THE EMBO JOURNAL

IDH2 stabilizes HIF-1α-induced metabolic reprogramming and promotes chemoresistance in urothelial cancer

Keisuke Shigeta, Masanori Hasegawa, Takako Hishiki, Yoshiko Naito, Yuto Baba, Shuji Mikami, Kazuhiro Matsumoto, Ryuichi Mizuno, Akira Miyajima, Eiji Kikuchi, Hideyuki Saya, Takeo Kosaka, and Mototsugu Oya
DOI: 10.15252/embj.2022110620

Corresponding author(s): Takeo Kosaka (takemduro@gmail.com) , Takeo Kosaka (takemduro@gmail.com), Mototsugu Oya (moto-oya@z3.keio.jp), Masanori Hasegawa (hasem@tsc.u-tokai.ac.jp)

Review Timeline:

Submission Date:	8th Jan 22
Editorial Decision:	9th Feb 22
Appeal Received:	15th Feb 22
Editorial Decision:	23rd Feb 22
Revision Received:	30th Jul 22
Editorial Decision:	16th Sep 22
Revision Received:	26th Nov 22
Accepted:	29th Nov 22

Editor: Daniel Klimmeck

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision **9th Feb 2022**

Dear Dr Kosaka,

Thank you for submitting your manuscript for consideration by the EMBO Journal. Please accept my apologies for the extended duration of the review process of the manuscript due protracted reviewer input as well as to detailed discussions in the team. Your study has been sent to three referees with expertise in cancer metabolism, and we have received reports from all of them, which I copy below. In light of these comments, I am afraid we decided that we cannot offer publication in The EMBO Journal.

As you will see the referees raise major concerns with the analysis that I am afraid preclude publication here. While referee #1 is overall more positive, referees #2 and #3 point to substantial issues with preceding work as well as insufficient support for the claims made on a role of increased wild-type IDH2 expression and reductive TCA in resistance of urothelial carcinoma. They in essence state that these findings are interesting, but too prematurely developed.

Given these negative opinions from good experts in the field, and considering that we only offer a single major round of revisions at the EMBO Journal, I am afraid we have decided that we cannot pursue this manuscript further towards publication.

However as to your preference at submission, we encourage you to transfer your study to our not-for-profit open-access sister journal, Life Science Alliance (LSA). We shared your manuscript and the accompanying reviews with LSA Executive Editor, Eric Sawey, who is interested in these findings, and would like to invite further consideration of this manuscript at LSA, revised to address the Reviewer comments.

We understand that such a revision might need to be re-reviewed, in which case, Dr. Sawey will walk the Reviewers through our transfer process.

We encourage you to use the link below to transfer your manuscript to LSA. You do not need to revise the manuscript before transferring it to LSA. Once you transfer, Dr. Sawey will email you an invitation to revise and resubmit, listing the same revision requests as mentioned above. Please feel free to reach out at e.sawey@life-science-alliance.org if you have any questions about the LSA journal, the transfer process or the revisions requested.

I realize that this negative decision will be disappointing, but I am afraid that the reports at hand did not allow for a different conclusion in this case.

I hope you will view the possibility of a transfer favourably. If this is the case, please use the link below to transfer the manuscript directly. I again apologize for the unusual delay.

Kind regards,

Daniel Klimmeck

Daniel Klimmeck, PhD
Senior Editor
The EMBO Journal

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Referee #1:

In this manuscript, Shigeta and colleagues address how metabolism is rewired in cancer cells with acquired resistance to the Gemcitabine (GEM) chemotherapy drug. They find increased steady-state glycolysis intermediated in GR cells, which relates to increased mRNA expression of glycolytic enzymes. They also observe (i) overall increase in PPP intermediates, which again relates to increased mRNA expression of PPP enzymes + CTPS1 at the entry point of pyrimidine biosynthesis, and (ii) increase in some TCA intermediates. They perform a better analysis by tracing glutamine, and they find: increased conversion of glutamine to isocitrate, increased routing of glutamine carbons into +3 malate (cytoplasmic derivative of cytoplasmic isocitrate), coupled to decreased routing of glutamine carbons into +4 malate (mitochondrial derivative of aKG into the TCA). So, it appears these cells perform less glutamine anaplerosis through the TCA cycle, and rather use the reverse aKG/isocitrate reaction. In line, they again find increased expression of IDH2 and to some extent IDH1 enzymes mediating such aKG/isocitrate reverse reaction. Surprisingly, the authors then find that by knocking down IDH2, also the glycolytic and CTPS1 enzymes are decreased, indicating that the aKG/isocitrate reaction mediated by IDH2 is the key trait driving metabolic rewiring in response to GEM

treatment. Indeed IDH2 inhibition rescues GEM sensitivity in vitro and perhaps in vivo. Finally, they show that such rewiring also affects NADPH production and glutathione regeneration, to such an extent that GR cells are also more resistant to oxidative stress elicited by cisplatin (CDDP) treatment, whose activity is known to depend on glutathione levels.

Overall this manuscript is well done, the data are based on two independent cell systems, and the findings bring about many interesting observations. The main novelty of the paper, which remains insufficiently developed, is the observation that resistant cells shift to a pseudo-hypoxic state that depends on the use of IDH2 in the reverse reaction (aKG to isocitrate) and, likely, on the subsequent activation of HIF1a. The fact that this is observed in cells with (apparently) WT IDH1/2 goes however against the current models (see my comment 4), and requires some more data to be solid, and some more insights to understand how such IDH2-mediated reverse reaction then occurs in response to GEM treatment.

MAJOR POINTS:

1) The experiment in Figure 2G does not demonstrate that intracellular dCTP can compete for GEM and mitigate its efficacy, because the authors did not demonstrate:
a) that such dCTP is efficiently taken up by cells and contributes to the internal pool (which is not something so well established in the literature);
b) that GEM is similarly taken up by cells, i.e. that such competition does not occur at the level of a common transporter;
c) that the increase of dCTP obtained by using such a high amount of extracellular dCTP matches the metabolic increase observed in Resistant cells, which is only 2-3 fold. This is important because there could be a competition effect with very high amounts of dCTP, but this is maybe not relevant at just 2-3 fold the physiological levels.

2) The authors claim that knocking down IDH2 restores GEM sensitivity. HOWEVER there is a problem in the pertaining panel Fig. 4C (maybe just a labeling problem?), because the graph indicates GR cells were used, but then in the graph there are WT and GEM cells. Is WT the non-resistant? Is GEM the same as GR? And then to which cell line does siIDH2 alone (empty triangles) refers to? In general, the data should be presented based on the two independent IDH2 siRNAs to exclude off-target effects.

3) The authors use what they call "IDH2-i" inhibitor, which is in reality AG-221 or Enasidenib (please use its real name in the text and figures so that this is clear to anyone). However, this inhibitor was specifically developed against MUTATED neomorphic IDH2, and in the original publication they showed much reduced activity against the WT enzyme, with an in vitro IC50 on the purified enzyme of 1,8microM (10.1158/2159-8290.CD-16-1034). The authors should provide some direct evidence that the IDH2-1 is really inhibiting the WT enzyme in their cells at the 1microM dose they are using, otherwise it is very hard to explain the effect of such drug.

4) given the nice effects obtained in vivo with the IDH2-i, the authors should re-sequence IDH2 and IDH1 in their GR cells to test whether GEM treatment selected for mutant IDH. They only provide a single read of the genomic DNA, but it remains hard to understand where does this comes from. It is possible for example that the GR population is mixed, and that they only sequenced a single pick? Did the authors carefully checked the sequencing peaks for double peaks indicative of heterogeneous mutation in the cell population? In principle, all their phenotypes are consistent with mutant IDH, because GR cell lines show evidence for pseudo-hypoxia with HIF1a stabilization at 20% oxygen conditions (Fig 2F!!!!), which depends on IDH2 (Fig 4B!!!), and which other showed in many papers depends on the oncometabolite 2HG produced by the mutant IDH enzymes. This would also explain why these cells reduce glutamine anaplerosis - this is a typical hypoxic response. In addition, the authors should measure production of the 2HG oncometabolite in WT and GR cells, and use WT cells transfected with a mutated IDH2 as a control.

5) Whatever the underlying mechanism, it would be important to provide some functional data on the relevance of HIF1a stabilization for the main phenotypes observed here, including enzyme expression, GEM and CDDP sensitivity in cells with HIF1a knockdown. If so, then the title should shift from IDH2 to WT IDH2-dependent stabilization of HIF1a.

6) The experiment in 4e lacks of the effect of IDH2-i alone, which is fundamental to understand whether the combination effect is due to sensitization to GEM, as claimed by the authors, or just to IDH2-i alone.

7) Data on cross-sensitivity to CDDP can make sense in light of the observed metabolic rewiring. However, the experiment showing that IDH2 inhibition also rescues this phenotype is hard to swallow - or at least requires an explanation to the reviewer and to the audience - in light of the fact that IDH2 uses up NADPH in the aKG/isocitrate reverse reaction, so its inhibition should provide more NADPH to cells, and more GSH, not less. Very likely, this is because IDH2 inhibition indirectly (i.e. by regulating mRNA expression of PPP enzymes) brings back the PPP to low levels observed in the WT cells, and as a consequence the PPP does not suppor NADPH production. This model can be easily demonstrated by showing that PPP inhibition should sensitize more GR cells than WT cells to CDDP treatment.

8) However, the experiment on CDDP also raises (again - see above the comment of IDH2 mutation) the issue that everything seems to start from IDH2, including rewiring of the PPP pathway. If IDH2 is really wild-type in these cells, then two main questions remain unanswered here:

1) what determines the use of glutamine in the reverse IDH2 reaction in GR cells? If it is an issue of expression levels, then is over expression of WT IDH2 sufficient to recapitulate such whole metabolic rewiring?
2) how does reverse IDH2 functioning (but in absence of 2HG production) stabilizes HIF1a and regulates transcription, so that it also rewires glycolysis and the PPP pathway?
Otherwise, the authors cannot provide an explanation for such "mimicry" of mutant IDH2 by a WT IDH2 enzyme, which is ultimately one of the main novelties of the paper.

Minor:

To test for increased glycolysis, they test growth in hypoxia. The authors should just measure OCR/ECAR in standard conditions and show whether the ability of cells to perform glycolysi is different.

Figure 2b, is sigmaPPP a mislabeling of PRPP? If not, a direct measure of the PRPP precursor of dNTPs would be helpful.

Figure 2c and 2d: the panels just show the endpoint purine and pyrimidine compounds, not the biosynthetic intermediates. This should be made clear in the figure and text.

The tracing of glutamine is coherent with the model, but the observation that the contribution of glutamine carbons to cytoplasmic malate is increased bears the question of whether the aKG/isocitrate reverse reaction occurs in mitochondria or (also) in the cytosol via IDH1. Why the authors exclude this? Increased mRNA expression of IDH2 but not IDH1 is weak, because enzymes often work more/less without the need to change their expression levels.

In the methods:
- provide the technique used to calculate relative gene expression levels based on qPCR
- provide the sequences of the siRNAs used in the manuscript in full

Referee #2:

Bladder cancer is the most common malignancy involving the urinary system and the majority of all bladder cancers are of the urothelial carcinoma (UC) histologic type. Systemic chemotherapy (a combination of gemcitabine and cisplatin) is the standard approach for treatment of patients with metastatic disease, which occurs in a significant proportion of UC patients. Unfortunately, chemotherapy resistance is a major barrier limiting patient survival. In the current study, Shigeta and colleagues employ UC cell line models of acquired chemotherapy resistance to investigate the metabolic alterations contributing to resistance, with a goal to identify pathways that can be targeted to enhance the efficacy of chemotherapy. The authors demonstrate that alterations in glycolytic metabolism and the pentose phosphate pathway contribute to gemcitabine resistance by facilitating increased production of dCTP. The authors then proceed to demonstrate an association between chemotherapy resistance and increased expression of isocitrate dehydrogenase 2 (IDH2) and suggest that IDH2-mediated reductive carboxylation contributes to chemotherapy resistance. The study addresses an issue that is of substantial clinical significance however the impact of the findings is limited. This is in large part due to the fact that each of the three mechanisms of resistance described in this study are not investigated in sufficient depth. The majority of the data is quite preliminary in nature and does not provide adequate support for many of the conclusions.

Major concerns that must be addressed are outlined below:
• The ability of T24GR and UMUC3GR cells to proliferate under hypoxia (Figure 1E) does not confirm glycolytic dependency in these cells versus the parental or CR counterparts. As an alternative, the impact of glucose limitation/deprivation on proliferation should be examined.
• The rationale for examining hypoxic conditions in Figure 1E/2E/2F/2G is lacking and confuses the story. Either the rationale should be explained, and hypoxic conditions should be included in a greater number of experiments, or hypoxic conditions should not be included in any of the experiments.
• The authors suggest that increased pyrimidine synthesis contributes to gemcitabine resistance but have not provided sufficient evidence to support this conclusion. For example, the authors should examine the impact of a pyrimidine synthesis inhibitor (e.g. leflunomide) and a purine synthesis inhibitor (e.g. mizoribine) on the cellular response to gemcitabine.
• The authors use the terms deoxycytidine and dCTP (deoxycytidine triphosphate) interchangeably and this has made the experiments in Figure 2G/H difficult to interpret. Please confirm if cells were supplemented with deoxycytidine or dCTP in Figure 2g and whether deoxycytidine or dCTP was measured in Figure 2h.
• Given the authors demonstrate glutamine carbon contributes to reductive carboxylation in T24GR and UMUC3GR cells (Figure 3D), the authors should determine the impact of glutamine deprivation and/or glutaminase inhibitor treatment on cellular sensitivity to gemcitabine.
• Figures 3-6 relate to the contribution of IDH2 to chemotherapy resistance in UC. This focus stems from the observation that IDH2 is more highly expressed in gemcitabine-resistant UC cell lines (T24GR and UMUC3GR) than parental UC cell lines (T24 and UMUC3). The authors employ enasidenib (AG-221) to demonstrate that IDH2 inhibition sensitizes T24GR and UMUC3GR cells to gemcitabine in vitro and in vivo. However, given that the cell lines do not harbour IDH2 neomorphic mutations (Supp Fig

1) and enasidenib is a selective inhibitor of mutant IDH2, these results are unexpected. The onus is on the authors to show that, at the doses employed in this study, AG-221 inhibits wild-type IDH2 in the UC cell lines.
• In Figure 5H/K, DCF is employed to measure ROS. However, changes in DCF fluorescence are not specific to changes in ROS levels and can occur in response to loss of cell viability. A more specific measure of ROS should be employed (e.g. CellROX).
• The authors provide evidence that GSH levels are higher in GR cells and suggest that the decrease in GSH observed following IDH depletion increases sensitivity to cisplatin. The authors should determine whether or not inhibition of GSH synthesis by buthionine sulfoximine (BSO) sensitizes GR cells to cisplatin.

Minor concerns that should be addressed are outlined below:
• The authors should explain what sigmaPPP and sigmaTCA represent in Figure 2B and 3A respectively. How were these values calculated?
• Both IDH2 siRNA constructs should be employed in Figure 4c and Figure 5i/k.

Referee #3:

Shigeta et al. investigate metabolic reprogramming as a mechanism that promotes chemoresistance in urothelial carcinomas. When investigating changes in metabolite levels after long-term selection in cis-platin or gemcitabine, the authors identify increased levels in various intermediates of glycolysis and the PPP, which correlates with increased expression of enzymes in these metabolic pathways as well as HIF1-a. Gemcitabine-resistant cells display increased levels of dCTP, and addition of dCTP to non-resistant cells promotes gemcitabine resistance. Gemcitabine-resistant cells also display increased reductive TCA cycle flux and NADPH levels, which depends on upregulation of IDH2. Genetic or pharmacological inhibition of IDH2 reverts the metabolic changes resensitizes cells to gemcitabine, and, interestingly, to cisplatin-induced ROS production. Finally, IDH2 levels are increased in patients with acquired chemoresistance, suggesting that IDH2 could be a promising therapeutic target.

