μM, or 40 μM DHE under indicated temperature for 5 min, respectively, followed by western blotting analysis of

His-PADI4 protein levels by His antibody. (**e**) Purified His-PADI4 proteins were incubated with indicated concentration of DHE under 70℃ for 5 min, followed by western blotting analysis of His-PADI4 protein levels by His antibody. (**f**) Pull-down of His-PADI4 by GST-HIF-1α in the presence of DMSO, or 10 μM, 20 μM, or 40 μM DHE. (**g**) An *in vitro* citrullination assay was performed by incubating purified GST-HIF-1α and His-PADI4 proteins in the presence of DMSO or 10 μM, 20 μM, or 40 μM DHE at 37 ℃ for 1 h followed by western blotting analysis of HIF-1α^{R698Cit}, GST, and His protein levels. (**h**) HEK293T cells transfected with Flag-EV or Flag-HIF-1α^{WT} plasmids together with HA-Ub plasmids were treated with DMSO or 25 μM DHE for 24 h and MG132 for 6 h. Cell lysates were immunoprecipitated with either IgG or anti-Flag antibodies and analyzed by western blotting. (**i**) Diagram showing that DHE represses the PADI4-HIF-1α interaction and HIF-1α^{R698} citrullination and promotes HIF-1α proteasomal degradation. **Figure R12f-i** are the same data as **Figure 5d, 5e, 5g, and 5h** in the original manuscript, respectively. We have included **Figure R12b, R12d,** and **R12e** as **Fig. S4d, Fig. 5c,** and **5d** in the revised manuscript, respectively.

Suggestions on how to improve the manuscript:

1) Hypoxia induced PADI4 protein levels (Figure 3B). This is interesting and should be discussed.

Response: Thanks. The upregulation of PADI4 by hypoxia is consistent with the study by Wang *et al*[10]. Per the reviewer's suggestion, we discussed this interesting issue in the third paragraph of **Discussion section** in the revised manuscript as follows: "...Interestingly, a recent study showed that hypoxia induced PADI4 expression in a HIF-dependent manner and that PADI4-mediated histone citrullination was required for HIF-1 transcriptional activity[46]. Combined with our finding that PADI4 catalyzes HIF-1α citrullination to maintain HIF-1α stability, these studies collectively indicate that the HIF-1α-PADI4 axis is a positive feedback loop that coordinates tumour development and would be a promising target of tumour therapy ".

2) In this study Dihydroergotamine is proposed as a drug preventing/inhibiting HIF-1α and PADI4 interaction. However, this drug does not inhibit free unbound PADI4 or HIF-1α or those bound to other proteins. This limitation should be clearly discussed. Some statements in the manuscript text should be corrected such as Figure 5 label: dihydroergotamine is an antagonist of PADI4 and represses HIF-1α expression.

Response: This point is well taken. Through screening and validation, we found that DHE occupies the enzymatic pocket of PADI4, and thus preventing HIF-1α binding as shown in **Figure S4a/b** and **5a/d/e** in the original manuscript. Experimentally, we proved that DHE directly binds to PADI4 through *in vitro* TSA assays (**Figure R12d, e**). It is quite reasonable for the reviewer to point out the limitations, which was discussed in the third paragraph in the **Discussion section** of the revised

manuscript as follows: "… In summary, our data proved the binding of DHE to PADI4 and the subsequent inhibitory effects on PADI4-HIF-1α interaction and HIF-1α stability, making it possible to clinically target HIF-1α for tumour treatment, but more comprehensive studies are required to better elucidate the precise mechanism by which DHE targets PADI4 in the future". In addition, we have corrected some statements in **Figure 5** label of the revised manuscript according to the reviewer's suggestions as follows: "Dihydroergotamine mesylate disrupts PADI4-HIF-1α interaction and represses HIF-1α expression", and fourth paragraph in the **Introduction section** of the revised manuscript as follows: " ...we demonstrate that dihydroergotamine mesylate (DHE), which disrupts the interaction of PADI4 and HIF-1α...".

3) More discussion should be provided on how these new data fit into current knowledge in the field. Citrullination of the HCC is not well reported, but there are studies documenting citrullination of colorectal cancer liver metastasis. Also, a thorough discussion of this highly relevant paper should be performed PMID: 29484680.

Response： The excellent work of Yuzhalin *et al.* on the role of citrullination in liver metastasis of colorectal cancer were discussed in the second paragraph of **Discussion section** in the revised manuscript as follows: "A few studies have revealed the role of PADI4 in cancer cells[30]. For example, Yuzhalin *et al.* found that the extracellular matrix in liver metastases contains a large proportion of citrullinated proteins and the levels of PADI4 were higher in colorectal cancer (CRC) liver metastases than primary CRC, or adjacent colon or liver, demonstrating that PADI4 is essential for liver metastases from CRC[43]. Positive effects of NETs on tumour proliferation, metastasis and awakening dormant tumour cells also indicate the tumour promoting role of PADI4[44]."

Moreover, citrullinated proteins induced by hypoxia and HIF-1α was discussed in the third paragraph of **Introduction section** as follows: " Yu *et al.* found that hypoxia upregulates PADI2 and citrullinated protein in HIF-1α-dependent manner in human fibroblast-like synoviocytes, proposing hypoxia involved in the mechanism of RA[36]".

Minor Critiques:

1) In the sentence "To test these possibilities, ectopically expressed Flag-tagged HIF-1α was immunoprecipitated and detected with an anti-pancitrullination antibody in HEK293T and Hep3B cells" please change detected for probed.

Response: Per the reviewer's suggestion, we have changed "detected" for "probed" in the revised manuscript.

2) In Figure 1G legend, the authors claim that they performed "probing with an anti pancitrulline antibody". But the methods section states that they used The Anti-Citrulline (Modified) Detection Kit which is not an anti pancitrulline antibody. Please clarify.

Response: We apologize for the confusion. In this study, we used the anti-pan citrulline antibody (Invitrogen, MA5-27573) and we have corrected the statements in the **Methods section**.

3) Most experiments in this study were performed in mouse cell lines. Therefore, mouse protein and gene nomenclature should be used throughout.

Response: This is a good point. Accordingly, we have modified the nomenclature of mouse protein (eg: HIF-1α, PADI4 and Actin) and gene (eg: *Hif-1α* and *Padi4*) throughout the manuscript.

4) All bar graphs should display individual data points, and it should be indicated in the figure legends whether each dot means a technical replicate or a culture dish.

Response: Thanks. Follow the reviewer's suggestion, we have provided all bar graphs with individual data points, and described the biological replicate in figure legends in the revised manuscript.

Reviewer #2 (Remarks to the Author):

The authors describe a novel mechanism for the regulation of HIF-1a. by PADI4-mediated citrullination of R698. This novel modification interferes with the binding of VHL, thus contribution to the stabilization of HIF-1a under hypoxic conditions. This finding will be of moderate significance to the field and builds on an already large body of work describing the regulation of HIF-1a by hypoxia and VHL. This was a well-written paper, and the data as a whole largely support the conclusions drawn. To increase the rigor and applicability of the findings to the field, some additional data are required:

Responses: We appreciate the positive comments on our study and the reviewer's insightful suggestions that helped us strengthen the quality of this study substantially.