The identification of metabolic alterations that confer resistance to chemotherapeutics including cross-resistance to different drugs are highly relevant for cancer therapy. As such, the general concept of the study is interesting. The mechanism of gemcitabine resistance - upregulation of HIF1-a, glucose uptake and the non-oxidative PPP to increase nucleotide production - was reported in pancreatic cancer by Shukla et al., 2017. The novelty of this part of the paper is thus largely confirmatory for urothelial carcinomas, which is still relevant, given substantial metabolic differences between individual cancer types. Metabolic reprogramming through increased expression of IDH2 is a novel resistance mechanism. While these findings are of potentially high impact, several experiments lack relevant controls and the authors tend to overinterpret their results, often based on correlative data or inferences. A substantial amount of additional work would be required to support the key conclusions of the manuscript.

Major points:
1. The authors passage two cell lines for 12 - 18 months in genotoxic drugs to generate chemoresistant cells. Does this treatment lead to reproducible phenotypes? Were the control cells passaged over the same period of time?

2. The manuscript including the title focuses on IDH2, but the connection of IDH2 to observed metabolic changes is often unclear. Why does IDH2 depletion reduce levels of glycolytic enzymes? Does overexpression of IDH2 in control cells recapitulate the metabolic phenotypes of resistant cells? The authors propose that the IDH1/2 shuttle generates cytosolic NADPH for GSH synthesis - do IDH1 and IDH2 inhibitors produce similar phenotypes? Does IDH2 inhibition cause the changes in intermediates of glycolysis and the PPP, which are proposed to confer gemcitabine resistance?

3. The authors demonstrate increased expression of multiple metabolic enzymes in resistant cells (glycolysis, PPP, TCA cycle) and conclude that these changes increase pathway flux and mediate therapy resistance. With the exception of genetic / pharmacological inhibition of IDH2, these data are entirely correlative and should be supported by data where enzyme levels are experimentally increased or decreased. This is critical to establish functional relevance, because culturing cells in chemotherapeutics for over a year likely induces many other alterations.

4. Fig. 1: The authors identify increased levels of various glycolysis and PPP intermediates in resistant cells. To demonstrate increased pathway flux, at least lactate production should be quantified.

5. The connection between the PPP and increased levels of dCTP are unclear to me. The PPP generates ribose, which is incorporated into all nucleotides. Are there other metabolite changes that help explain why only dCTP is elevated?

6. Fig. 4e - h: The tumor growth data do not include treatment with the IDH2 inhibitor alone. However, the manuscript mentions that tumor volume after treatment with IDH2 inhibitor alone was 232 +-77 m^3 - this would be comparable to the tumor volume after treatment with gemcitabine + IDH2-i. To evaluate the efficacy of the co-treatment, it is essential to include data for each inhibitor separately in the figures.

7. Fig. 5: The authors demonstrate increased intermediates of the oxidative PPP, which is a major source of cytosolic NADPH. It is unclear why the authors focus on mitochondrial NADPH generation by reductive TCA. Does inhibition of the oxidative PPP resensitize cells to cisplatin?

8. The authors speculate that glutamine-dependent metabolism in the TCA cycle contributes to therapy resistance. To provide experimental evidence for this, the authors should compare the effects of inhibitors against glutaminase or IDH2 on NADPH levels, ROS levels and drug resistance.

Minor points:
1. The metabolomics data show large variations within the experimental groups. Are these technical replicates or independently derived cell lines?

2. The font size in some figures is rather difficult to read.

3. Fig. 5c: Figure legend text and colors are very difficult to decipher.

4. Fig. 5: For the IDH2 siRNA + cisplatin experiments, please also show effects on cell viability.

5. Fig. 5: Can the resensitization to cisplatin by IDH2 siRNA be blocked by antioxidants?

6. Fig. 5: Do the cisplatin-resistant cells shown in Fig. 1 display similar changes in NADPH levels, ROS and IDH2 dependency as the gemcitabine-resistant cells? Or do these cells acquire resistance to cisplatin by a different mechanism?

7. The authors repeatedly refer to anaerobic metabolism. In experiments where cells are cultured in 21 % oxygen, it would be more appropriate to refer to reductive metabolism and aerobic glycolysis.

February 14, 2021

Dear Editor of Embo Journal,

We submitted our manuscript entitled EMBOJ-2022-110620 "Wild-Type IDH2 Induces Anaerobic Metabolic Reprogramming in Chemo-resistant Urothelial Carcinoma", which we submitted on Jan 8th, 2022 and received the decision on Feb 9th according to the external review.

We like to thank all the reviewers for their constructive comments, which would help us to improve our manuscript. Following their suggestions, we received a positive comments from Reviewer 1, and may have impacted potential interest from Reviewer 2 and 3. Since there were some critical comments that reviewers were anxious about because of our insufficient explanation regarding of our experimental methods and data, we would like to inform to the chief editor how we truly intended in detail. The potential interest of our finding which comprise certain novelty is that IDH2 gain of function without IDH2 mutation in UC cells would also comprise the potential to reprogram their intracellular metabolism to adapt themselves to hypoxic condition. Furthermore, if IDH2 becomes the promising target for chemo-resistant urothelial carcinoma, IDH2 inhibitor can be introduced in actual clinical setting within rapid process since IDH2 inhibitor, Enadesinib (AG221), has already been utilized in second line treatment of acute myeloid leukemia. We do recognize Enadesinib (AG221) was developed for chemo-relapsed AML patients who has IDH2 mutations, but we aimed to choose AG221 for IDH2 inhibitor to resensitize chemoresistance via inhibiting metabolic rewiring since this medicine has already introduced to human level, which would speed up for making breakthrough so as to defeat the poor patient survival in chemoresistant UC patients.

We kindly request to have the opportunity to revise our manuscript so as to support all reviewer's comment for providing more rigorous additional information to make our experimental model more concrete and reproducible.

Point-to-point response to main concerns:

Referee #1:

Overall this manuscript is well done, the data are based on two independent cell systems, and the findings bring about many interesting observations. The main novelty of the paper, which remains insufficiently developed, is the observation that resistant cells shift to a pseudo-hypoxic state that depends on the use of IDH2 in the reverse reaction (aKG to isocitrate) and, likely, on the subsequent activation of HIF1a. The fact that this is observed in cells with (apparently) WT IDH1/2 goes however against the current models (see my comment 4), and requires some more data to be solid, and some more insights to understand how such IDH2-mediated reverse reaction then occurs in response to GEM treatment.

Main concern:

1) The experiment in Figure 2G does not demonstrate that intracellular dCTP can compete for GEM and mitigate its efficacy, because the authors did not demonstrate:

a) that such dCTP is efficiently taken up by cells and contributes to the internal pool (which is not something so well established in the literature);

b) that GEM is similarly taken up by cells, i.e. that such competition does not occur at the level of a common transporter;

c) that the increase of dCTP obtained by using such a high amount of extracellular dCTP matches the metabolic increase observed in Resistant cells, which is only 2-3 fold. This is important because there could be a competition effect with very high amounts of dCTP, but this is maybe not relevant at just 2-3 fold the physiological levels.

Response) Thank you for critical comments. From the metabolome analysis, we observed that dCTP is about 3 times larger than WT cells (see Extended Figure 1A). We agree that the extra cellular level of dCTP needs high amount for WT cells for weakening the cytotoxic effect of GEM. However, the discrepancy may have occurred because metabolome analysis held for WT and GR cells were not conducted under GEM exposure, which may have reflected the baseline dCTP level in GR cells compared to WT cells.

Therefore, we can try conducting CE-MS metabolome analysis to compare the dCTP level after GEM exposure upon both WT and GR cells, whether it shows big increase of intracellular dCTP in GR cells.

2) The authors claim that knocking down IDH2 restores GEM sensitivity. HOWEVER there is a problem in the pertaining panel Fig. 4C (maybe just a labeling problem?), because the graph indicates GR cells were used, but then in the graph there are WT and GEM cells. Is WT the non-resistant? Is GEM the same as GR? And then to which cell line does siIDH2 alone (empty triangles) refers to? In general, the data should be presented based on the two independent IDH2 siRNAs to exclude off-target effects.

Response) Thank you for your critical suggestions. We apologize for the confusion representing both GR and WT cells' cell survival.
We changed the graph for comparing the GEM ＋ siNTC (non-targeting control), GEM+siIDH2#1, and GEM+siIDH2#2 in both GR cells (Extended Figure 1B).

3) The authors use what they call "IDH2-i" inhibitor, which is in reality AG-221 or Enasidenib (please use its real name in the text and figures so that this is clear to anyone). However, this inhibitor was specifically developed against MUTATED neomorphic IDH2, and in the original publication they showed much reduced activity against the WT enzyme, with an in vitro IC50 on the purified enzyme of 1,8microM (10.1158/2159-8290.CD-16-1034). The authors should provide some direct evidence that the IDH2-1 is really inhibiting the WT enzyme in their cells at the 1microM dose they are using, otherwise it is very hard to explain the effect of such drug.
Response) Thank you for your suggestions and we apologize not showing the figure of IDH2-inhibitor alone. We added the result of cytotoxic effect of IDH2-I alone to the previous Figure 4 e-h (Extended Figure C).
As the reviewer pointed out, it is true that Enasidenib have selectivity upon IDH2 mutant cells compared to WT cells (Chen et al. Cell Communication and Signaling (2020) https://doi.org/10.1186/s12964-020-00536-7). In the literature, however, the IC50 is 1.8μM for $IDH2^{WT}$ TF1 cells which suggests that Enasidenib would also show certain cytotoxic effect upon I $IDH2^{WT}$ cancer cells. Regarding to UC cells, we found that IC50 of Enasidenib was 1.2μM for T24GR and for 1.9μM for UMUC3GR cells , which was not so different from 1.8μM for TF1 cells (erythroblast) .
We agree that Enasidenib itself may not be the most promising drug for expecting the new cytotoxic drug agent upon chemo-resistant UC cells since there is no IDH2 mutation. However, Guo-Fang Zhang et al. (Cell metabolism (2021) doi: 10.1016/j.cmet.2020.11.020.) have recently introduced that IDH2 inhibitor (AGI-6780) showed to suppress the reductive TCA cycle function which restore insulin secretion in pancreatic cells. Needless to say, these pancreatic islet cells and *in vivo* experiments have no IDH2 mutation, but IDH2 inhibitor

showed significant suppression from reductive carboxylation to normalize into oxidative carboxylation. Since AGI6780 is the pro-drug of Enasidenib (AG-221) that inhibits both mutated and wild-type IDH2, this article proved that IDH2 inhibitor would inhibit reductive TCA in wild-type IDH2 cells.

Thus, we will try additional IDH2 inhibitor (AGI-6780: prodrug of AG221) to confirm that IDH2 inhibitors and/or with combination of GEM whether it inhibits the extire metabolic rewiring and results in recovering the chemosensitivity in chemoresistant UC cells.

4) given the nice effects obtained in vivo with the IDH2-i, the authors should re-sequence IDH2 and IDH1 in their GR cells to test whether GEM treatment selected for mutant IDH. They only provide a single read of the genomic DNA, but it remains hard to understand where does this comes from. It is possible for example that the GR population is mixed, and that they only sequenced a single pick? Did the authors carefully checked the sequencing peaks for double peaks indicative of heterogeneous mutation in the cell population? In principle, all their phenotypes are consistent with mutant IDH, because GR cell lines show evidence for pseudo-hypoxia with HIF1a stabilization at 20% oxygen conditions (Fig 2F!!!!), which depends on IDH2 (Fig 4B!!!), and which other showed in many papers depends on the oncometabolite 2HG produced by the mutant IDH enzymes. This would also explain why these cells reduce glutamine anaplerosis - this is a typical hypoxic response. In addition, the authors should measure production of the 2HG oncometabolite in WT and GR cells, and use WT cells transfected with a mutated IDH2 as a control.

Response) Thank you for your critical comment. As our manuscript showed, we agree our manuscript has certain weakness proving whether our cell lines have IDH mutations or not. We did try DNA sanger sequence to confirm mutations by using DNA primers covering IDH1 and IDH2 (IDH1-R132 and IDH2-R140-R172).

We will try DNA exon sequence of our two cell lines (T24WT, T24GR, UMUC3WT, and UMUC3GR) to confirm IDH1/2 mutations or other major DNA change among these 4 cells.

For additional information, we measured 2HG for both WT and GR cells, and found that certain 2HG accumulation were observed in both GR cells compared to WT (Extended Figure 2A). However, the increase rate was milder than we expected, (i.e. they are 3-4 times higher in GR cells than WT cells, since the change would be much higher with IDH2 mutation cells).

We will try inducing IDH2 transfection to $IDH2^{WT}$ UC cell lines (5637 or J82 cells) and would conduct metabolome analysis to confirm whether the metabolic reprogramming would become reproducible by IDH2 gain of function or not.

5) Whatever the underlying mechanism, it would be important to provide some functional data on the relevance of HIF1a stabilization for the main phenotypes observed here, including enzyme expression, GEM and CDDP sensitivity in cells with HIF1a knockdown. If so, then the title should shift from IDH2 to WT IDH2-dependent stabilization of HIF1a.

Response)

Thank you for your critical suggestions. We have not conducted down regulation of HIF1A neither in vitro nor in vivo yet.

We can add functional analysis of HIF1A knock down whether it regulates the zanaerobic enzymes expression, and whether it change GEM/CDDP sensitivity.

6) The experiment in 4e lacks of the effect of IDH2-i alone, which is fundamental to understand whether the combination effect is due to sensitization to GEM, as claimed by the authors, or just to IDH2-i alone.

Response) Thank you for your suggestions and we apologize not showing the figure of IDH2-inhibitor alone. We added the result of cytotoxic effect of IDH2-I alone. (Extended Figure 1C)

7) Data on cross-sensitivity to CDDP can make sense in light of the observed metabolic rewiring. However, the experiment showing that IDH2 inhibition also rescues this phenotype is hard to swallow - or at least requires an explanation to the reviewer and to the audience - in light of the fact that IDH2 uses up NADPH in the aKG/isocitrate reverse reaction, so its inhibition should provide more NADPH to cells, and more GSH, not less. Very likely, this is because IDH2 inhibition indirectly (i.e. by regulating mRNA expression of PPP enzymes) brings back the PPP to low levels observed in the WT cells, and as a consequence the PPP does not suppor NADPH production. This model can be easily demonstrated by showing that PPP inhibition should sensitize more GR cells than WT cells to CDDP treatment.

Response) Thank you for your critical comment. We observed that whole cell NADPH level and GSH levels were significantly higher in both GR cells, which are known as strong reducing agents to detoxify ROS accumulation. As the review raise the critical point, we agree to the reviewer's insight that IDH2 inhibition indirectly brings back the metabolic pathway in glycolysis and weakens PPP bypass by downregulating the key PPP enzymes. To answer this question, we will try using G6PD inhibitor and G6PD inhibitor combination

with CDDP to show PPP inhibition would sensitize GR cells to CDDP treatment or not.