1. The authors elegantly outline a novel mechanism of regulation of HIF-1a during hypoxia. Since HIF-2a is regulated by similar mechanisms (PHDs and VHL) and also plays an important pro-

tumorigenic role in HCC and other cancer types, the authors should demonstrate whether PADI4 also stabilize HIF-2a and whether citrullination plays a similar role in regulating the stability of HIF-2a.

Response: We truly appreciate our reviewer for the very insightful comment. It's known that HIF-1α and HIF-2α are regulated by similar mechanisms and play roles in tumor development. To investigate whether PADI4 and citrullination regulate HIF-2α stability, co-immunoprecipitation (co-IP) assay was performed and showed that PADI4 interacts with HIF-2α, but it is weaker than the binding of PADI4 and HIF-1α (**Figure R13a, lane 3 *vs* lane 4**). Next, the immunoprecipitated HIF-2α was also found to have citrullinated modification probed with anti-pan citrulline antibody (**Figure R13b**). Furthermore, PADI4 knockdown also resulted in decreased HIF-2α protein levels (**Figure R13c**). Collectively, These results suggest that HIF-2α can also bind PADI4 and undergo citrullination modification, but it appears to be weaker than HIF-1α. In addition, PADI4 also regulates the protein level of HIF-2α, which will be a very interesting direction for future research.

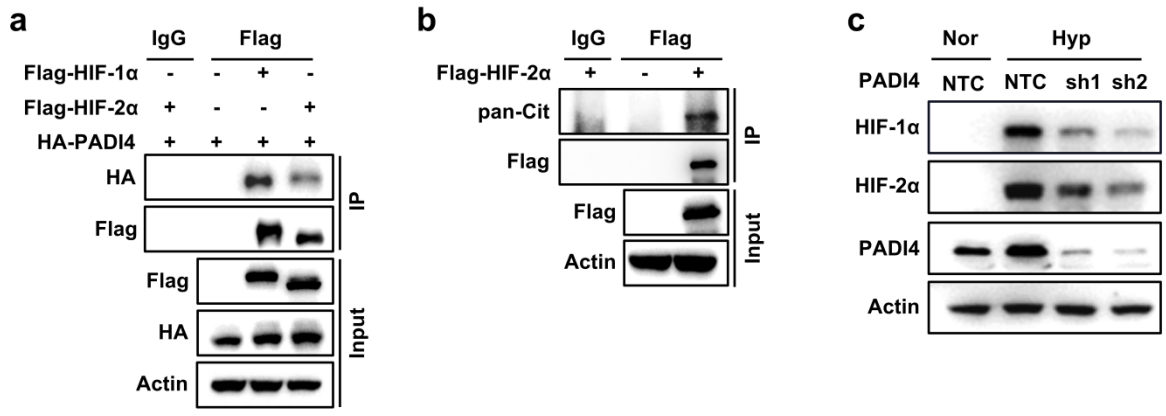


Figure R13. PADI4 and citrullination may regulate the stability of HIF-2α. (**a**) HEK293T cells co-transfected with Flag-EV, Flag-HIF-1α or Flag-HIF-2α plasmids together with HA-PADI4 plasmids were immunoprecipitated with either IgG or anti-Flag antibodies followed by western blotting analysis. (**b**) HEK293T cells transfected with Flag-EV or Flag-HIF-2α plasmids were culture under hypoxic conditions for 6 h. Cell lysates were harvested and immunoprecipitated with either IgG or anti-Flag antibodies, followed by western blotting analysis to measure the levels of citrullinated HIF-2α. (**c**) Hep3B cells expressing NTC or PADI4 shRNAs were cultured under normoxic or hypoxic conditions for 6 h, cell lysates were harvested and the protein levels of HIF-1α and HIF-2α were measured by western blotting. Actin served as the loading control.

2. The author suggest that citrullination only occurs when the HIF-1a hydroxylation by the PHDS are inhibited, either by hypoxia, or by expression of a HIF-1a mutant that cannot by hydroxylated. The authors should supplement this finding using shPADI4 in combination with other inhibitors of the PHDs (eg using iron chelators such as DFO or CoCL2) to verify that this effect is PHD, rather than

hypoxia dependent.

Response: Following this suggestion, Hep3B-shPADI4 and HepG2-shPADI4 cells were treated under hypoxic conditions or DFO treatment for 6 h, respectively. Western blotting results showed that DFO treatments leads to obvious accumulation of HIF-1α proteins (**Figure R14a, lane 5 vs lane 1**), while PADI4 knockdown could weaken this effect (**Figure R14a, lane 6/7 vs lane 5**), which is similar to hypoxic treatment (**Figure R14a, lane 3/4 vs lane 2**). This suggested that PADI4 maintains HIF-1α stability when HIF-1α hydroxylation is inhibited. Our IP results showed that DFO could efficiently repress HIF-1α hydroxylation, accompanied by enhanced interaction of HIF-1α and PADI4 as well as increased HIF-1α^{R698} citrullination levels under normoxia (**Figure R14b**). These results were consistent with the enhanced interaction of PADI4 with HIF-1α^{DM} (the P402/564A double mutant lacks residues that can be hydroxylated) compared to wild type HIF-1α under normoxia (**Figure 4b** in the original manuscript). Collectively, these data concluded that HIF-1α hydroxylation regulated by PHDs disrupts HIF-1α-PADI4 interaction as well as PADI4-mediated HIF-1α citrullination. We have included **Figure R14a, b** as **Fig. 4d,c** in the revised manuscript, respectively.