8) However, the experiment on CDDP also raises (again - see above the comment of IDH2 mutation) the issue that everything seems to start from IDH2, including rewiring of the PPP pathway. If IDH2 is really wild-type in these cells, then two main questions remain unanswered here:
1) what determines the use of glutamine in the reverse IDH2 reaction in GR cells? If it is an issue of expression levels, then is over expression of WT IDH2 sufficient to recapitulate such whole metabolic rewiring?
2) how does reverse IDH2 functioning (but in absence of 2HG production) stabilizes HIF1a and regulates transcription, so that it also rewires glycolysis and the PPP pathway?
Otherwise, the authors cannot provide an explanation for such "mimicry" of mutant IDH2 by a WT IDH2 enzyme, which is ultimately one of the main novelties of the paper.

Response) Thank you for pointing out our biggest founding in our paper. We did suspect IDH2 mutation in GEM-resistant UC cells, since the aerobic glycolysis, promotion of PPP bypass, and reductive TCA were observed from our experiment. However, 2-HG accumulation level were only 3-4 times higher in GR cells than WT cells (Extended figure 2A), which is not as high as the amount which recent literatures reported in IDH2 mutated cell lines. In addition, DNA Sanger sequence targeting IDH1/2 did not show any IDH1/2 mutations in GR cell lines. On the other hand, the recent study revealed that reductive TCA cycle metabolism can be observed in pancreatic islet cells via IDH2 stimulation and IDH2 was shown as the key enzyme for maintaining the reductive TCA glucose stimulated insulin secretion (doi: 10.1016/j.cmet.2020.11.020). Moreover, downregulation or pharmacologic suppression of IDH2 resulted in inhibiting insulin secretion with lowering NADPH levels. These pancreatic islet cells are normal cells without IDH1/2 mutations, and therefore, we suspect that IDH1/2 gain of function does not always derive from IDH1/2 mutations.
To verify the IDH2 gain of function promotes metabolic rewiring even in $IDH2^{WT}$, we will try overexpressing IDH2 in $IDH2^{WT}$ UC cell lines and would conduct metabolome analysis to confirm whether the metabolic reprogramming would become reproducible or not and whether IDH2 gain of function correlates with chemoresistance for UC cells.
Moreover, as we showed the 2-HG level in GR cells, we can confirm the m-RNA and/or protein levels of PHD2, Hif1A, whether IDH2 regulates the entire metabolic reprogramming or not by stabilizing Hif1α.

Referee #2:

Bladder cancer is the most common malignancy involving the urinary system and the majority of all bladder cancers are of the urothelial carcinoma (UC) histologic type. Systemic chemotherapy (a combination of gemcitabine and cisplatin) is the standard approach for treatment of patients with metastatic disease, which occurs in a significant proportion of UC patients. Unfortunately, chemotherapy resistance is a major barrier limiting patient survival. In the current study, Shigeta and colleagues employ UC cell line models of acquired chemotherapy resistance to investigate the metabolic alterations contributing to resistance, with a goal to identify pathways that can be targeted to enhance the efficacy of chemotherapy. The authors demonstrate that alterations in glycolytic metabolism and the pentose phosphate pathway contribute to gemcitabine resistance by facilitating increased production of dCTP. The authors then proceed to demonstrate an association between chemotherapy resistance and increased expression of isocitrate dehydrogenase 2 (IDH2) and suggest that IDH2-mediated reductive carboxylation contributes to chemotherapy resistance. The study addresses an issue that is of substantial clinical significance however the impact of the findings is limited. This is in large part due to the fact that each of the three mechanisms of resistance described in this study are not investigated in sufficient depth. The majority of the data is quite preliminary in nature and does not provide adequate support for many of the conclusions.

Main concern:

1) The ability of T24GR and UMUC3GR cells to proliferate under hypoxia (Figure 1E) does not confirm glycolytic dependency in these cells versus the parental or CR counterparts. As an alternative, the impact of glucose limitation/deprivation on proliferation should be examined.

Response) Thank you for the comment. We would confirm the cell survival of WT and GR cells under glucose free, glutamine free, and glucose$^-$glutamine$^-$ free conditions.

2) The rationale for examining hypoxic conditions in Figure 1E/2E/2F/2G is lacking and

confuses the story. Either the rationale should be explained, and hypoxic conditions should be included in a greater number of experiments, or hypoxic conditions should not be included in any of the experiments.

Response) Thank you for your suggestion. We aimed to show the key enzymes expression levels (both mRNA and protein) under normoxia and hypoxia since some of the key enzymes which functions under hypoxia conditions may be degraded under normoxia condition during the process of collecting the lysate (i.e. Hif1-α). Thus, we aimed to create Figures1E/2E/2F/2G under 21%O_2 and 1% O_2 to emphasize showing the gradation of expression levels of anaerobic enzymes.

3) The authors suggest that increased pyrimidine synthesis contributes to gemcitabine resistance but have not provided sufficient evidence to support this conclusion. For example, the authors should examine the impact of a pyrimidine synthesis inhibitor (e.g. leflunomide) and a purine synthesis inhibitor (e.g. mizoribine) on the cellular response to gemcitabine.

Response) Thank you for the critical comment. We agree there was certain weakness for concluding the association between increased pyrimidine synthesis contributes to GEM resistance.

We are happy to conduct to examine the impact of a pyrimidine synthesis inhibitor (e.g. leflunomide) and a purine synthesis inhibitor (e.g. mizoribine) on the cellular response to gemcitabine in both GR cells.

4) The authors use the terms deoxycytidine and dCTP (deoxycytidine triphosphate) interchangeably and this has made the experiments in Figure 2G/H difficult to interpret. Please confirm if cells were supplemented with deoxycytidine or dCTP in Figure 2g and whether deoxycytidine or dCTP was measured in Figure 2h.

Response) Thank you for your critical comment. As the reviewer pointed out, all the experiments were conducted for measuring dCTP in both Figure 2g and 2h. Thus, we corrected our graph in Figure 2h.

5) Given the authors demonstrate glutamine carbon contributes to reductive carboxylation in T24GR and UMUC3GR cells (Figure 3D), the authors should determine the impact of glutamine deprivation and/or glutaminase inhibitor treatment on cellular sensitivity to gemcitabine.

Response) Thank you for the comment. As the reviewer pointed out, we will try evaluating the cell survival rates under glutamine deprivation condition and glutaminase inhibitor treatment.

6) Figures 3-6 relate to the contribution of IDH2 to chemotherapy resistance in UC. This focus stems from the observation that IDH2 is more highly expressed in gemcitabine-resistant UC cell lines (T24GR and UMUC3GR) than parental UC cell lines (T24 and UMUC3). The authors employ enasidenib (AG-221) to demonstrate that IDH2 inhibition sensitizes T24GR and UMUC3GR cells to gemcitabine in vitro and in vivo. However, given that the cell lines do not harbour IDH2 neomorphic mutations (Supp Fig 1) and enasidenib is a selective inhibitor of mutant IDH2, these results are unexpected. The onus is on the authors to show that, at the doses employed in this study, AG-221 inhibits wild-type IDH2 in the UC cell lines.

Response) same as reviewer 1 (3).

7) In Figure 5H/K, DCF is employed to measure ROS. However, changes in DCF fluorescence are not specific to changes in ROS levels and can occur in response to loss of cell viability. A more specific measure of ROS should be employed (e.g. CellROX).

Response) Thank you for the comment.

We will try using CellROX for more specific measurement of ROS accumulation.

8) The authors provide evidence that GSH levels are higher in GR cells and suggest that the decrease in GSH observed following IDH depletion increases sensitivity to cisplatin. The authors should determine whether or not inhibition of GSH synthesis by buthionine sulfoximine (BSO) sensitizes GR cells to cisplatin.

Response) Thank you for your comment. We added the results using the BSO upon both GR cells (Extended Figure 2B)

9) Both IDH2 siRNA constructs should be employed in Figure 4c and Figure 5i/k.

Response) Thank you for your comment. We added the results of siIDH2#1 and siIDH2#2 transfection upon NADPH, GSH, and ROS measurements. (Extended Figure 2C)

Referee #3:

The identification of metabolic alterations that confer resistance to chemotherapeutics including cross-resistance to different drugs are highly relevant for cancer therapy. As such, the general concept of the study is interesting. The mechanism of gemcitabine resistance - upregulation of HIF1-a, glucose uptake and the non-oxidative PPP to increase nucleotide production - was reported in pancreatic cancer by Shukla et al., 2017. The novelty of this part of the paper is thus largely confirmatory for urothelial carcinomas, which is still relevant, given substantial metabolic differences between individual cancer types. Metabolic reprogramming through increased expression of IDH2 is a novel resistance mechanism. While these findings are of potentially high impact, several experiments lack relevant controls and the authors tend to overinterpret their results, often based on correlative data or inferences. A substantial amount of additional work would be required to support the key conclusions of the manuscript.

Main concern:

1. The authors passage two cell lines for 12 - 18 months in genotoxic drugs to generate chemoresistant cells. Does this treatment lead to reproducible phenotypes? Were the control cells passaged over the same period of time?

Response) Thank you for the suggestion. We have cultured same amount of time in control cells (T24WT and UMUC3WT) during the generation of T24GR and UMUC3GR. In the real clinical situation, GC therapy would acquire chemoresistance We conducted STR（short tandem repeat）analysis after generating GR cells and showed consistency with WT cells. Thus, we consider that our experimental cell model would reflect the real clinical setting.

2. The manuscript including the title focuses on IDH2, but the connection of IDH2 to observed metabolic changes is often unclear. Why does IDH2 depletion reduce levels of glycolytic enzymes? Does overexpression of IDH2 in control cells recapitulate the metabolic phenotypes of resistant cells? The authors propose that the IDH1/2 shuttle generates cytosolic NADPH for GSH synthesis - do IDH1 and IDH2 inhibitors produce similar

phenotypes? Does IDH2 inhibition cause the changes in intermediates of glycolysis and the PPP, which are proposed to confer gemcitabine resistance?

Response) Thank you for the critical comments. We suspect that IDH2 gain of function triggers glutaminolysis and would produce 2-HG, which is widely known as oncometabolite. 2-HG stabilizes Hif1-α, which is the master regulator of promoting anaerobic glycolysis and PPP shunt. As a matter of fact, we observed 2-HG accumulation in GR cells compared to WT cells (Extended Figure 2A).

To verify the IDH2 gain of function promotes metabolic rewiring even in $IDH2^{WT}$, we will try overexpressing IDH2 in $IDH2^{WT}$ UC cell lines and would conduct metabolome analysis to confirm whether the metabolic reprogramming would become reproducible or not and whether IDH2 gain of function correlates with chemoresistance for UC cells.

We also will try measuring the amounts of intermediate metabolites, NADPH, GSH, and ROS levels under using IDH2 inhibitor.

3. The authors demonstrate increased expression of multiple metabolic enzymes in resistant cells (glycolysis, PPP, TCA cycle) and conclude that these changes increase pathway flux and mediate therapy resistance. With the exception of genetic / pharmacological inhibition of IDH2, these data are entirely correlative and should be supported by data where enzyme levels are experimentally increased or decreased. This is critical to establish functional relevance, because culturing cells in chemotherapeutics for over a year likely induces many other alterations.

Response) Thank you for the comment. To answer this question, we will try conducting CE-MS to measure intermediate metabolites under IDH2 overexpression and under the use of IDH2 inhibitor.

4. Fig. 1: The authors identify increased levels of various glycolysis and PPP intermediates in resistant cells. To demonstrate increased pathway flux, at least lactate production should be quantified.

Response) Thank you for your comment. In specific, the lactate production level were 900727.8±134650.9nmol/kg in T24GRcells which was significantly higher than that of WT cells (533755.1 ± 53254.46328 nmol/kg, p=0.010). Referring to UMUC3, the lactate production level were 1853622.0 ± 422404.5nmol/kg in UMUC3GRcells which was significantly higher than that of WT cells (832021.0±123099.1 nmol/kg, p=0.048).

5. The connection between the PPP and increased levels of dCTP are unclear to me. The PPP generates ribose, which is incorporated into all nucleotides. Are there other metabolite

changes that help explain why only dCTP is elevated?

Response) Thank you for your comment. We looked over the results of metabolome analysis and once confirmed that dCTP is selectively increased in GR cells compared to other dNTPs (Extended figure 1A). As we looked the intermediate metabolites of purine and pyrimidine biosynthesis (Figure 2c and 2d), it revealed that CTP (a precursor of dCTP) were significantly higher in GR cells than WT cells. Finally, CTP synthase 1 (CTP1) showed significantly higher mRNA and protein expression in GR cells, which is a key enzyme for dCTP biosynthesis. These results completely follow the Schukla's (doi: 10.1016/j.ccell.2017.06.004.) report, and we suspect that GR cells comprise high dCTP level to achieve molecular competition against GEM.

6. Fig. 4e - h: The tumor growth data do not include treatment with the IDH2 inhibitor alone. However, the manuscript mentions that tumor volume after treatment with IDH2 inhibitor alone was 232 +-77 m^3 - this would be comparable to the tumor volume after treatment with gemcitabine + IDH2-i. To evaluate the efficacy of the co-treatment, it is essential to include data for each inhibitor separately in the figures.

Response) Thank you for your suggestions and we apologize not showing the figure of IDH2-inhibitor alone. We added the result of cytotoxic effect of IDH2-I alone in Extended Figure 1C .

7. Fig. 5: The authors demonstrate increased intermediates of the oxidative PPP, which is a major source of cytosolic NADPH. It is unclear why the authors focus on mitochondrial NADPH generation by reductive TCA. Does inhibition of the oxidative PPP resensitize cells to cisplatin?

Response) Thank you for your critical comment. We observed that whole cell NADPH level and GSH levels were significantly higher in both GR cells, which are known as strong reducing agents to detoxify ROS accumulation. As the review raise the critical point, we agree to the reviewer's insight that IDH2 inhibition indirectly brings back the metabolic pathway in glycolysis and weakens PPP bypass by downregulating the key PPP enzymes. To answer this question, we will try using G6PD inhibitor and G6PD inhibitor with CDDP to GR and WT cells to show PPP inhibition would sensitize GR cells to CDDP treatment.

8. The authors speculate that glutamine-dependent metabolism in the TCA cycle contributes to therapy resistance. To provide experimental evidence for this, the authors should compare the effects of inhibitors against glutaminase or IDH2 on NADPH levels, ROS levels and drug resistance.

Response) Thank you for your critical comment.

We can add results by using IDH2 inhibitors (or glutaminase inhibitor) and measure NADPH and ROS levels to confirm the drug resistance.

We feel grateful if the editor team can reconsider our manuscript as one of the candidate papers for publication for “Embo Journal”, by answering all the reviewers’ comments.

Sincerely,

Takeo Kosaka, MD, PhD (lead contact)
Department of Urology, Keio University School of Medicine
35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan.
Phone: 81-3-5363-3824, Fax: 81-3-3225-1985,
E-mail: takemduro@gmail.com

1st Revision - Editorial Decision **23rd Feb 2022**

Dear Dr Kosaka,

Thank you for following up on our recent decision regarding your manuscript EMBOJ-2022-110620, which was rejected post review on Febr 9th, 2022 and sharing a detailed point-by-point response to the referees' critique. I have now carefully considered your rebuttal and discussed the matter in detail in the editorial team.

We appreciate that the additional results presented and complementary experiments suggested might be appropriate to address a substantial portion of the critique and significantly improve the robustness and relevance of the study. While we note that the extent of revision work would exceed the regular theme our journal follows, we can overall encourage you to work towards a revised version along the lines sketched in your outline, which we would be able to editorially consider and potentially share with the referees for re-evaluation. However, please note that given the open outcome of key experiments requested, and the substantial major issues stated regarding robustness and depth of the insights provided, the burden on convincing the editorial team and experts would lie entirely on you in this case, and we consider the outcome of such re-evaluation open at this point.

I share below information on the formatting requirements for EMBO Journal manuscripts which you would need to adhere to at resubmission of the revised study.

Please let me know if you are interested in working towards a revised version of your study, in which case I would keep the entry open in our system.

Kind regards,

Daniel Klimmeck

Daniel Klimmeck, PhD
Senior Editor | The EMBO Journal
d.klimmeck@embojournal.org

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Daniel Klimmeck, PhD
Senior Editor
The EMBO Journal

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

As you know, we generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Instruction for the preparation of your revised manuscript:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point response to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/Author Checklist%20-%20EMBO%20J-1561436015657.xlsx). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript.

6) It is mandatory to include a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see https://www.embopress.org/page/journal/14602075/authorguide#datadeposition).
In case you have no data that requires deposition in a public database, please state so in this section. Note that the Data Availability Section is restricted to new primary data that are part of this study.
*** Note - All links should resolve to a page where the data can be accessed. ***

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data can be provided as individual .xls or .csv files (including a tab describing the data). For 'blots' or microscopy, uncropped images should be submitted (using a zip archive or a single pdf per main figure if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at .

9) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online (see examples in https://www.embopress.org/doi/10.15252/embj.201695874). A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc. in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: .

- Additional Tables/Datasets should be labelled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

10) When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
http://bit.ly/EMBOPressFigurePreparationGuideline

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11) For data quantification: please specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. The figure legends should contain a basic description of n, P and the test applied. Graphs must include a description of the bars and the error bars (s.d., s.e.m.).

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 24th May 2022.

Link Not Available

Referee #1:

Main concern:

1) The experiment in Figure 2G does not demonstrate that intracellular dCTP can compete for GEM and mitigate its efficacy, because the authors did not demonstrate:
a) that such dCTP is efficiently taken up by cells and contributes to the internal pool (which is not something so well established in the literature);
b) that GEM is similarly taken up by cells, i.e. that such competition does not occur at the level of a common transporter;
c) that the increase of dCTP obtained by using such a high amount of extracellular dCTP matches the metabolic increase observed in Resistant cells, which is only 2-3 fold. This is important because there could be a competition effect with very high amounts of dCTP, but this is maybe not relevant at just 2-3 fold the physiological levels.

Response) Thank you for pointing out this crucial issue.

Metabolomic analysis revealed that GR cells showed significant increases in the levels of final pyrimidine biosynthesis compounds (CTP and UTP), and we also found that compared to the levels of dATP, dTTP, and dGTP, the level of dCTP was specifically increased in GR cells (Figure R1-1a).

We measured the baseline dCTP levels in WT and GR cells. The results are shown in Figure R1-1b. The baseline dCTP levels were significantly higher in T24GR and UMUC3GR cells (447.65 nmol/μg±17.8 nmol/μg and 584.65 nmol/μg±53.2 nmol/μg, respectively) than in T24WT and UMUC3WT cells (114.59 nmol/μg±15.5 nmol/μg and 343.93 nmol/μg±37.3 nmol/μg, p=0.001 and p=0.004, respectively).

Although we attempted to measure the amount of intracellular gemcitabine (GEM) taken up by cells, we could not quantify the GEM concentration because it was below the limit of detection.

Thus, we confirmed that GR cells have increased expression of SLC28A3 and SLC29A1, which are known as the principal transporters for GEM and deoxycytidine analog uptake. These results may suggest that both dCTP and GEM are efficiently taken up by GR cells and contribute to the internal pool (Figure R1-1d).

We compared the therapeutic response to 1μM GEM observed upon treatment with other nucleoside analogs, such as 2-deoxyadenosine monohydrate, 2-deoxyguanosine monohydrate, and thymidine, to that observed upon treatment with dCTP in WT cells. WT cells treated with dCTP at concentrations_of 10 μM or higher showed decreased GEM

sensitivity, while other nucleoside analogs did not show any molecular competition with GEM (Figure R1-1c).

Finally, we measured intracellular dCTP levels in WT and GR cells treated with or without dCTP (10 μM) and GEM (1 μM). CE-MS analysis revealed that the dCTP levels were largely maintained in GR cells and WT cells even under GEM treatment (approximately 2-3-fold). Indeed, there were no significant differences in viability upon treatment with various concentrations of GEM between GR cells and WT cells treated with 10 μM dCTP. (Figure R1-1e and 1f).

These results may support the evidence that the dCTP level was specifically increased in GR cells and that high baseline intracellular dCTP levels may contribute to molecular competition with GEM.

We added the above results and explanation as Revised Figure 2g-l and Figure EV1c in the Results section of our revised manuscript.

2) The authors claim that knocking down IDH2 restores GEM sensitivity. HOWEVER there is a problem in the pertaining panel Fig. 4C (maybe just a labeling problem?), because the graph indicates GR cells were used, but then in the graph there are WT and GEM cells. Is WT the non-resistant? Is GEM the same as GR? And then to which cell line does siIDH2 alone (empty triangles) refers to? In general, the data should be presented based on the two independent IDH2 siRNAs to exclude off-target effects.

Response) Thank you for your important suggestions. We apologize for the confusion generated by our showing both the GR and WT cell survival rates in the same graph. We changed the graph to show the viability of GR cells exposed to various concentrations of GEM for 48 hr after transfection with NTC and IDH2-targeting (siIDH2#1 and #2) siRNA (left: T24 cells; right: UMUC3 cells) (Revised Figure 4h).

We replaced Figure 4c with Revised Figure 4h in the Results section of our revised manuscript.

3) The authors use what they call "IDH2-I" inhibitor, which is in reality AG-221 or Enasidenib (please use its real name in the text and figures so that this is clear to anyone). However, this inhibitor was specifically developed against MUTATED neomorphic IDH2, and in the original publication they showed much reduced activity against the WT enzyme, with an in vitro IC50 on the purified enzyme of 1.8microM (10.1158/2159-8290.CD-16-1034). The authors should provide some direct evidence that the IDH2-1 is really inhibiting the WT enzyme in their cells at the 1microM dose they are using, otherwise it is very hard to explain

the effect of such drug.

Response) Thank you for your suggestions. As the reviewer pointed out, it is true that enasidenib has selectivity for IDH2 mutant cells compared to WT cells (Chen *et al*, 2020). In the literature, however, the IC50 is reported as 1.8 μM in WT IDH2 TF1 cells, which suggests that enasidenib would also show an appreciable cytotoxic effect on WT IDH2 cancer cells. Regarding UC cells, we found that the IC50 of enasidenib was 1.2 μM in T24GR cells and 1.9 μM in UMUC3GR cells, which was not greatly different from the reported IC50 of 1.8 μM in TF1 cells.

However, we agree with the reviewer's criticism that using enasidenib was not appropriate for demonstrating the therapeutic effect of IDH2 inhibition on GR cells, because enasidenib specifically targets neomorphic mutant IDH2 (mutant IDH2), but the current study focused on wild-type IDH2 (WT IDH2) gain-of-function-induced chemoresistance. Therefore, we repeated the *in vitro* and *in vivo* experiments with AGI6780 (an IDH2 inhibitor targeting both mutant and WT IDH2) in GR cell lines and the murine UC xenograft model (Revised Figure 7a-m).

To determine the pharmacological effect of an IDH2 inhibitor on GR cells, we used the IDH2 inhibitor AGI-6780, a chemical compound known to potently inhibit both mutant and WT IDH2. We evaluated its impact on cell viability and proliferation. We found that the IC50 values of AGI6780 were 6.4 μM in T24GR cells and 8.9 μM in UMUC3GR cells. Thus, we treated T24GR and UMUC3GR cells with 10 μM AGI6780 in combination with various concentrations of GEM to evaluate whether AGI6780 restores GEM sensitivity. A significant cytotoxic effect was observed with the combination of GEM and AGI6780 compared to GEM alone (Revised Figure 7a). Measurement of 2-HG production revealed significantly lower 2-HG levels in both GR cells with AGI6780 (0.632-fold lower in T24GR cells and 0.518-fold lower in UMUC3GR cells compared to vehicle control-treated cells) (Revised Figure 7b). Similarly, significantly lower dCTP levels were observed in GR cells treated with AGI6780 (0.529-fold lower in T24GR cells and 0.191-fold lower in UMUC3GR cells compared to vehicle control-treated cells) (Revised Figure 7c).

Regarding CDDP sensitivity, we further treated T24GR and UMUC3GR cells with 10 μM AGI6780 in combination with various concentrations of CDDP. Similar to the GEM experiment, AGI6780 combined with CDDP showed a significant cytotoxic effect on both GR cell lines (Revised Figure 7d). Less NADPH/NADP production and higher ROS generation were observed in the AGI6780-treated group (Revised Figure 7e and 7f).

To verify the therapeutic effect of AGI6780 *in vivo*, we performed an animal study to evaluate the therapeutic effects of AGI6780 alone and in combination with GEM. On Day 25

after the start of treatment, the tumor volume (mean ± standard deviation (SD)) in mice treated with AGI6780 alone was 360.0±43.9 mm3, which was significantly lower than that in mice treated with vehicle control (859.8±178.9 mm3, p=0.006). Furthermore, the tumor volume in mice treated with the combination of GEM and AGI6780 was 200.2±47.8 mm3, which was significantly lower than that in mice treated with GEM alone (758.4± 145.8 mm3, p=0.002) (Figure R1-2c or Revised Figure 7g). There were no apparent toxic effects, such as a decrease in body weight, in mice in any treatment group (Figure R1-2e or Revised Figure 7i). IHC revealed that the expression of IDH2, CAIX (a surrogate marker of hypoxia, similar to Hif1-α), TIGAR, TKT, and CTPS1 was decreased in the AGI6780 and GEM/AGI6780 combination treatment groups compared to the control group (Figure R1-2f or Revised Figure 7j). Likewise, similar results were obtained regarding AGI6780 in combination with CDDP in the murine UC xenograft model (the tumor volume (mean ±SD) on Day 25 in mice treated with CDDP and AGI6780 was 226.4±45.6mm3, which was significantly lower than that in mice treated with CDDP alone (658.0±100.1 mm3, p=0.001) (Figure R1-2g-i or Revised Figure 7k-m).

We added the above results and explanation as Revised Figure 7a-m in the Results section of our revised manuscript.

4) given the nice effects obtained in vivo with the IDH2-i, the authors should re-sequence IDH2 and IDH1 in their GR cells to test whether GEM treatment selected for mutant IDH. They only provide a single read of the genomic DNA, but it remains hard to understand where does this comes from. It is possible for example that the GR population is mixed, and that they only sequenced a single pick? Did the authors carefully checked the sequencing peaks for double peaks indicative of heterogeneous mutation in the cell population? In principle, all their phenotypes are consistent with mutant IDH, because GR cell lines show evidence for pseudo-hypoxia with HIF1a stabilization at 20% oxygen conditions (Fig 2F!!!!), which depends on IDH2 (Fig 4B!!!), and which other showed in many papers depends on the oncometabolite 2HG produced by the mutant IDH enzymes. This would also explain why these cells reduce glutamine anaplerosis（補充反応） - this is a typical hypoxic response. In addition, the authors should measure production of the 2HG oncometabolite in WT and GR cells, and use WT cells transfected with a mutated IDH2 as a control.

Response) Thank you for your insightful comment. We agree that as evident in our manuscript, it has a weakness in proving whether our cell lines have IDH mutations.

Thus, we performed whole-exome sequencing (WES) on DNA from four cell lines (T24WT, T24GR, UMUC3WT, and UMUC3GR), and the results are shown in Figure EV2. Several

genetic differences were found between WT and GR cells, but no IDH1/2 mutations were found by WES analysis of either GR cell line (Revised Figure EV2).

Although our current study focused on a phenotype consistent with that in mutant IDH2 cell lines, several recent articles have demonstrated that reductive TCA metabolic flux is observed after WT IDH2 gain of function, leading to cancer cell proliferation *in vitro and in vivo (Barnabas et al, 2021; Shi et al, 2020; Zeng et al, 2022).* According to these reports, the potential mechanisms of WT IDH2 involve aerobic glycolysis, glutamine-dependent reductive carboxylation, and/or 2-HG production. One novel study by Zeng et al. recently confirmed that WT IDH2 gain of function in acute myelocyte leukemia (AML) patients plays a critical role in activating reductive TCA metabolic flux and thus promotes the survival of AML cells and leads to poor prognosis (Zeng *et al.*, 2022). These new findings support our findings in that metabolic reprogramming may also begin with WT IDH2 gain of function and therefore stimulate aerobic glycolysis and PPP bypass via Hif1 stabilization, which leads to GEM resistance.

As the reviewer pointed out, evaluating 2-hydroxyglutarate (2-HG) production in GR cells is one of the key experiments to implicate IDH2-dependent Hif1-α stabilization. Many previous articles have demonstrated that IDH2 mediates 2-HG accumulation and stabilizes Hif1-α expression by suppressing the expression of EGLN/PHD family members, which generally hydroxylate Hif1-α and lead to its degradation under normoxic conditions (Du & Hu, 2021).

To confirm the levels of 2-HG production, we measured 2-HG levels in both WT and GR cells (Figure R1-3a). We found that 2-HG production was certainly increased in GR cells compared to WT cells in both cell lines. Specifically, T24GR cells produced 2.15 times more 2-HG than the corresponding WT cells ($p=0.027$), while UMUC3GR cells produced 3.51 times more 2-HG than the corresponding WT cells ($p=0.008$). Comparison of WT cells with and without transfection of IDH2 (T24WT IDH2ox and UMUC3WT IDH2ox) indicated significant increases in 2-HG in both cell lines (T24WT IDH2ox cells showed a 2.94-fold higher 2-HG level than T24WT cells; UMUC3WT IDH2ox cells showed a 6.40-fold higher 2-HG level than UMUC3WT cells). Likewise, WT IDH2-overexpressing J82 (a third UC cell line used for IDH2 overexpression) cells (J82-IDH2ox cells) also showed a significant increase in 2-HG production compared to the corresponding WT cells (4.27-fold higher 2-HG level) (Figure R1-3b).

Next, we conducted RT-PCR and WB analyses to determine whether PHD2 expression is suppressed in GR cells. The results are shown in Figures R1-3c and 3d. We confirmed that EGLN1 (the gene encoding PHD2) was significantly suppressed in GR cells. WB analysis also showed a significant decrease in PHD2 protein expression. In cells with IDH2

downregulation, we confirmed that the significantly higher level of EGLN1 in both GR cell lines led to a decrease in Hif1-α expression (Figure R1-3e and 3f).

These results suggest that GR cells demonstrate WT IDH2 gain of function-mediated reductive carboxylation, which leads to increased 2-HG production and results in Hif1-α stabilization via suppression of EGLN1/PHD2 expression.

We added the above results and explanation as Revised Figure EV2 and Revised Figure 3f, 3h, and 3i in the Results section of our revised manuscript.

5) Whatever the underlying mechanism, it would be important to provide some functional data on the relevance of HIF1a stabilization for the main phenotypes observed here, including enzyme expression, GEM and CDDP sensitivity in cells with HIF1a knockdown. If so, then the title should shift from IDH2 to WT IDH2-dependent stabilization of HIF1a.

Response) Thank you for your important suggestions. As shown by the above results, we found that WT IDH2 gain of function stimulates Hif1-α expression via 2-HG production.