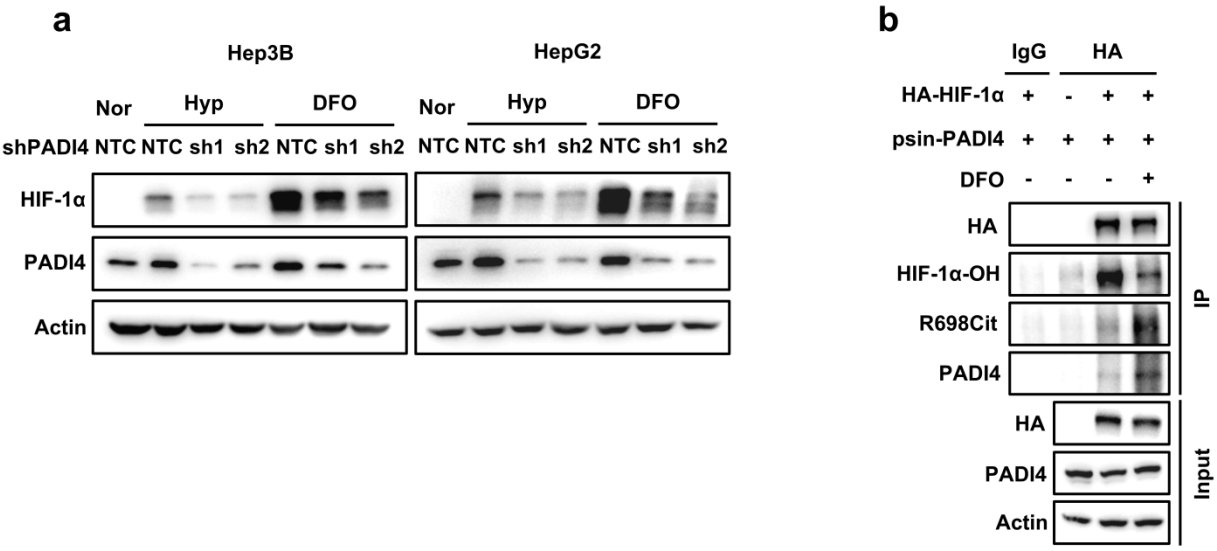


Figure R14. PADI4 knockdown suppresses HIF-1α protein levels under hypoxia or DFO treatment. (**a**) Hep3B (left panel) and HepG2 (right panel) cells expressing NTC or PADI4 shRNAs were treated by hypoxia or DFO for 6 h. Cell lysates were harvested and the protein levels of HIF-1α were measured by western blotting. Actin served as the loading control. (**b**) HEK293T cells were transfected with HA-EV or HA-HIF-1α plasmids together with psin-PADI4 plasmids with or without DFO in the presence of MG132 under normoxic conditions for 6 h. Cell lysates were immunoprecipitated with IgG or anti-HA antibodies, followed by western blotting analysis. Actin served as the loading control. **Figure R14b** is the same data as **Figure R10**. We have included **Figure R14a,b** as **Fig. 4d,c** in the revised manuscript, respectively.

3. In Fig. 1C, the authors show co-localization of HIF-1a and PADI4 in hypoxic HEP3B cells. It is surprising that HIF-1a is appears to be expressed throughout the cell (both cytoplasmic and nuclear) under these hypoxic conditions, when it is expected that HIF-1a should be largely nuclear. Similarly, no staining validations controls are shown for PADI4. Thus, the authors should provide validation of the HIF-1a and PADI4 staining conditions used for used for ICC (for example using normoxia vs hypoxia or siRNA knockdown) to validate that the signals seen are indeed specific for the proteins indicated.

Response: We thank the referee for pointing this out. The similar concern is also raised by Reviewers 1&3. Following the reviewer's suggestion, we optimized the dilution ratio of primary antibodies, and found obvious nuclear localization of HIF-1α under hypoxic conditions (**Figure R15a, left panel**). PADI4 antibody was also validated in Hep3B-shPADI4 cells, nuclear and cytoplasm localization of PADI4 was observed in NTC control cells which is consistent with previous report[2,3], almost no signal was detected when we further knock down PADI4 (**Figure R15a, right panel**). We further optimized the experimental conditions and observed high quality colocalization between HIF-1α and PADI4 (**Figure R15b**), which is consistent with our original conclusion. We have included **Figure R15b** as **Fig. 1c** in the revised manuscript.

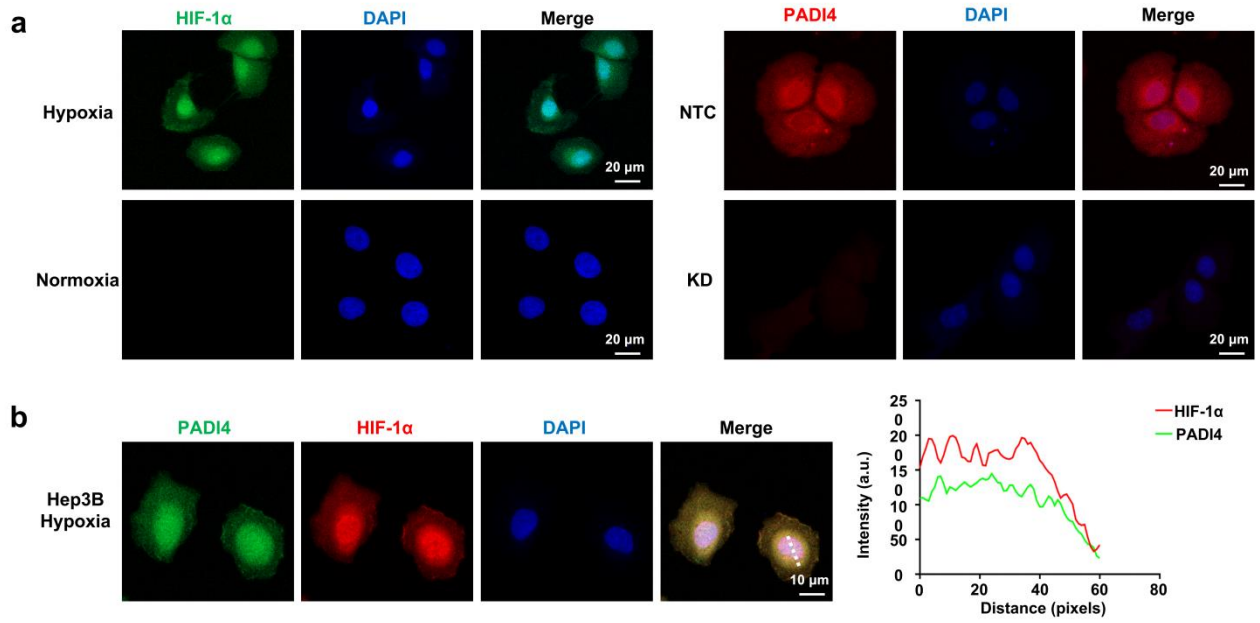


Figure R15. IF staining of PADI4 and HIF-1α. (**a**) Specificity validation of HIF-1α and PADI4 staining. Hep3B cells cultured under hypoxic or normoxic conditions for 6 h were used for the endogenous HIF-1α staining (left panel). Hep3B cells expressing NTC or shPADI4 were used for the endogenous PADI4 staining (right panel). Scare bar: 20 μm. (**b**) IF analysis of the colocalization of PADI4 and HIF-1α in Hep3B cells treated under hypoxic conditions for 6 h. Scale bars: 10 μm (left panel). Intensity profiles of each line were quantified by ImageJ software

and drawn with GraphPad Prism 7.0 (right panel). We have included **Figure R15b** as **Fig. 1c** in the revised manuscript, respectively.

Additional minor comments

1. Line 139 Page 6: The authors state that PADI4 significantly increased the citrullination levels of HIF-1α in a dose-dependent manner (Fig. 1g). Authors should provide statistical analysis to justify this statement or modify statement accordingly

Response: Thanks. We have repeated this experiment for another three times. Similar trends have been observed (**Figure R16a**), and following the referee's suggestion, we also added the corresponding quantitative results (**Figure R16b**), which are consistent with the original conclusion. To avoid confusion, we have replaced **Fig. 1g** with the 2nd time data in the revised manuscript.