To clarify the relationship between Hif1-α expression and chemoresistance, we further conducted Hif1-α knockdown by using HIF1A-targeting siRNAs (siHIF1A#1: s6539 and siHiF1A#2: s6541) in both GR cell lines. The mRNA expression levels of glycolytic enzymes (GLUT1, TIGAR, G6PD, TKT, CTPS1, PKM2, and LDHA) decreased significantly with HIF1A downregulation. Similar results were obtained in the WB analysis, indicating that the expression of key metabolic enzymes (TIGAR, TKT, and CTPS1) involved in glycolysis and the PPP shift was significantly suppressed by HIF1A downregulation. Finally, we confirmed that HIF1A knockdown GR cells showed clear restoration of the cytotoxic effect with GEM administration. Treating cells with the combination of 1 μM digoxin (which is known as a Hif1-α inhibitor) and GEM revealed that GEM sensitivity was restored in both T24GR and UMUCGR cells (Figure R1-4 a-d).

These results indicate that HIF1-α expression under normoxic conditions is regulated by IDH2 expression, which suggests that all the observed metabolic reprogramming results from WT IDH2-dependent stabilization of HIF1-α. Given the findings in 4) and 5), we agree with the reviewer that all the observed metabolic reprogramming results from WT IDH2-dependent stabilization of HIF1-α.

Thus, we changed our main title to "**Wild-Type IDH2-dependent Stabilization of HIF1a Induces Metabolic Reprogramming for Acquisition of Chemoresistance in Urothelial Carcinoma**".

We added the above results and explanation as Revised Figure 4a-c in the Results section of our revised manuscript.

6) The experiment in 4e lacks of the effect of IDH2-i alone, which is fundamental to understand whether the combination effect is due to sensitization to GEM, as claimed by the authors, or just to IDH2-i alone.

Response) Thank you for your suggestions; we apologize for not showing the effect of the IDH2 inhibitor alone in the figure. We repeated the *in vivo* experiment with AGI6780 (an IDH2 inhibitor targeting both mutant and wild-type IDH2) in the murine UC xenograft model. The results are shown in Revised Figure 7g-m and include the effect of AGI6780 alone.

7) Data on cross-sensitivity to CDDP can make sense in light of the observed metabolic rewiring. However, the experiment showing that IDH2 inhibition also rescues this phenotype is hard to swallow - or at least requires an explanation to the reviewer and to the audience - in light of the fact that IDH2 uses up NADPH in the aKG/isocitrate reverse reaction, so its inhibition should provide more NADPH to cells, and more GSH, not less. Very likely, this is because IDH2 inhibition indirectly (i.e. by regulating mRNA expression of PPP enzymes) brings back the PPP to low levels observed in the WT cells, and as a consequence the PPP does not support NADPH production. This model can be easily demonstrated by showing that PPP inhibition should sensitize more GR cells than WT cells to CDDP treatment.

Response) Thank you for your insightful comment. We observed that the whole-cell NADPH levels and GSH levels were significantly higher in both lines of GR cells than in the corresponding WT cells. As the reviewer raised this critical point, we asked why the total NADPH level was increased in GR cells, since IDH2 consumes NADPH in the reverse reaction converting αKG to isocitrate. However, we appreciate the reviewer's keen insight that NADPH is mainly generated via the oxidative PPP, which is the major source of cytosolic NADPH (Chen et al, 2019). In fact, G6PD was overexpressed in GR cells compared to WT cells (Revised Figure 2e and 2f).

Considering the reviewer's insightful comment, we used a G6PD inhibitor (G6PD-i), which is known as a major PPP inhibitor, to confirm whether NADPH is dependent on the oxidative PPP. Surprisingly, we found that CDDP sensitivity was restored in both GR cell lines by combined treatment with the G6PD inhibitor and CDDP (Figure R1-5a and R1-5b). Moreover, we confirmed that the NADPH/NADP ratio and GSH level in GR cells were significantly decreased by G6PD-I administration, which suggests that NADPH production results mostly from oxidative PPP stimulation (Figure R1-5c-e). On the other hand, as Figure R1-5b shows, we did not find significant effects on GEM sensitivity by adding G6PD-i

to GEM in GR cells. This result may suggest that GEM resistance is also dependent upon the nonoxidative PPP, which is stimulated by TKT and TIGAR expression.

These results explain the mechanisms of the NADPH increase in GR cells and indicate how they contribute to cross-sensitivity to CDDP. We found that the increase in the NADPH/NADP ratio resulting from PPP stimulation may contribute to further GSH production and decrease reactive oxygen species (ROS) generation, which reduces the therapeutic effect of CDDP by reinforcing the antioxidant defense (Revised Figure 6e).

We added the above results and explanation as Revised Figure 6e and Revised Figure EV4a-e in the Results section of our revised manuscript.

8) However, the experiment on CDDP also raises (again - see above the comment of IDH2 mutation) the issue that everything seems to start from IDH2, including rewiring of the PPP pathway. If IDH2 is really wild-type in these cells, then two main questions remain unanswered here:
1) what determines the use of glutamine in the reverse IDH2 reaction in GR cells? If it is an issue of expression levels, then is over expression of WT IDH2 sufficient to recapitulate such whole metabolic rewiring?
2) how does reverse IDH2 functioning (but in absence of 2HG production) stabilizes HIF1a and regulates transcription, so that it also rewires glycolysis and the PPP pathway?
Otherwise, the authors cannot provide an explanation for such "mimicry" of mutant IDH2 by a WT IDH2 enzyme, which is ultimately one of the main novelties of the paper.

Response) Thank you for pointing out the most critical and the principal finding that our study should emphasize. As we discussed above, several articles have revealed the metabolic association between WT IDH2 gain of function and reductive glutamine metabolism under normoxia conditions. Reductive glutamine metabolism is suspected to be induced by WT IDH2 in breast cancer and AML (Barnabas *et al.*, 2021; Zeng *et al.*, 2022) and could thus be a potential therapeutic target. To clarify whether IDH2 overexpression induces metabolic reprogramming, we performed a gain-of-function experiment by overexpressing WT IDH2 in J82 cells, which exhibit low IDH2 expression. The results are shown in Figure R1-6. CE-MS metabolome analysis revealed that WT IDH2-overexpressing J82 (J82-IDH2ox) cells showed significant increases in glutamine, α-KG, isocitrate and citrate levels. The labeling analysis also showed increased α-KG (+5) and decreased malate (+4) levels, suggesting clear activity of reductive glutamine metabolism. Furthermore, RT–PCR and WB analyses showed increases in IDH2 and Hif1-α expression along with 2-HG accumulation

(Figure R1-6e). CE-MS metabolome analysis revealed that the intermediate metabolites of glycolysis and PPP intermediates were enhanced in IDH2ox cells (Figure R1-6h and 6i). Finally, a significantly high level of dCTP was found in J82-IDH2ox cells (Figure R1-6j), and compared to WT cells, J82-IDH2ox cells showed GEM resistance (Figure R1-6k).

The current results demonstrate that WT IDH2 overexpression recapitulates the reductive TCA cycle, stabilizing Hif1-α expression via 2-HG production and resulting in stimulation of aerobic glycolysis and PPP bypass. Our study revealed that 2-HG production was increased in GR cells, and this phenomenon was consistent with the observations in J82-IDH2ox cells. Therefore, we hypothesize that WT IDH2 gain of function stabilizes Hif1-α expression by increasing 2-HG production via metabolic reprogramming to glutamine-dependent reductive metabolism. Stabilized Hif1-α under normoxic conditions promotes the expression of glycolytic and PPP-related enzymes, which leads to stimulation of aerobic glycolysis and the PPP. We consider this to be the mechanism by which UC cells acquire chemoresistance to adapt themselves for maintained survival.

We added the above results and explanation as Revised Figure 5a-k and Revised Figure EV5a-f in the Results section of our revised manuscript.

Reviewer1: minor

Minor:

1）To test for increased glycolysis, they test growth in hypoxia. The authors should just measure OCR/ECAR in standard conditions and show whether the ability of cells to perform glycolysi is different.

Response) Thank you for your suggestion. We conducted XF24 extracellular flux analysis to measure the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) and compare these rates between WT and GR cells. In comparison with the WT cell lines, GR cells demonstrated an increased ECAR and OCR. As a result, GR cells showed a lower OCR/ECR ratio than WT cells (T24: OCR/ECR, 5.35 ±1.98 in WT cells and 3.45 ±0.75 in GR cells. UMUC3: OCR/ECR, 5.23 ±2.69 in WT cells and 2.40 ±1.47 in GR cells). These results suggest that aerobic glycolysis is upregulated in GR cells compared with WT cells (Figure R1-7a-c).

We added the above results and explanation as Revised Figures 1f and 1g in the Results section and added the following details in the Materials and Methods section of our revised manuscript.

XF24 Extracellular Flux Analysis

The extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) analyses were performed with an XF24 extracellular flux analyzer (Seahorse Biosciences, North Billerica, MA). A total of 4.0*104 cells per well were seeded in 24-well cell culture plates (Seahorse Biosciences, North Billerica, MA) in RPMI 1640 medium supplemented with 10% FBS and incubated at 37 °C overnight in 5% CO2 incubator. The next day, the growth medium was replaced with bicarbonate-free RPMI 1640 medium, and the cells were incubated at 37 °C for 1 hr in a CO2-free incubator to equilibrate the medium temperature and pH. By utilizing a Seahorse XF24 analyzer, ECAR and OCR were measured under baseline conditions and under treatment with 2-deoxyglucose (2-DG; 50 μM) and rotenone (1 μM). The values are presented as the means ± standard errors of the mean.

2)Figure 2b, is sigmaPPP a mislabeling of PRPP? If not, a direct measure of the PRPP precursor of dNTPs would be helpful.

Response) Thank you for your suggestion. We aimed to show ΣPPP as the summed content of intermediates of the PPP metabolites shown in the graph, but this presentation was confusing. We deleted the sigma PPP data, since this value is likely to confuse the reader. Instead, we added the amount of PRPP to all graphs (Revised Figure 2b, Revised Figure 5i, and Figure EV5e) so that it could help to describe the stimulation of nucleoside biosynthesis.

3)Figure 2c and 2d: the panels just show the endpoint purine and pyrimidine compounds, not the biosynthetic intermediates. This should be made clear in the figure and text.

Response) Thank you for your suggestion. We agree with the reviewer's comment and therefore changed the sentence and graphs in all figures to show only the end product purine and pyrimidine compounds.

4)The tracing of glutamine is coherent with the model, but the observation that the contribution of glutamine carbons to cytoplasmic malate is increased bears the question of whether the aKG/isocitrate reverse reaction occurs in mitochondria or (also) in the cytosol via IDH1. Why the authors exclude this? Increased mRNA expression of IDH2 but not IDH1 is weak, because enzymes often work more/less without the need to change their expression levels.

Response)

Thank you for your response; this is a challenging question to answer at this stage. Although our results demonstrated the induction of reductive glutamine metabolism by IDH2 gain of function, we could not specify how IDH1 functions to stimulate or suppress this phenomenon. We attempted to use an IDH1 inhibitor (AGI5198) for preliminary experiments and found a lower therapeutic effect induced on GR cells by this inhibitor than by an IDH2 inhibitor (AGI6780). On the other hand, AGI5198 reduced the NADPH/NADP ratio and GSH level in GR cells to some degree (Figure R1-7c-e). Thus, we agree that the interaction between mitochondrial IDH2 and cytosolic IDH1 contributes to reductive carboxylation and overall metabolic reprogramming, influencing chemoresistance in UC cells. In the current study, however, we could not determine the degree to which these two key metabolic molecules interact to restore chemoresistance.

Since the interaction of AGI5198 and AGI6780 becomes complicated and would need much more discussion, we declare this point as a limitation and would like to leave clarification of the mechanism by which UC cells acquire chemoresistance as the next major topic.

In the methods:

- provide the technique used to calculate relative gene expression levels based on qPCR

Response) Thank you for your comment. We provided the details of the approach for calculating gene expression based on qPCR in the Methods section of our revised manuscript.

Gene Expression Analysis by Real-Time PCR (qPCR)

Total RNA was isolated by using RNA easy columns (QIAGEN) following the manufacturer's instructions. Total RNA was reverse transcribed to cDNA by using a High Capacity Reverse Transcription Kit (Applied Biosystems, Tokyo, Japan). qPCR was performed with gene-specific primers with 40 cycles at 95 °C for 10 sec and 60 °C for 60 sec in a 10 μL reaction mixture containing 1 μL of cDNA, 1 μL of each primer and 8 μL of TaqMan® Fast Universal PCR Master Mix (2×) (Applied Biosystems, Waltham, MA, US). Beta-actin was used as the internal control. Quantification was performed with the ΔΔCt method (Schmittgen *et al*, 2008). The primers used for reverse transcription and amplification are listed in the Supplementary Table.

- provide the sequences of the siRNAs used in the manuscript in full

Response) Thank you for your suggestion. We used 5 types of siRNAs, including a

nontargeting control, and we added the information shown below to the Methods section.

1.siRNA non-targeting control (AM4611)
sense: 5’-UAGCGACUAAACACAUCAA-3’
antisense: 5'-UUGAUGUGUUUAGUCGCUA- 3'
2.siRNA#1(s6539) HIF1A
sense: 5’-CCAUAUAGAGAUACUCAAA-3’
antisense: 5’-UUUGAGUAUCUCUAUAUGG-3
3.siRNA#2(s6541) HIF1A
sense: 5’-CCUCAGUGUGGGUAUAAGA-3’
antisense: 5’-UCUUAUACCCACACUGAGG-3’
4. siRNA#2(s227) IDH2
sense: 5’-CAAGAACACCAUACUGAAA-3’
antisense: 5’-UUUCAGUAUGGUGUUCUUG-3’
5. siRNA#2(106706) IDH2
sense: 5’-GGAGCAGUGCGUUUUACCU-3’
antisense: 5’AGGUAAAACGCACUGCUCCtg-3’

Referee #2:

Main concern:

1) *The ability of T24GR and UMUC3GR cells to proliferate under hypoxia (Figure 1E) does not confirm glycolytic dependency in these cells versus the parental or CR counterparts. As an alternative, the impact of glucose limitation/deprivation on proliferation should be examined.*

Response) Thank you for the comment. We cultured cells under glucose limitation and deprivation conditions. We compared the survival of three types (WT, CR, and GR) of two cell lines after 72 hr under these conditions (Figure R2-1a). For the glucose limitation medium, we used 1000 mg/L D-glucose, half the concentration of glucose in standard medium (2000 mg/L D-glucose). The glucose deprivation medium contained 0mg/L D-glucose. We confirmed that GR cells showed tolerance under both glucose limitation and glucose deprivation conditions in both cell lines, while CR and WT cells could not survive**. From this result, we assumed that GR cells were able to survive without glucose since glutamine metabolism was stimulated as an alternative source of energy production.** Thus, we also evaluated the change in cell survival under glutamine deprivation conditions. We confirmed that GR cells still showed higher cell survival rates than CR/WT cells in glucose+/glutamine- (glu+ gln-) medium. However, no parental or resistant cells could survive under both glucose- and glutamine deprivation.

From these results, we confirmed that different metabolic mechanisms are used for survival in GR cells, suggesting the induction of both glucose and glutamine dependence relative to CR and WT cells.

We added the above results and explanation as Revised Figure 1e in the Results section of our revised manuscript.

2) The rationale for examining hypoxic conditions in Figure 1E/2E/2F/2G is lacking and confuses the story. Either the rationale should be explained, and hypoxic conditions should be included in a greater number of experiments, or hypoxic conditions should not be included in any of the experiments.

Response) Thank you for your suggestion. We agree with the reviewer that Figure 1E/2E/2F/2G would confuse the readers. Given the reviewer's opinion, we deleted the hypoxia data from Figure 1E and Figure 2e to prevent confusion. In Figure 2F, since the key transcription factor Hlf1-α is often degraded under normoxic conditions during collection of the lysate, we continue to show the WB results in 21% O_2 and 1% O_2. In the rest of the

figures, we decided not to include any data under hypoxic conditions.

We deleted Figure 1E/2G and replaced Figure 2E with Revised Figure 2e in the Results section of our revised manuscript.

3) The authors suggest that increased pyrimidine synthesis contributes to gemcitabine resistance but have not provided sufficient evidence to support this conclusion. For example, the authors should examine the impact of a pyrimidine synthesis inhibitor (e.g. leflunomide) and a purine synthesis inhibitor (e.g. mizoribine) on the cellular response to gemcitabine.

Response) Thank you for the insightful comment. We agree that our conclusion that the association between increased pyrimidine synthesis contributes to GEM resistance is weak. As the reviewer pointed out, we examined the cytotoxic effect of a pyrimidine synthesis inhibitor (leflunomide) and a purine synthesis inhibitor (mizoribine) combined with GEM in GR cells. The results are shown in Figure R2-1b. There were no significant differences in cell survival between WT and GR cells treated with leflunomide or mizoribine alone. Since the IC50 values of leflunomide and mizoribine were 22.1 μM and 20.1 μM, respectively, in T24GR cells, we examined the combination of 25 μM leflunomide with GEM, and showed a significant decrease in cell survival in combination with 1 μM GEM (T24GR cell viability rate with 1 μM GEM vs. 1 μM GEM combined with 25μM leflunomide: 86.1±6.1% vs. 28.1±6.8%, $p<0.001$). In contrast, compared to1 μM GEM alone, the combination of 25 μM mizoribine with 1 μM GEM did not significantly affect the cell viability rate (T24GR cell viability rate with 1μM GEM vs. 1μM GEM combined with 25μM leflunomide: 80.9±6.4% vs. 67.7±9.2%). Similar results were obtained in UMUC3GR cells (Figure R2-1c and 1d).

These results demonstrate that pyrimidine synthesis is more dependent upon the cellular response to GEM than is purine synthesis, suggesting that increased pyrimidine synthesis contributes to GEM resistance.

We added the above results and explanation as Revised Figure 2g and 2h, Figure EV1a and EV1b in the Results section of our revised manuscript.

4) The authors use the terms deoxycytidine and dCTP (deoxycytidine triphosphate) interchangeably and this has made the experiments in Figure 2G/H difficult to interpret. Please confirm if cells were supplemented with deoxycytidine or dCTP in Figure 2g and whether deoxycytidine or dCTP was measured in Figure 2h.

Response) Thank you for your insightful comment. We apologize for the confusion in Figures 2g and 2h. First, the drug supplemented was 2'-deoxycytidine-5-triphosphate

(dCTP).

Since previous articles have reported that GEM participates in molecular competition with intracellular dCTP, we treated WT cells with dCTP to determine whether this compound may show molecular competition with GEM.

To clarify the relationship between the dCTP increase and GEM resistance, we took a few steps. First, by CE-MS metabolome analysis, we found that compared to those of other nucleosides, the level of dCTP was specifically increased in GR cells (Figure R1-1a). We next measured the baseline dCTP levels in WT and GR cells. The results are shown in Figure R1-1b. The baseline dCTP levels were significantly higher in T24GR and UMUC3GR cells (447.65 nmol/μg±17.8 nmol/μg and 584.65 nmol/μg±53.2 nmol/μg, respectively) than in T24WT and UMUC3WT cells (114.59 nmol/μg±15.5 nmol/μg and 343.93 nmol/μg±37.3 nmol/μg, p=0.001 and p=0.004).

We further compared the therapeutic response to 1μM GEM observed upon treatment with other nucleoside analogs, such as 2-deoxyadenosine monohydrate, 2-deoxyguanosine monohydrate, and thymidine, to that observed upon treatment with dCTP in WT cells. We found that WT cells treated with dCTP at concentrations higher than 10 μM dCTP showed decreased GEM sensitivity, while other nucleoside analogs did not show any molecular competition with GEM (Figure R1-1c).

We also measured intracellular dCTP levels in WT and GR cells treated with and without dCTP and GEM. CE-MS analysis revealed that the dCTP level was largely maintained in GR cells compared to WT cells even under GEM treatment. Indeed, there were no significant differences in viability with GEM concentration between GR cells and WT cells treated with 10 μM dCTP. (Figure R1-1e and 1f)

These results may support the evidence that the dCTP level is specifically increased in GR cells and that a high baseline intracellular dCTP level may contribute to molecular competition with GEM.

We added the above results and explanation as Revised Figure 2g-l and Figure EV1c in the Results section of our revised manuscript.

5) Given the authors demonstrate glutamine carbon contributes to reductive carboxylation in T24GR and UMUC3GR cells (Figure 3D), the authors should determine the impact of glutamine deprivation and/or glutaminase inhibitor treatment on cellular sensitivity to gemcitabine.

Response) Thank you for the comment. As the reviewer pointed out, we first confirmed the impact of glutamine deprivation on cellular sensitivity to GEM. The results are shown in

Figure R2-1e. The results showed decreased survival of GR cells under glutamine deprivation conditions. We also confirmed that GEM sensitivity was restored by treatment with a glutaminase inhibitor. We used the glutaminase inhibitor BPTES (10 μM) and found that BPTES in combination with GEM restored GEM sensitivity to some degree (Figure R2-1f).

From these results, we confirmed that glutamine metabolism contributes to GEM resistance stimulated by WT IDH2 gain of function. However, GEM sensitivity was only partially restored under these conditions, probably because GR cells also show increased glucose flux (as Revised Figure 1e-g shows); therefore, the core mechanism of GEM resistance in UC cells is dependent on the metabolism of both glutamine and glucose.

6) Figures 3-6 relate to the contribution of IDH2 to chemotherapy resistance in UC. This focus stems from the observation that IDH2 is more highly expressed in gemcitabine-resistant UC cell lines (T24GR and UMUC3GR) than parental UC cell lines (T24 and UMUC3). The authors employ enasidenib (AG-221) to demonstrate that IDH2 inhibition sensitizes T24GR and UMUC3GR cells to gemcitabine in vitro and in vivo. However, given that the cell lines do not harbour IDH2 neomorphic mutations (Supp Fig 1) and enasidenib is a selective inhibitor of mutant IDH2, these results are unexpected. The onus is on the authors to show that, at the doses employed in this study, AG-221 inhibits wild-type IDH2 in the UC cell lines.

Response) Thank you for your suggestions. As the reviewer pointed out, it is true that enasidenib has selectivity for IDH2 mutant cells compared to WT cells (Chen *et al.*, 2020). In the literature, however, the IC50 is reported as 1.8 μM in WT IDH2 TF1 cells, which suggests that enasidenib would also show an appreciable cytotoxic effect on WT IDH2 cancer cells. Regarding UC cells, we found that the IC50 of enasidenib was 1.2 μM in T24GR cells and 1.9 μM in UMUC3GR cells, which was not greatly different from the reported IC50 of 1.8 μM in TF1 cells.

However, we agree with the reviewer's criticism that using enasidenib was not appropriate for demonstrating the therapeutic effect of IDH2 inhibition on GR cells, because enasidenib specifically targets neomorphic mutant IDH2 (mutant IDH2), but the current study focused on wild-type IDH2 (WT IDH2) gain-of-function-induced chemoresistance. Therefore, we repeated the *in vitro* and *in vivo* experiments with AGI6780 (an IDH2 inhibitor targeting both mutant and WT IDH2) in GR cell lines and the murine UC xenograft model (Revised Figure 7a-m).

To determine the pharmacological effect of an IDH2 inhibitor on GR cells, we used the IDH2 inhibitor AGI-6780, a chemical compound known to potently inhibit both mutant and WT IDH2. We evaluated its impact on cell viability and proliferation. We found that the IC50 values of AGI6780 were 6.4 μM in T24GR cells and 8.9 μM in UMUC3GR cells. Thus, we treated T24GR and UMUC3GR cells with 10 μM AGI6780 in combination with various concentrations of GEM to evaluate whether AGI6780 restores GEM sensitivity. A significant cytotoxic effect was observed with the combination of GEM and AGI6780 compared to GEM alone (Revised Figure 7a). Measurement of 2-HG production revealed significantly lower 2-HG levels in both GR cells with AGI6780 (0.632-fold lower in T24GR cells and 0.518-fold lower in UMUC3GR cells compared to vehicle control-treated cells) (Revised Figure 7b). Similarly, significantly lower dCTP levels were observed in GR cells treated with AGI6780 (0.529-fold lower in T24GR cells and 0.191-fold lower in UMUC3GR cells compared to vehicle control-treated cells) (Revised Figure 7c).

Regarding CDDP sensitivity, we further treated T24GR and UMUC3GR cells with 10 μM AGI6780 in combination with various concentrations of CDDP. Similar to the GEM experiment, AGI6780 combined with CDDP showed a significant cytotoxic effect on both GR cell lines (Revised Figure 7d). Less NADPH/NADP production and higher ROS generation were observed in the AGI6780-treated group (Revised Figure 7e and 7f).

To verify the therapeutic effect of AGI6780 *in vivo*, we performed an animal study to evaluate the therapeutic effects of AGI6780 alone and in combination with GEM. On Day 25 after the start of treatment, the tumor volume (mean ± standard deviation (SD)) in mice treated with AGI6780 alone was 360.0±43.9 mm3, which was significantly lower than that in mice treated with vehicle control (859.8±178.9 mm3, p=0.006). Furthermore, the tumor volume in mice treated with the combination of GEM and AGI6780 was 200.2±47.8 mm3, which was significantly lower than that in mice treated with GEM alone (758.4± 145.8 mm3, p=0.002) (Figure R1-2c or Revised Figure 7g). There were no apparent toxic effects, such as a decrease in body weight, in mice in any treatment group (Figure R1-2e or Revised Figure 7i). IHC revealed that the expression of IDH2, CAIX (a surrogate marker of hypoxia, similar to Hif1-α), TIGAR, TKT, and CTPS1 was decreased in the AGI6780 and GEM/AGI6780 combination treatment groups compared to the control group (Figure R1-2f or Revised Figure 7j). Likewise, similar results were obtained regarding AGI6780 in combination with CDDP in the murine UC xenograft model (the tumor volume (mean ±SD) on Day 25 in mice treated with CDDP and AGI6780 was 226.4±45.6mm3, which was significantly lower than that in mice treated with CDDP alone (658.0±100.1 mm3, p=0.001) (Figure R1-2g-i or Revised Figure 7k-m).

We added the above results and explanation as Revised Figure 7a-m in the Results

section of our revised manuscript.

7) *In Figure 5H/K, DCF is employed to measure ROS. However, changes in DCF fluorescence are not specific to changes in ROS levels and can occur in response to loss of cell viability. A more specific measure of ROS should be employed (e.g. CellROX).*

Response) Thank you for the comment. We re-evaluated intracellular ROS levels by using CellROX Orange (C10443; Invitrogen, USA). ROS levels were quantified by measuring the fluorescence at 545/565 nm with a 2300 EnSpire Multilabel Plate Reader (PerkinElmer, Waltham, MA, US).
Figure 5h and 5K were modified to Revised Figure 6h and 6k according to the quantitative amounts determined by CellROX Orange staining. Similar to the previous findings, a significant increase in ROS levels was observed in T24WT and UMUC3 WT cells under treatment with 10 μM CDDP, while the increase was nonsignificant in GR cell lines. However, with pharmacological IDH2 inhibition or IDH2 downregulation, significant increases in ROS levels compared to those in the control GR cells were observed (Revised Figure 6k and Revised Figure 7f).

8) *The authors provide evidence that GSH levels are higher in GR cells and suggest that the decrease in GSH observed following IDH depletion increases sensitivity to cisplatin. The authors should determine whether or not inhibition of GSH synthesis by buthionine sulfoximine (BSO) sensitizes GR cells to cisplatin.*

Response) Thank you for your comment. We added the results of buthionine sulfoximine treatment (BSO) in both GR cell lines. We found a significant cytotoxic effect on GR cells treated with the combination of CDDP and 50 μM BSO (Figure R2-2a). **This result suggests that GSH plays a critical role as a strong reducing agent in the development of CDDP resistance.**

9) Both IDH2 siRNA constructs should be employed in Figure 4c and Figure 5i/k.
Response) Thank you for your comment. We added the results related to siIDH2#1 and siIDH2#2 transfection to the NADPH, GSH, and ROS measurements (Figure R2-2b to 2f).

We replaced Revised Figure 4c to Revised Figure 4h, Figure 5i to Revised Figure 6 i-l in the Results section of our revised manuscript.

Review2: minor

Minor concerns that should be addressed are outlined below:

• The authors should explain what sigmaPPP and sigmaTCA represent in Figure 2B and 3A respectively. How were these values calculated?

Response) Thank you for your comment. Sigma PPP and sigma TCA show the summed content of intermediate metabolites of the PPP and TCA cycle determined by CE-MS. Since these parameters are less common than other measures and may confuse the readers, we decided to delete them.

Referee #3:

Main concern:

1. *The authors passage two cell lines for 12 - 18 months in genotoxic drugs to generate chemoresistant cells. Does this treatment lead to reproducible phenotypes? Were the control cells passaged over the same period of time?*

Response) Thank you for the suggestion. GR cells were cultured with increasing concentrations of GEM over a period of approximately 18 months. At the same time, we cultured control cells (T24WT and UMUC3WT) for the same amount of time. By carefully following the procedure described by Shukla et al (Shukla *et al*, 2017), we determined the resistance status at each treatment by calculating the IC50 of GEM using MTT cytotoxicity assays, as shown in Figure 1b.

2. The manuscript including the title focuses on IDH2, but the connection of IDH2 to observed metabolic changes is often unclear. Why does IDH2 depletion reduce levels of glycolytic enzymes? Does overexpression of IDH2 in control cells recapitulate the metabolic phenotypes of resistant cells? The authors propose that the IDH1/2 shuttle generates cytosolic NADPH for GSH synthesis - do IDH1 and IDH2 inhibitors produce similar phenotypes? Does IDH2 inhibition cause the changes in intermediates of glycolysis and the PPP, which are proposed to confer gemcitabine resistance?

Response) Thank you for the insightful comments. As we first report in the current study, **we propose that WT IDH2 gain of function stimulates reductive glutamine metabolism to induce accumulation of 2-hydroxyglutarate (HG), which is an oncometabolite, to stabilize Hif1-α expression. Our story then proceeds to discuss the aerobic glycolysis and PPP bypass stimulation induced by Hlf1-α stabilization, resulting in an increase in the intracellular dCTP level for chemoresistance acquisition.** To confirm the entire phenomenon, we conducted WT IDH2 gain-of-function experiments by overexpressing IDH2 in J82 cells, a UC cell line without IDH2 gene mutation (Figure R1-6). By conducting CE-MS metabolome analysis, we found that J82-IDH2ox cells (IDH2-overexpressing cells; Figure R1-6a and 6b) showed increased α-KG and citrate production, which an indicator of reductive glutamine metabolism. Indeed, 2-HG production was significantly increased in J82-IDH2ox cells compared to the corresponding WT cells (Figure R1-6g). J82-IDH2ox cells also showed significant increases in the levels of intermediate metabolites involved in glycolysis and the PPP, showing the stimulation of aerobic glycolysis and PPP bypass. The

RT–PCR and WB results also supported the increased expression of key enzymes, including Hif1-α, G6PD, TIGAR, TKT, and CTPS1, which are crucial for PPP bypass and for specific pyrimidine biosynthesis. Finally, we confirmed the significantly high level of dCTP, which contributes to the molecular competition with GEM, in J82-IDH2 cells.