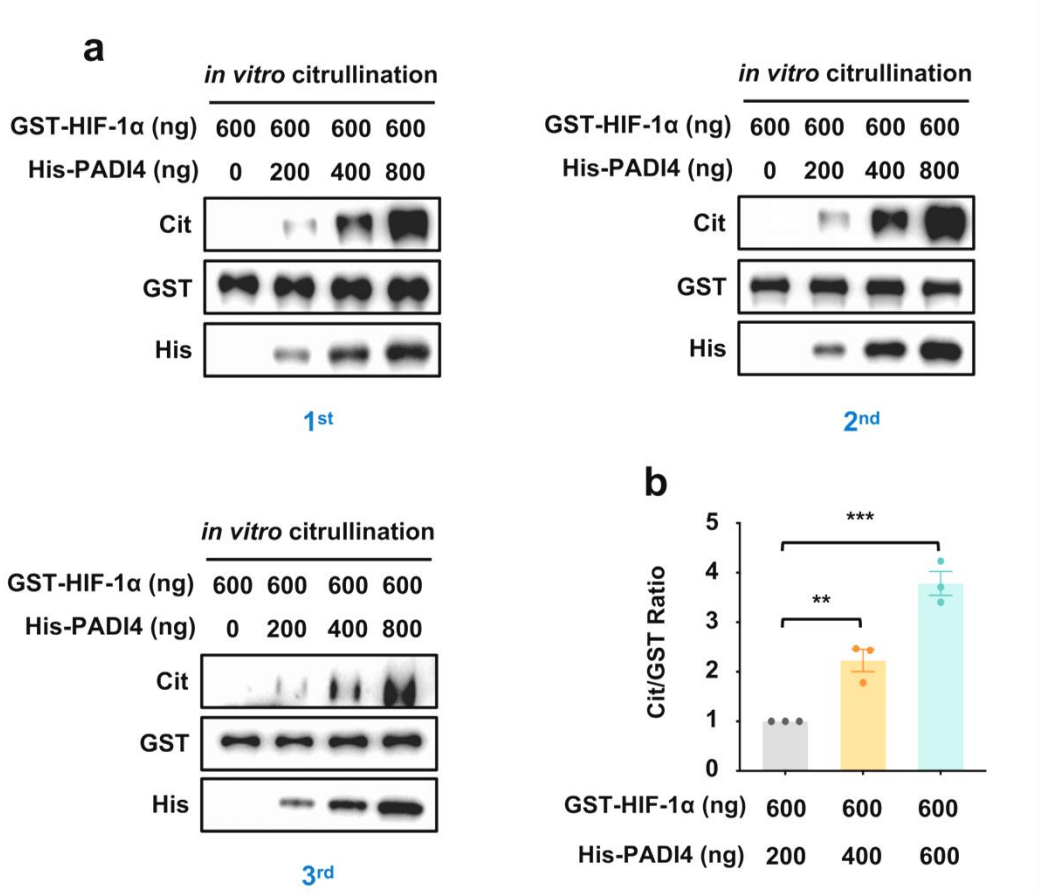


Figure R16. PADI4 directly catalyzes HIF-1α citrullination. (**a**) *In vitro* citrullination assays were performed by incubating purified His-PADI4 and GST-HIF-1α proteins at 37 °C for 1 h in catalytic buffer and probing with an anti-pancitrulline antibody. The results of three independent experiments are presented. (**b**) Quantification of HIF-1α-Cit protein levels relative to GST-HIF-1α protein levels. Data are presented as the mean ± SEM of 3

independent experiments (n=3 biological replicates). Group differences are analyzed by ordinary one-way ANOVA test. We have replaced **Fig. 1g** with the 2nd time data in the revised manuscript.

2. Line 225 page 10:"The authors state that PADI4 significantly blocked VHL binding to HIF-1α under both normoxic and hypoxic conditions (Fig. 4c) Authors should provide statistical analysis to justify this statement or modify statement accordingly.

Response: Thanks. We have repeated this experiment for three times. Similar trends have been observed (**Figure R17a**), and following the referee's suggestion, we also added the corresponding quantitative results (**Figure R17b**), which are consistent with the original conclusion.

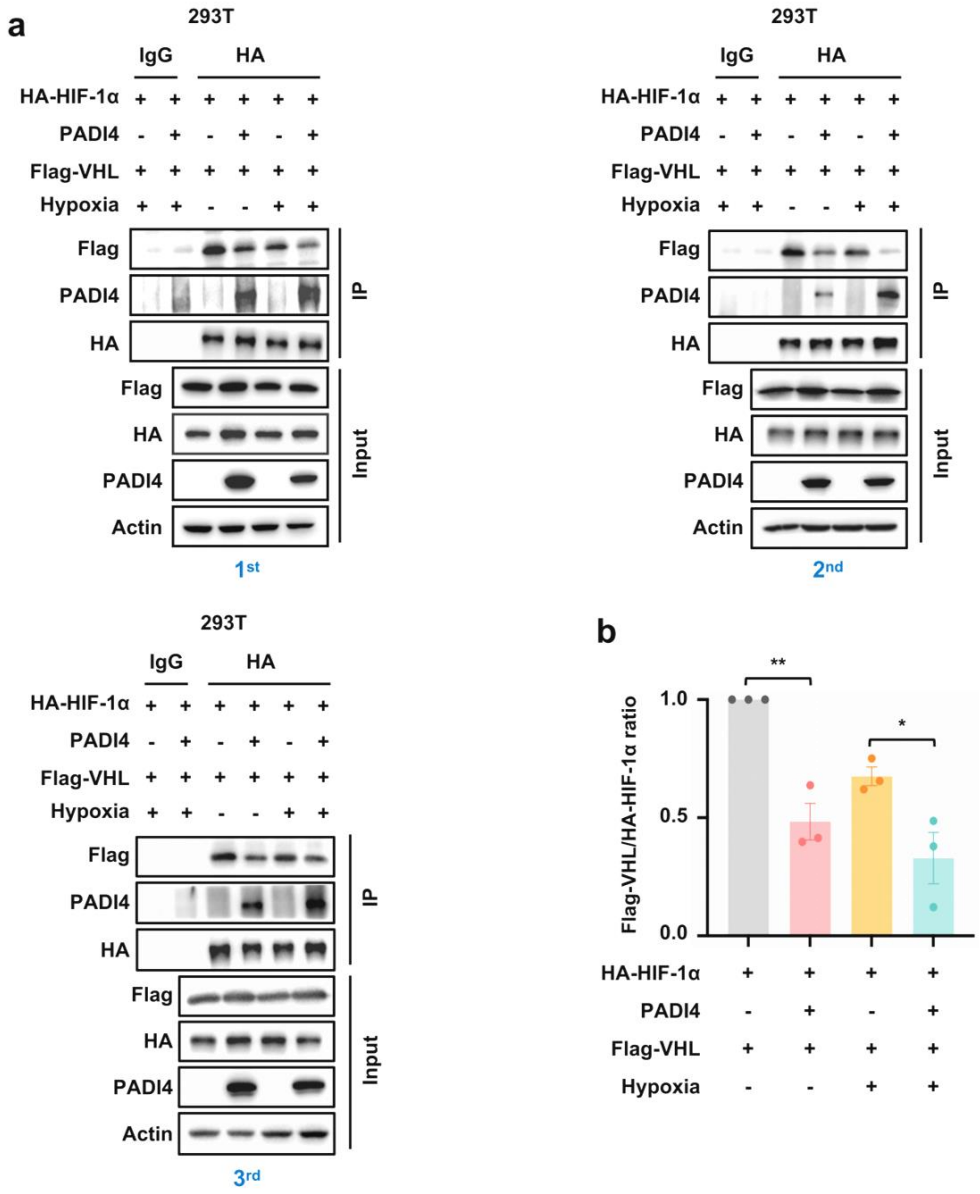


Figure R17. PADI4 blocks VHL binding to HIF-1α under normoxic and hypoxic conditions. (a)

HEK293T cells transfected with EV or PADI4 plasmids together with HA-HIF-1α and Flag-VHL plasmids were cultured under normoxic or hypoxic conditions in the presence of 10 μM MG132 for 8 h. Cell lysates were immunoprecipitated with either IgG or anti-HA antibodies, followed by western blotting analysis. Actin served as the loading control. The results of three independent experiments are presented. (**b**) Quantification of Flag-VHL protein levels relative to HA-HIF-1α protein levels. Data are presented as the mean ± SEM of 3 independent experiments (*n*=3 biological replicates). Group differences are analyzed by ordinary one-way ANOVA test.