These data support that overexpression of WT IDH2 may reproduce the metabolic phenotypes of GR cells (Figure R1-6).

We further conducted metabolome analysis comparing the intermediate metabolites of the TCA cycle, glycolysis and PPP between J82-IDH2ox cells treated with and without the IDH2 inhibitor AGI6780. We found that the levels of most intermediates of glycolysis and the PPP pathway were decreased by AGI6780 treatment (Figure R3-1). In addition, a decrease in the dCTP level was observed after AGI6780 treatment (Figure R3-1d).

Regarding IDH1 and IDH2 inhibitors, the largest difference between IDH1 and IDH2 inhibitors was that the IDH-1 inhibitor (AGI5198) did not show a significant cytotoxic effect on GR cells, while a significant cytotoxic effect was observed for the IDH2 inhibitor in combination with GEM (Figure R1-7c). We measured the NADPH/NADP ratio after treatment with IDH1 and IDH2 inhibitors (AGI5198 and AGI6780, respectively). We found that both inhibitors decreased the NADPH/NADP ratio in T24GR and UMUC3GR cells (Figure 1-7d). Likewise, the GSH level also showed a significant decrease with both AGI5198 and AGI6780 (Figure R1-7e). These data indicate that NADPH production mainly relies on cytosolic NADPH production, as previously reported by Chen et al. (Chen *et al.*, 2019).

From these experiments, we confirmed two crucial points**. First, the WT IDH2 gain-of-function experiment revealed that IDH2 overexpression contributes to reprogramming the entire cell metabolism by stimulating reductive glutamine metabolism along with aerobic glycolysis and PPP bypass in UC cell lines. Second, the increases in intracellular reducing agents such as NADPH and/or GSH result from oxidative PPP activity. Thus, IDH2 inhibition suppresses overall metabolic reprogramming and decreases the antioxidant ability, which results in sensitization of chemoresistant cells.**

We added the above results and explanation as Revised Figure 5a-k and Figure EV5a-f in the Results section of our revised manuscript.

3. The authors demonstrate increased expression of multiple metabolic enzymes in resistant cells (glycolysis, PPP, TCA cycle) and conclude that these changes increase pathway flux and mediate therapy resistance. With the exception of genetic / pharmacological inhibition of IDH2, these data are entirely correlative and should be supported by data where enzyme

levels are experimentally increased or decreased. This is critical to establish functional relevance, because culturing cells in chemotherapeutics for over a year likely induces many other alterations.

Response) Thank you for the comment. To prove the entire metabolic reprogramming phenomenon in UC cells with GEM resistance, we took three steps.

1) We first evaluated the functional relationship between Hif1-α and chemoresistance. We demonstrated the expression of key enzymes in GR cells with HIF1a knockdown (Figure R1-4). We transfected GR cells with two siRNAs (siHif1A#1 and siHif1A#2) and found that the expression of key enzymes, including TIGAR, TKT, and CTPS1, was significantly decreased at both the mRNA and protein levels. We also confirmed that GEM sensitivity was restored with Hif1A knockdown in GR cells (Figure R1-4c). We also found that treatment with 1 μM digoxin (a representative Hif1-α inhibitor) combined with GEM showed significant cytotoxicity in GR cells (Figure R1-4d). Since GR cells showed stabilized Hif1-α expression even in an environment of 20% O_2 (Figure 2f), we found that Hif1-α regulates downstream metabolic enzymes to stimulate aerobic glycolysis and the PPP.

2) We conducted whole-exome sequencing (WES) of DNA from T24WT/GR and UMUC3WT/GR cells to confirm whether these cells harbored IDH1/2 mutations. Although several differences were confirmed between WT and GR cells, no mutations were found in the IDH1 or IDH2 gene (Figure EV2).

3) We overexpressed WT IDH2 in another UC cell line (J82) without IDH2 mutations. We confirmed that most glycolytic and PPP enzymes, including Hif1-α, were overexpressed with IDH2 overexpression (Revised Figure 5a-k).

4) Finally, we confirmed that downregulation of both HIF1A and IDH2 contributes to the restoration of chemosensitivity in UC cells, suggesting that all the observed metabolic reprogramming results from WT IDH2-dependent stabilization of HIF1-α (Revised Figure 4a-h).

These results support the hypothesis that WT IDH2-dependent stabilization of HIF1-α induces metabolic reprogramming for chemoresistance acquisition in UC cells.

4. Fig. 1: The authors identify increased levels of various glycolysis and PPP intermediates in resistant cells. To demonstrate increased pathway flux, at least lactate production should be quantified.

Response) Thank you for your comment. Specifically, the lactate concentration was 953935.1±261415.9 nmol/kg in T24GR cells, which was significantly higher than that in the corresponding WT cells (533755.1±53254.5 nmol/kg, p=0.047). Regarding UMUC3 cells, the lactate concentration was 1853622.0±422404.5 nmol/kg in UMUC3GR cells, which was significantly higher than that in the corresponding WT cells (832021.0±123100.0 nmol/kg, p=0.032).
We listed the specific quantified amounts of glycolytic intermediates in Supplementary Table A

5. The connection between the PPP and increased levels of dCTP are unclear to me. The PPP generates ribose, which is incorporated into all nucleotides. Are there other metabolite changes that help explain why only dCTP is elevated?

Response) Thank you for your comment. We looked over the results of metabolome analysis and confirmed that dCTP was specifically increased in GR cells compared to other dNTPs (Figure R1-1a). First, we added the results of Phosphoribosyl pyrophosphate (PRPP) increase in GR cells, which is the biochemical intermediates for purine and pyrimidine synthesis (Revised Figure 2b). Next, as we checked the intermediate metabolites of purine and pyrimidine biosynthesis (Figure 2c and 2d), we found that the final compounds of pyrimidine synthesis (CMP, CDP, CTP, UMP, UDP, and UTP) showed higher amounts in GR cells compared to WT cells. In contrast, no significant increases were seen in purine biosynthesis compounds (Figure 2c). Finally, CTP synthase 1 (CTPS1) showed significantly higher mRNA and protein expression in GR cells, which is known as a key enzyme for converting UTP to CTP and leads to an increase of dCTP pools.

To clarify the relationship between the dCTP increase and GEM resistance, we took a few steps. We found that compared to those of other nucleosides, the level of dCTP was specifically increased in GR cells (Figure R1-1a). We next measured the baseline dCTP levels in WT and GR cells. The results are shown in Figure R1-1b. The baseline dCTP levels were significantly higher in T24GR and UMUC3GR cells (447.65 nmol/μg±17.8 nmol/μg and 584.65 nmol/μg±53.2 nmol/μg, respectively) than in T24WT and UMUC3WT cells (114.59 nmol/μg±15.5 nmol/μg and 343.93 nmol/μg±37.3 nmol/μg, p=0.001 and p=0.004).

We further compared the therapeutic response to 1μM GEM observed upon treatment with other nucleoside analogs, such as 2-deoxyadenosine monohydrate, 2-deoxyguanosine monohydrate, and thymidine, to that observed upon treatment with dCTP in WT cells. We found that WT cells treated with dCTP at concentrations higher than 10 μM dCTP showed decreased GEM sensitivity, while other nucleoside analogs did not show any molecular

competition with GEM (Figure R1-1c).

We also measured intracellular dCTP levels in WT and GR cells treated with and without dCTP and GEM. CE-MS analysis revealed that the dCTP level was largely maintained in GR cells compared to WT cells even under GEM treatment. Indeed, there were no significant differences in viability with GEM concentration between GR cells and WT cells treated with 10 μM dCTP. (Figure R1-1e and 1f)

These results may support the evidence that the dCTP level is specifically increased in GR cells and that a high baseline intracellular dCTP level may contribute to molecular competition with Gem.

We added the above results and explanation as Revised Figure 2g-l and Figure EV1c in the Results section of our revised manuscript.

6. Fig. 4e - h: The tumor growth data do not include treatment with the IDH2 inhibitor alone. However, the manuscript mentions that tumor volume after treatment with IDH2 inhibitor alone was 232 +-77 m³ - this would be comparable to the tumor volume after treatment with gemcitabine + IDH2-i. To evaluate the efficacy of the co-treatment, it is essential to include data for each inhibitor separately in the figures.

Response) Thank you for your suggestions and we apologize not showing the figure of IDH2-inhibitor alone. Since the current study focused upon WT-IDH2 expression and chemoresistance, we received a criticism by other reviewer that using enasidenib was not appropriate for demonstrating the therapeutic effect of IDH2 inhibition on GR cells, because enasidenib specifically targets neomorphic mutant IDH2 (mutant IDH2) (Chen *et al.*, 2020), but the current study focused on wild-type IDH2 (WT IDH2) gain-of-function-induced chemoresistance. Therefore, we repeated the *in vitro* and *in vivo* experiments with AGI6780 (an IDH2 inhibitor targeting both mutant and WT IDH2) in GR cell lines and the murine UC xenograft model (Revised Figure 7a-m).

To determine the pharmacological effect of an IDH2 inhibitor on GR cells, we used the IDH2 inhibitor AGI-6780, a chemical compound known to potently inhibit both mutant and WT IDH2. We evaluated its impact on cell viability and proliferation. We found that the IC50 values of AGI6780 were 6.4 μM in T24GR cells and 8.9 μM in UMUC3GR cells. Thus, we treated T24GR and UMUC3GR cells with 10 μM AGI6780 in combination with various concentrations of GEM to evaluate whether AGI6780 restores GEM sensitivity. A significant cytotoxic effect was observed with the combination of GEM and AGI6780 compared to GEM alone (Revised Figure 7a). Measurement of 2-HG production revealed significantly lower 2-HG levels in both GR cells with AGI6780 (0.632-fold lower in T24GR cells and 0.518-fold

lower in UMUC3GR cells compared to vehicle control-treated cells) (Revised Figure 7b). Similarly, significantly lower dCTP levels were observed in GR cells treated with AGI6780 (0.529-fold lower in T24GR cells and 0.191-fold lower in UMUC3GR cells compared to vehicle control-treated cells) (Revised Figure 7c).

Regarding CDDP sensitivity, we further treated T24GR and UMUC3GR cells with 10 μM AGI6780 in combination with various concentrations of CDDP. Similar to the GEM experiment, AGI6780 combined with CDDP showed a significant cytotoxic effect on both GR cell lines (Revised Figure 7d). Less NADPH/NADP production and higher ROS generation were observed in the AGI6780-treated group (Revised Figure 7e and 7f).

To verify the therapeutic effect of AGI6780 *in vivo*, we performed an animal study to evaluate the therapeutic effects of AGI6780 alone and in combination with GEM. On Day 25 after the start of treatment, the tumor volume (mean ± standard deviation (SD)) in mice treated with AGI6780 alone was 360.0±43.9 mm3, which was significantly lower than that in mice treated with vehicle control (859.8±178.9 mm3, p=0.006). Furthermore, the tumor volume in mice treated with the combination of GEM and AGI6780 was 200.2±47.8 mm3, which was significantly lower than that in mice treated with GEM alone (758.4± 145.8 mm3, p=0.002) (Figure R1-2c or Revised Figure 7g). There were no apparent toxic effects, such as a decrease in body weight, in mice in any treatment group (Figure R1-2e or Revised Figure 7i). IHC revealed that the expression of IDH2, CAIX (a surrogate marker of hypoxia, similar to Hif1-α), TIGAR, TKT, and CTPS1 was decreased in the AGI6780 and GEM/AGI6780 combination treatment groups compared to the control group (Figure R1-2f or Revised Figure 7j). Likewise, similar results were obtained regarding AGI6780 in combination with CDDP in the murine UC xenograft model (the tumor volume (mean ±SD) on Day 25 in mice treated with CDDP and AGI6780 was 226.4±45.6mm3, which was significantly lower than that in mice treated with CDDP alone (658.0±100.1 mm3, p=0.001) (Figure R1-2g-i or Revised Figure 7k-m).

We added the above results and explanation as Revised Figure 7a-m in the Results section of our revised manuscript.

7. Fig. 5: The authors demonstrate increased intermediates of the oxidative PPP, which is a major source of cytosolic NADPH. It is unclear why the authors focus on mitochondrial NADPH generation by reductive TCA. Does inhibition of the oxidative PPP resensitize cells to cisplatin?

Response) Thank you for your insightful comment. We observed that the whole-cell NADPH levels and GSH levels were significantly higher in both lines of GR cells than in the corresponding WT cells. As the reviewer raised this critical point, we asked why the total

NADPH level was increased in GR cells, since IDH2 consumes NADPH in the reverse reaction converting αKG to isocitrate. However, we appreciate the reviewer's keen insight that NADPH is mainly generated via the oxidative PPP, which is the major source of cytosolic NADPH (Chen *et al.*, 2019). In fact, G6PD was overexpressed in GR cells compared to WT cells (Revised Figure 2e and 2f).

Considering the reviewer's insightful comment, we used a G6PD inhibitor (G6PD-i), which is known as a major PPP inhibitor, to confirm whether NADPH is dependent on the oxidative PPP. Surprisingly, we found that CDDP sensitivity was restored in both GR cell lines by combined treatment with the G6PD inhibitor and CDDP (Figure R1-5a and R1-5b). Moreover, we confirmed that the NADPH/NADP ratio and GSH level in GR cells were significantly decreased by G6PD-I administration, which suggests that NADPH production results mostly from oxidative PPP stimulation (Figure R1-5c-e). On the other hand, as Figure R1-5b shows, we did not find significant effects on GEM sensitivity by adding G6PD-i to GEM in GR cells. This result may suggest that GEM resistance is also dependent upon the nonoxidative PPP, which is stimulated by TKT and TIGAR expression.

These results explain the mechanisms of the NADPH increase in GR cells and indicate how they contribute to cross-sensitivity to CDDP. We found that the increase in the NADPH/NADP ratio resulting from PPP stimulation may contribute to further GSH production and decrease reactive oxygen species (ROS) generation, which reduces the therapeutic effect of CDDP by reinforcing the antioxidant defense (Revised Figure 6e).

We added the above results and explanation as Revised Figure EV4a-e in the Results section of our revised manuscript.

8. The authors speculate that glutamine-dependent metabolism in the TCA cycle contributes to therapy resistance. To provide experimental evidence for this, the authors should compare the effects of inhibitors against glutaminase or IDH2 on NADPH levels, ROS levels and drug resistance.

Response) Thank you for your insightful comment. First, we cultured cells under glucose and glutamine deprivation conditions. We compared the survival of three types (WT, CR, and GR) of two cell lines under these conditions. We confirmed that GR cells showed tolerance under glucose deprivation conditions (glucose-/glutamine+) in both cell lines (Figure R2-1a), while CR and WT cells could not survive. We also evaluated the change in cell survival under glutamine deprivation conditions and confirmed that GR cells showed significantly higher cell survival rates than CR/WT cells in glucose+/glutamine- medium.