2. Page 16 Line 379: The authors state that "In addition to the long-held view that HIF-1α is stable owing to the decreased activities of PHDs under hypoxic conditions, we provide evidence based on epigenetic modification findings that PADI4-mediated citrullination of HIF-1 is equally critical for HIF-1 stabilization and activity under hypoxic conditions". This statement is not justified by the data. No comparison was made between the contribution of PHDs versus citrullination for the stabilization of HIF-1a during hypoxia. In addition, the authors suggest that citrullination may only occur in non-hydroxylated HIF suggesting that citrullination requires inhibition of the PHDs (by hypoxia).

Response: We appreciate the referee for the critical comments. To be more rigorous and objective, we have rewritten the statement as “In addition to the long-held view that HIF-1α is stable owing to the decreased activities of PHDs under hypoxic conditions, we provide an extra mechanism based on epigenetic modification findings that PADI4-mediated citrullination of HIF-1α is critical for HIF-1α stabilization and activity under hypoxic conditions”.

Reviewer #3 (Remarks to the Author):

In this study, the researchers investigated the role of peptidyl arginine deiminase 4 (PADI4) in regulating tumor progression and its potential therapeutic significance. Through a series of in vitro and in vivo experiments, the researchers demonstrated that the enzymatic activity of PADI4 contributes to tumor growth. Mechanistically, PADI4-mediated citrullination of HIF-1α blocks VHL binding, thereby preventing VHL-mediated HIF-1α ubiquitination and subsequent proteasome degradation. Overall, the results of the study reveal citrullination as a previously unrecognized posttranslational modification related to HIF-1α stability and suggest that targeting PADI4-mediated HIF-1α citrullination could be a promising therapeutic strategy for cancers with elevated HIF-1α expression.

Responses: We thank the reviewer for the positive comments that have well summarized the major findings and significance of this study. We also appreciate the reviewer for the constructive suggestions that are truly helpful for us to improve this manuscript.

Question 1: According to the literature, the PAD4 protein has a nuclear localization signal and is expressed in the nucleus. However, in the authors' immunofluorescence images, PAD4 is distributed throughout the entire cytoplasm. Considering that the authors already have the Flag-PAD4 vector, this reviewer suggest the authors use a Flag antibody for immunofluorescence to test the localization of PAD4 in normal and hypoxia conditions. In Fig. 1c and Fig. 2h, please perform control experiments to stain the cells with HIF-1α and PAD4 antibodies separately.

Response: We appreciate the reviewer for the constructive suggestion. We optimized the dilution ratio of primary antibodies and validated PADI4 antibody in Hep3B-shPADI4 cells, nuclear and cytoplasm localization of PADI4 was observed in NTC control cells, and almost no signal was detected when we further knockdown PADI4 (**Figure R18a**). Following our reviewer's suggestion, IF assays were performed in Hep3B-Flag-PADI4 cells using Flag antibody. The results showed that PADI4 was localized in both cytoplasm and nucleus under normoxic and hypoxic conditions, which was consistent with previous reports[2,3] (**Figure R18b**). In addition, we stained Hep3B cells with PADI4 and HIF-1α antibodies, respectively, and found both nuclear and cytoplasm localization of endogenous PADI4 and HIF-1α proteins under hypoxia (**Figure R18c**).

By employing these positive and negative controls suggested by our reviewers, experimental conditions of **Figure 1c** and **Figure 2h** in the original manuscript were further reperformed. There is a clear colocalization between HIF-1α, PADI4 and HIF-1α^{R698Cit} with high quality (**Figure R3a, b**), which is consistent with our original conclusion. We have included **Figure R3a, R3b** as **Fig. 1c** and **Fig. 2h** in the revised manuscript, respectively.

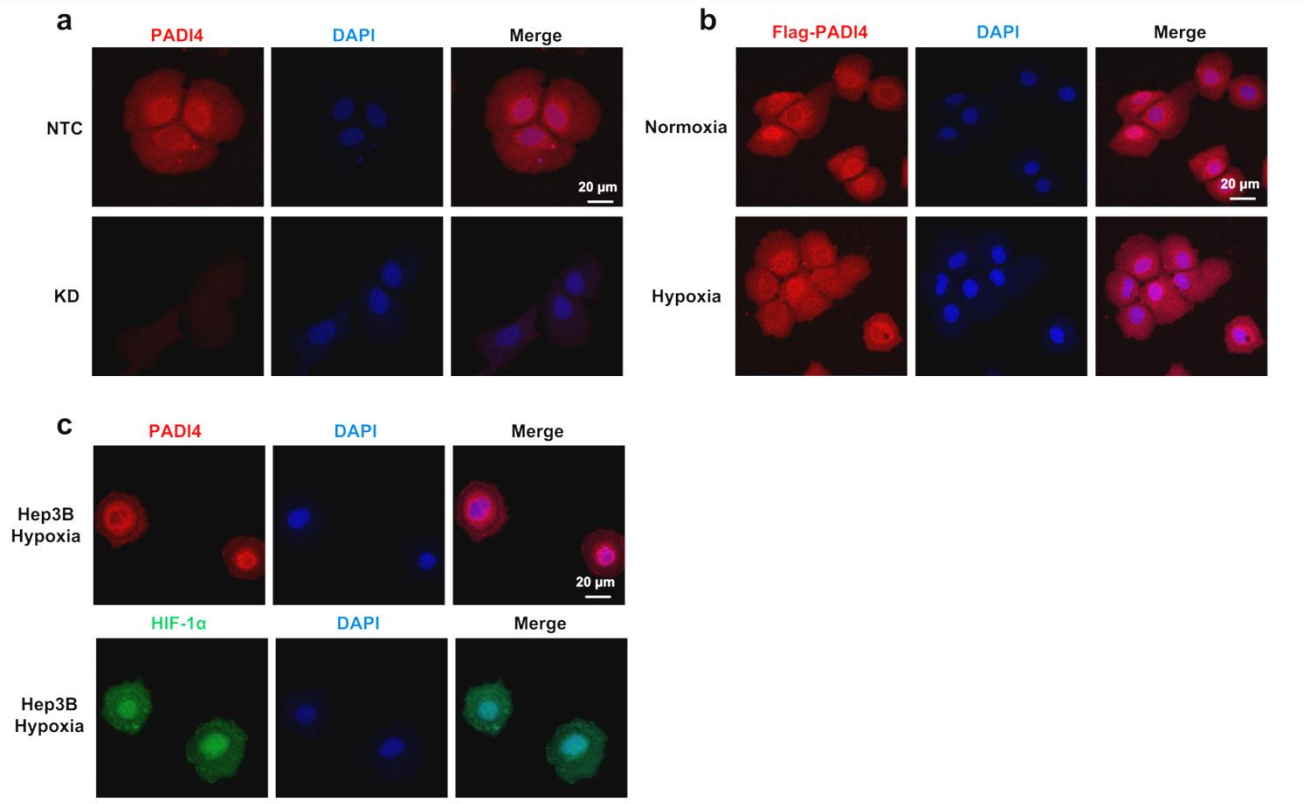


Figure R18. IF staining of PADI4, Flag-PADI4, PADI4 and HIF-1α. (**a**) Hep3B cells expressing NTC or shPADI4 were used for the endogenous PADI4 staining. Scare bar: 20 μm. (**b**) Subcellular localization analysis of exogenous Flag-tagged PADI4 in Hep3B cells cultured under normoxic or hypoxic conditions for 6 h using Flag antibody. Scare bar: 20 μm. (**c**) Hep3B cells cultured under hypoxic conditions for 6 h were stained with endogenous PADI4 and HIF-1α antibodies, respectively. Scare bar: 20 μm.

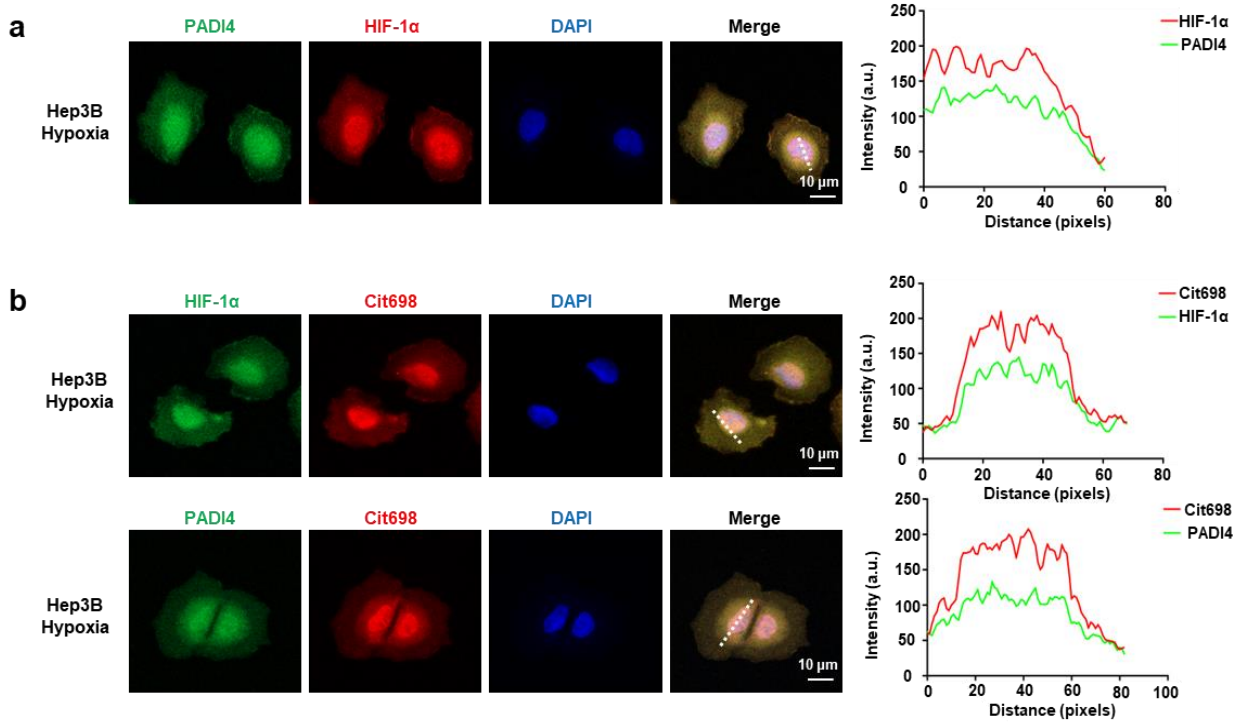


Figure R3. Analysis of IF staining of PADI4, HIF-1α and HIF-1α^{R698Cit} in Hep3B cells. (**a-b**) IF

analysis of the colocalization of PADI4 and HIF-1α (**a**), citrullinated HIF-1α^{R698} and HIF-1α or PADI4 (**b**) in Hep3B cells cultured under hypoxic conditions for 6 h (left panel). Scale bars: 10 μm. Intensity profiles of each line were quantified by ImageJ software and drawn with GraphPad Prism 7.0 (right panel). We have included **Figure R3a, R3b** as **Fig. 1c** and **Fig. 2h** in the revised manuscript, respectively.

Question 2: Please provide the molecular weight of PAD4 in the Western blot? In Figure 3-g, please provide the molecular weights of endogenous PAD4 and exogenous PAD4?

Response: Per the reviewer's suggestion (reviewer 1 raised similar concern), updated **Figure 3g and S3g** in the revised manuscript were appended below along with the source data with the protein ladder.

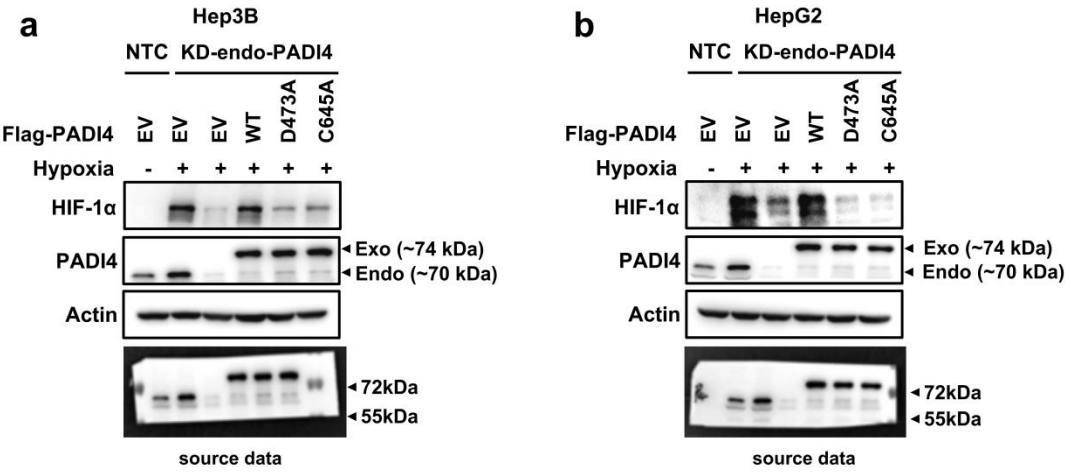


Figure R19. PADI4 enzymatic activity is essential for maintaining HIF-1α protein levels. (**a-b**) Endogenous PADI4-knockdown Hep3B cells (**a**) and HepG2 cells (**b**) were infected with viruses expressing Flag-EV, Flag-PADI4^{WT}, Flag-PADI4^{D473A}, or Flag-PADI4^{C645A} and further cultured under normoxic or hypoxic conditions for 6 h. Cell lysates were harvested, and the protein levels of HIF-1α were analyzed by western blotting. Source data of the exogenous and endogenous PADI4 with the protein ladder are shown. Actin served as the loading control. **Figure R19a,b** is the same data as **Figure R7c,d**. We have updated **Fig. 3g** and **S3g** with **Figure R19a** and **R19b** in the revised manuscript.

Question 3: In reference 39, it was showed that the expression levels of PAD4 and HIF-1α increase under hypoxic conditions. In Figure 5f, the expression level of PAD4 under hypoxic conditions is largely consistent with that under normal conditions; however, in Figure S3c-d, the expression level of PAD4 is significantly increased under hypoxia conditions. There seems to be a contradiction in the results of these two figures. Please clarify these data points.

Response: We apologize for the confusion. Wang *et al.* showed that the protein levels of PADI4 began to be upregulated after 5 hours of hypoxic treatment (Reference 39 in the original manuscript). Accordingly, we treated Hep3B cells with hypoxia for 4 to 24 hours and also found that the expression of endogenous PADI4 started to increase slightly at 4 hours and became obvious after 6 hours under

hypoxia (**Figure R20a**). To ensure the consistency of the data and avoid confusion, we reperformed the experiment of **Figure 5f** in the original manuscript and found that PADI4 is upregulated under hypoxic treatment for 6 h, DHE treatment decreases hypoxia-induced HIF-1α expression, without affecting PADI4 protein levels (**Figure R20b**). We have included **Figure R20b** as **Fig. 5i** in the revised manuscript.

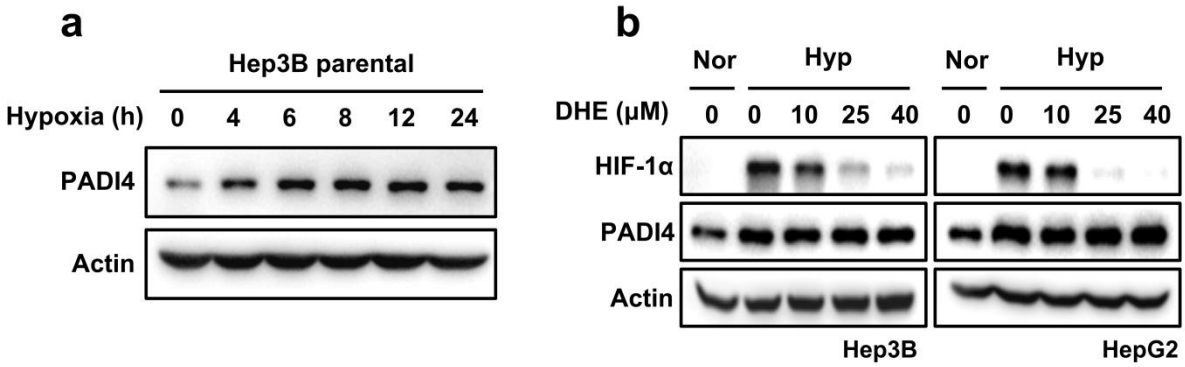


Figure R20. DHE treatments lead to the decrease of hypoxia-induced HIF-1α proteins. (**a**) Parental Hep3B cells were cultured under hypoxic conditions for indicated time, cell lysates were harvested and protein levels of PADI4 were measured by western blotting. Actin served as the loading control. (**b**) Hep3B (left panel) and HepG2 (right panel) cells were treated with DMSO or 10 μM, 25 μM, or 40 μM DHE for 24 h and cultured under normoxic or hypoxic conditions for another 6 h followed by western blotting analysis of HIF-1α and PADI4 protein levels. Actin served as loading control. **Figure R20a** is the same data as **Figure R9c**. We have included **Figure R20b** as **Fig. 5i** in the revised manuscript.

Question 4: The oral bioavailability of DHE is low, please test the serum and tumor DHE concentrations in the mouse model. Please offer rationale for choosing oral administration instead of intravenous administration.

Response: We truly appreciate our reviewer for this insightful comments. Following this suggesion, we performed additional animal study to measure the DHE concentration within the serum and tumor tissues. BALB/c nude mice were subcutaneously inoculated of Hep3B cells and were intragastrically administered (i.g.) of 75 mg/kg DHE every two days starting on Day 12. Consistent with our previous results as shown in **Figure 5k, l** in the original manuscript, the DHE administration significantly inhibited tumor growth *in vivo* without affecting body weight (**Figure R21a-d**). Furthermore, HPLC-MS/MS method was established to detect DHE concentration and the representative HPLC-MS/MS chromatograms of blank, 20 ng/ml and 200 ng/ml DHE were shown (**Figure R21e**). The linear equation, correlation coefficient and linear range of DHE are presented, which shows the DHE linear is well in the linear range with $R > 0.99$, meeting the requirements for the biological sample analysis

method (**Figure R21f**). Intratumral DHE concentration was 672.47 ng/ml, 274.71 ng/ml and 85.152 ng/ml after 4 h, 8 h and 12 h treatment, respectively (**Figure R21g**). To analyze the DHE concentration in serum, mice were treated with 75 mg/kg DHE by i.g every two days. After DHE treatments for four times, serum was collected at 1 h, 2 h, 4 h, 8 h and 12 h after DHE treatmet on the last day and DHE concentration was measured. The results showed that T_{max} (the time that drug concentrations reach the higheast peak) of DHE is about 2 h; C_{max} (the maximum drug concentration) of DHE is 2,500 ng/ml; AUC (Area under the concentration-time curve) of DHE is 8,547 ng/ml (**Figure R21h, i**). Overall, these data do detect the presence of DHE in both blood and tumors and hint at its effectiveness in tumor treatment.

Moreover, we were also aware of the low oral bioavailability of DHE, so the DHE employed in our study was actually dihydroergotamine mesylate (MCE, HY-B0670A), in which the mesylate salt could improve the chemical stability and bioavailability of the parent drug[13,14]. Sorry for our carelessness in not making it clear, we have changed “dihydroergotamine” to “dihydroergotamine mesylate” in the **Methods section** of revised manuscript. We fully agree with the reviewer that the DHE bioavailability of intravenous administration is higher than oral administration, but considering that intravenous DHE may cause venous thrombosis in patients with peripherally inserted central catheter (PICC) for chemotherapy[15], we finally choose oral administration to evaluate its tumour inhibitory effect.

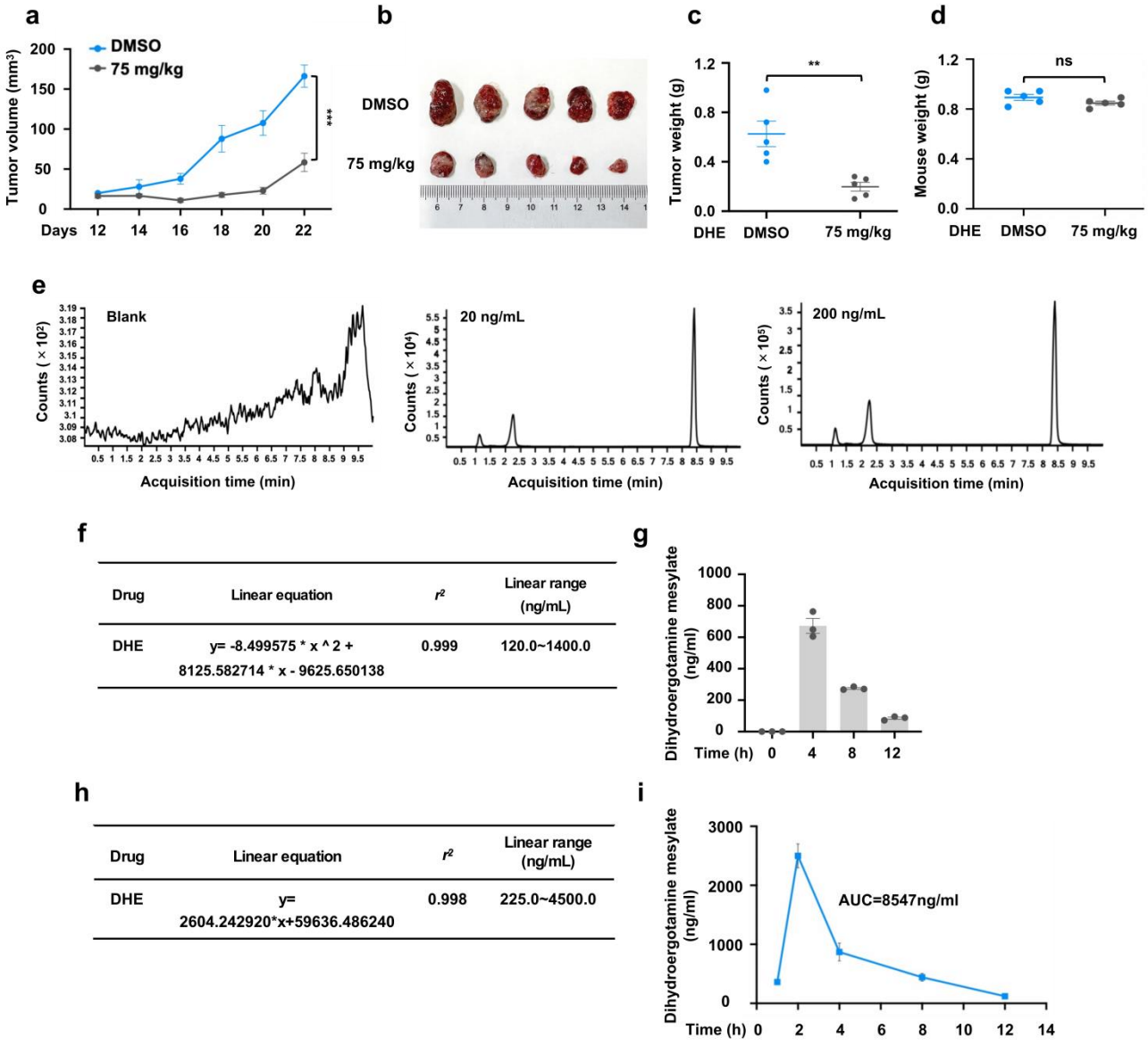


Figure R21. Detection of the DHE concentration in tumor and serum in BALB/c nude mouse inoculated with Hep3B cells. (**a-d**) Parental Hep3B cells were injected subcutaneously into the flanks of BALB/c nude mice. After 12 days, the mice were divided into two groups and treated with DMSO (*n*=5) or DHE 75 mg/kg (*n*=15) by i.g. every two days. Representative tumour volume (*n*=5 per group) was determined based on calliper measurements every two days starting on Day 12 (**a**). The mice were sacrificed on Day 22. Photographs show representative xenografts at the end of the experiment (Day 22) (**b**). Representative tumour weight and mouse weight (*n*=5 per group) was measured with an electronic scale (**c,d**). The data are presented as the mean ± SEM. (**e**) Representative HPLC-MS/MS chromatograms of a blank, 20 ng/ml DHE and 200 ng/ml DHE. (**f**) Standard curves, correlation coefficients (*r*) and linear ranges for assessment of DHE concentration in tumor tissues. (**g**) Detection of DHE concentration in tumor tissue of (*n*=3) after oral administration of DHE as indicated time. The data are presented as the mean ± SEM. (**h**) Standard curves, correlation (*r*) and linear ranges of DHE for assessment of DHE concentration in serum. (**i**) Detection of DHE concentration in serum (*n*=3) after oral administration of DHE as indicated time. The data are presented as the mean ± SEM.

Question 5: PAD4 plays a role in maintaining the stability of HIF-1α by citrullination. In animal experiments, DHE is also utilized to inhibit the citrullination of HIF-1α by PAD4. Please test if HIF-1α citrullination and histone citrullination is altered after DHE administration in the mouse model?

Response: Per the reviewer's suggestion, we examined the citrullination levels of HIF-1α and Histone H3 in the tumor tissue lysates from mouse model. Western blotting results showed that DHE administration led to a reduction of citrullination levels of HIF-1α and Histone H3 (**Figure R22**). We have included **Figure R22** as **Fig. S4g** in the revised manuscript.

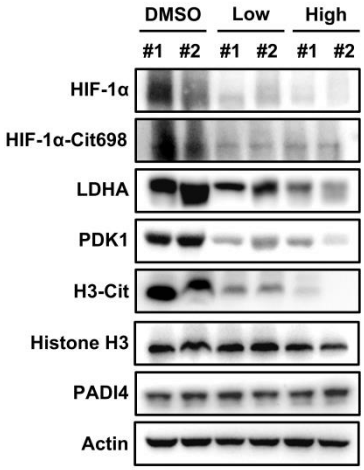


Figure R22. DHE administration decreased the citrullination levels of HIF-1α and Histone3 as well as HIF-1 targets in the tumor lysates. Western blotting analysis of HIF-1α, HIF-1α^{R698Cit}, LDHA, PDK1, Histone H3 (citrulline R2, R8, R17), Histone H3 and PADI4 protein levels in tumor tissues lysates described in Figure 5k. Actin served as the loading control. We have included **Figure R22** as **Fig. S4g** in the revised manuscript.

Reference:

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REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

All the comments have been adequately addressed.

Reviewer #3 (Remarks to the Author):

In the review of the submitted manuscript, the reviewer found the research to be well-conducted and the methodology to be sound. The results presented in the manuscript are clear and support the conclusions drawn by the authors. The findings contribute valuable insights to the field.This reviewer agree with publication of the revised manuscript.

Reviewer #4 (Remarks to the Author):

The major concerns were all addressed and the authors have conclusively shown a novel mechanism of HIF regulation.

Point-by-point response to the comments of the Reviewers

Reviewer #1 (Remarks to the Author):

All the comments have been adequately addressed.

Response: We are grateful for our reviewer's positive comments.

Reviewer #3 (Remarks to the Author):

In the review of the submitted manuscript, the reviewer found the research to be well-conducted and the methodology to be sound. The results presented in the manuscript are clear and support the conclusions drawn by the authors. The findings contribute valuable insights to the field. This reviewer agrees with publication of the revised manuscript.

Response: We appreciate our reviewer for the positive and encouraging comments.

Reviewer #4 (Remarks to the Author):

The major concerns were all addressed and the authors have conclusively shown a novel mechanism of HIF regulation.

Response: We thank our reviewer for the positive comments.