However, GR cells could not survive under deprivation of both glucose and glutamine (glucose-/glutamine-). **These results support the hypothesis that glutamine metabolism is also stimulated in GR cells as an alternative energy production pathway.**

Next, we confirmed the NADPH/NADP ratio, GSH level, ROS level, and cytotoxicity by using the glutaminase inhibitor BPTES and the IDH2 inhibitor AGI6780. The results are shown in Figure R3-2. Cells treated with AGI6780 showed a significant reduction in the NADPH/NADP ratio and GSH level but only slight decreased upon BPTES treatment (Figure R3-2b and 2c). Likewise, a slight increase in the ROS level was found with BPTES, while a significant increase in the ROS level was found with AGI6780. ROS accumulation was increased by combination treatment with CDDP (Figure R3-2d). Regarding drug resistance, the IC50 values of BPTES and AGI6780 were 10.3 μM and 6.4 μM, respectively, in T24GR cells, and we conducted WST assay to evaluate the cytotoxicity of GEM, GEM+BPTES, and GEM+AGI6780 treatment. Although an apparent cytotoxic effect was observed in T24GR cells treated with GEM+BPTES, no significant decrease in drug resistance was found with BPTES alone. In contrast, AGI6780 showed a significant cytotoxic effect in combination with both GEM and CDDP (Figure R3-2e).

These results support the hypothesis that glutamine-dependent metabolism contributes to drug resistance, but the landscape of metabolic reprogramming is expanded from reductive glutamine metabolism to glucose-dependent metabolism, including aerobic glycolysis and PPP bypass. The experimental results suggest that glutaminase inhibition is feasible for overcoming chemoresistance to some degree, but it seems that the entire landscape of metabolic reprogramming results from IDH2-dependent Hif1-α stabilization due to 2-HG accumulation.

Thus, targeting WT IDH2 function by AGI6780 showed a crucial effect on suppressing both reductive glutamine metabolism and glucose-dependent aerobic glycolysis with PPP bypass, which resulted in the acquisition of chemoresistance.

Reviewer3: minor

Minor points:

1. *The metabolomics data show large variations within the experimental groups. Are these technical replicates or independently derived cell lines?*

Response) Thank you for the question. Our chemo-resistant cells (CR and GR cell lines) are independently derived cell lines. We purchased T24 and UMUC3 cells from ATCC and cultured untreated WT cells, WT cells treated with increasing concentrations of CDDP, and WT cells treated with increasing concentrations of GEM. Upon acquisition of CDDP/GEM resistance, the cell lines were passaged independently and separately.

We conducted metabolome analysis again using n=4 replicates of cells, each cultured in 10 cm^2 dishes that were passaged at the same time. We replaced the heatmap in Figure 1C to show the differences in intermediate metabolites more clearly (Revised Figure 1c).

2. The font size in some figures is rather difficult to read.

Response) Thank you for the comment. We checked the amounts again and uniformly revised the font style to Arial with a size of at least 10 points.

3. Fig. 5c: Figure legend text and colors are very difficult to decipher.

Response) Thank you for your comment. We modified Figure 5c, focusing on the volume curves of the 4 tumors for simplification. We replaced Figure 5c with Revised Figure 6c in the Results section of our revised manuscript.

4. Fig. 5: For the IDH2 siRNA + cisplatin experiments, please also show effects on cell viability.

Response) Thank you for your suggestion. We added the cell viability results from the IDH2 siRNA + CDDP experiments (Figure R3-3a).

5. Fig. 5: Can the resensitization to cisplatin by IDH2 siRNA be blocked by antioxidants?

Response) Thank you for your suggestion. We used gallic acid (Selleck Chemicals, TX, USA) as an antioxidant agent. The results are shown in Figure R3-3b. We confirmed that GA reversed the cytotoxic effect of CDDP on GR cells transfected with IDH2. **This result suggests that the antioxidant level is responsible for the acquisition of CDDP resistance in GR cells, and that IDH2 downregulation thus decreases the antioxidant ability.**

6. Fig. 5: Do the cisplatin-resistant cells shown in Fig. 1 display similar changes in NADPH levels, ROS and IDH2 dependency as the gemcitabine-resistant cells? Or do these cells acquire resistance to cisplatin by a different mechanism?

Response) Thank you for the keen insight. As you pointed out, CDDP-resistant UC cells also show high intracellular GSH levels and lower ROS production with CDDP exposure. The mechanisms by which UC cells acquire cisplatin (CDDP) resistance were, however, previously reported by our group (Ogihara *et al*, 2019; Shigeta *et al*, 2020). In our previous

studies, we found that CR cells showed increased expression of the x-cystine/glutamate transporter (xCT), which is known as a cystine/glutamate transporter. We reported that CD44 variant isoforms stabilized xCT in a UC cell line, which enhanced intracellular GSH synthesis through the uptake of cystine and contributed to the suppression of ROS production(Hasegawa *et al*, 2016; Ogihara *et al.*, 2019). **Thus, we revealed that the antioxidant ability of CR cells can be enhanced via stimulation of xCT expression, which results in CDDP resistance.**

We evaluated the NADPH and NADP levels in WT, CR, and GR cells and observed that CR cells showed lower NADPH levels than WT and GR cells (Figure 3-3c). Moreover, no significant metabolic shift was observed in CR cells compared to WT cells (Revised Figure 1c). **From these results, we believe that the underlying mechanisms in CR and GR cells are different and therefore consider WT IDH2-dependent metabolic reprogramming a new insight for clarifying the mechanism of chemoresistance acquisition in UC cells.**

7. The authors repeatedly refer to anaerobic metabolism. In experiments where cells are cultured in 21 % oxygen, it would be more appropriate to refer to reductive metabolism and aerobic glycolysis.

Response) Thank you for your insightful suggestion. We thoroughly standardized the terms aerobic glycolysis and reductive metabolism (or carboxylation) in our manuscript to make it clearer for the readers.

We again thank the reviewers for their helpful suggestions. We hope that this manuscript now meets the standards for publication in The EMBO Journal.

Best regards,

Takeo Kosaka, MD, PhD
Department of Urology, Keio University School of Medicine,
35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan.
Phone: 81-3-5363-3824, Fax: 81-3-3225-1985,
Email: takemduro@gmail.com

Figures for reviewers removed

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2nd Revision - Editorial Decision **16th Sep 2022**

Dear Dr Kosaka,

Thank you for submitting your revised manuscript (EMBOJ-2022-110620R1) to The EMBO Journal, as well as for your patience with our feedback, which got protracted by delayed reviewer input and detailed discussion in the editorial team. Your amended study was sent back to the three referees for their re-evaluation, and we have received comments from all of them, which I enclose below.

As you will see, the experts stated that the work has been substantially improved by the complementary work and while referee #2 remains overall more critical, we have concluded that in light of the strong encouragement by referees #1 and #3 we can pursue your study further and are now in favour of publication, pending minor revision.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal.

Please consider the remaining minor comments of the reviewers regarding conceptual integration of your findings and technicalities carefully and amend the text and discussion accordingly where appropriate.

Also, we now need you to take care of a number of minor issues related to formatting and data presentation as detailed below, which should be addressed at re-submission.

Please contact me at any time if you have additional questions related to below points.

As you might have noted on our web page, every paper at the EMBO Journal now includes a 'Synopsis', displayed on the html and freely accessible to all readers. The synopsis includes a 'model' figure as well as 2-5 one-short-sentence bullet points that summarize the article. I would appreciate if you could provide this figure and the bullet points.

Thank you for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to your final revision.

Again, please contact me at any time if you need any help or have further questions.

with
Kind regards,

Daniel Klimmeck

Daniel Klimmeck PhD
Senior Editor
The EMBO Journal

Formatting changes required for the revised version of the manuscript:

>> Please limit the keywords on the manuscript to maximally five.

>> Restrict the abstract to maximally 175 words.

>> Please introduce ORCID IDs for all corresponding authors (M.H.) via our online manuscript system. See below additional information.

>> Consider additional changes and comments from our production team as indicated by the .doc file enclosed and leave changes in track mode.

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Thank you very much in advance.

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We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (15th Dec 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

In this resubmitted/revised manuscript, the authors carried a substantial amount of new experiments that now make sense of previous data and reconcile the paradoxical observation of a pseudo-hypoxic response driven by WT, and not mutant, IDH2 in GEM-resistant cancer cells. The new data show that WT IDH2 overexpression is sufficient to increase the "spurious" 2HG production (typically associated to mutant IDH2) as well as glutamine rewiring to support such production. 2HG then drives HIF1a stabilization, which is now found required to then coordinate the extensive metabolic rewiring observed in the GEM-resistant cells. This is relevant for treatment of GEM-resistant cancer cells with a small-molecule compound, providing preclinical evidence for a potential treatment to reduce the emergence of GEM resistance in patents.

One main point that remains unexplained is the effects of dCTP treatment on cells, which would need some more clarifications, or at least a clear discussion (see Main concern 1 below).

Main concern 1

The problem I had raised here is that GEM aka 2',2'-difluoro 2'-deoxycytidine (dFdC) is imported as a nucleoSide and subsequently modified by the cell via phosphorylation into nucleoTides dFdCMP, dFdCDP and finally dFdCTP. The authors provide data by treating cells with extracellular dCTP, which is NOT transported by facilitated nuceloSides transporters. So, how can extracellular dCTP save from GEM remains without an explanation. The authors used some nucleoSides, but not deoxycitosine (dC) which would have been the right experiment to do to test replenishment of the intracellular pool. So, the most likely explanation is that the biological rescue in the GEM+dCTP set-up is due to some kind of inhibition of GEM transport by extracellular dCTP, and not by an intracellular effect of dCTP. This is perhaps now secondary because knockdown of HIF1a (i.e. the main player that mediated this whole rewiring) is sufficient to restore GEM sensitivity. So, maybe this should just be toned down.

Main concern 2

OK the data are now fine.

Main concern 3

The authors provide a new set of data by using another IDH2 inhibitor, AGI6780, which also inhibits WT IDH2. This is of course very good as it complements previous data based on the AG221 inhibitor. The old data are however now missing from the submission. In my opinion, this should be reintroduced because (1) it is an independent reagent providing coherent results and (2) it may be useful in the future as it pinpoints that also WT IDH2 can produce 2HG (apparently accepted in the literature

https://www.liebertpub.com/doi/10.1089/ars.2019.7902) and in sufficient amount to have a biological effect - hence, the AG221 inhibitor could be useful against WT-IDH2 tumors as well, if metabolism is rewired in such a way to promote 2HG production. This might also be important for clinical studies on this compound, as the expected target is ONLY mutant IDH2, while the expectation from these results is that it may benefit also wt IDH2 patients.

Main concern 4

The authors provide direct evidence for 2HG production by WT IDH2. So, it becomes now clear why the cells show a pseudo-hypoxic phenotype like the one observed in mutant IDH2 cancer cells - both conditions display 2HG production which then triggers the phenotype. This is in line with other data (for example https://www.liebertpub.com/doi/10.1089/ars.2019.7902 - where however it was disputed whether the "intermediate" amount of 2HG produced by WT IDH2 would be sufficient to trigger a phenotype). The authors should cite the main papers where such 2HG production by WT IDH2 has been observed to reinforce their claims.

Main concern 5

The requirement for HIF1a is important, as it now links the observations into a logical model. Whether HIF1a is being truly stabilized by 2HG has not been proven, but the literature in support points in the right direction. The new title is now appropriate.

Main concern 6

The requested experiment was done in full.

Main concern 7

The new data now make a fair point on this secondary issue.

Main concern 8

The new experiments answer sufficiently well my doubts. Coupled to the above points, the data now make much more sense.

All minor points were addressed.

Referee #2:

I appreciate that the authors have made such an effort to improve their study. New data provide additional evidence supporting the conclusion that increased IDH2 expression, and subsequent HIF1a stabilisation, contributes to gemcitabine and cisplatin resistance in urothelial cancer models. However, the authors still fail to provide definitive evidence that IDH2/HIF1a-induced changes in dCTP abundance directly contribute to gemcitabine resistance. There is also a significant amount of overlap between the current study and a paper published in Cancer Cell (Shukla, 2017), which elegantly demonstrated that HIF1a upregulates pyrimidine biosynthesis to drive gemcitabine resistance in pancreatic cancer. Unfortunately, the current study provides an incremental advance in knowledge over what has already been published. Arguably the most significant finding in the current study is the association between elevated WT IDH2 expression, metabolic reprogramming and therapy resistance. It is therefore essential that the authors provide some explanation as to why IDH2 expression is increased in gemcitabine-resistant cells. The authors mention IDH2 "gain of function", but it is unclear what they mean by this. Additional concerns are outlined below.

It appears that WST-8 is utilised to monitor changes in cell viability throughout the study. Given that this assay measures changes in metabolic activity, an alternative assay should be used to confirm that the various perturbations employed throughout the study do indeed impact cell proliferation or cell survival.

Page 6/Line 16 and Page 25/Line 36 - the authors do not provide any evidence (i.e. stable isotope tracing) demonstrating increased glucose flux through the PPP. These statements should be rephrased.

With respect to Figure 2e, the authors should perform a more thorough analysis (either by RT-PCR or RNA-Seq) of differential

expression of enzymes involved in both purine and pyrimidine metabolism.

Figure 2k clearly demonstrates that supplementing cells with dCTP does not change the intracellular abundance of dCTP. Therefore, elevated dCTP abundance cannot explain the effect of dCTP supplementation on gemcitabine-induced changes in cell viability (Figure 2l). The authors fail to show, other than correlative evidence, that increased intracellular dCTP levels contribute to gemcitabine resistance. The authors should have employed deoxycytidine, which was effectively used by Shukla et al. (Cancer Cell, 2017) to demonstrate that increased levels of dCTP drive gemcitabine resistance in pancreatic cancer.

The authors must also demonstrate that deoxycytidine supplementation abrogates the changes in cellular sensitivity to gemcitabine observed following HIF1a/IDH2 knockdown and IDH2 inhibition.

In Figure 5c, it appears that purine AND pyrimidine species are increased in J82 IDHox cells relative to parental J82 cells. For this reason, the abundance of dATP and dTTP should be determined in addition to dCTP (Figure 5j). The authors should also determine the impact of both leflunomide and mizoribine on the sensitivity of J82 IDHox cells to gemcitabine.

The authors should determine whether or not genetic (siRNA) or pharmacological (AGI6780) inhibition of IDH2 sensitizes WT T24G or UMUC3 cells to gemcitabine/cisplatin.

What is the relative viability/proliferation rate of T24GR/UMUC3GR siIDH2 cells compared to T24GR/UMUC3GR siNTC cells?

Referee #3:

The authors have performed extensive additional experiments and satisfactorily addressed my concerns. The results provide interesting insights into a novel form of metabolic dysregulation through wild type IDH2.

 # Referee #1:

In this resubmitted/revised manuscript, the authors carried a substantial amount of new experiments that now make sense of previous data and reconcile the paradoxical observation of a pseudo-hypoxic response driven by WT, and not mutant, IDH2 in GEM-resistant cancer cells. The new data show that WT IDH2 overexpression is sufficient to increase the "spurious" 2HG production (typically associated to mutant IDH2) as well as glutamine rewiring to support such production. 2HG then drives HIF1a stabilization, which is now found required to then coordinate the extensive metabolic rewiring observed in the GEM-resistant cells. This is relevant for treatment of GEM-resistant cancer cells with a small-molecule compound, providing preclinical evidence for a potential treatment to reduce the emergence of GEM resistance in patents.

Response) Thank you for your positive comments. We appreciate your help in improving our manuscript to reach such a concrete conclusion.

One main point that remains unexplained is the effects of dCTP treatment on cells, which would need some more clarifications, or at least a clear discussion (see Main concern 1 below).

Main concern 1
The problem I had raised here is that GEM aka 2',2'-difluoro 2'-deoxycytidine (dFdC) is imported as a nucleoSide and subsequently modified by the cell via phosphorylation into nucleoTides dFdCMP, dFdCDP and finally dFdCTP. The authors provide data by treating cells with extracellular dCTP, which is NOT transported by facilitated nuceloSides transporters. So, how can extracellular dCTP save from GEM remains without an explanation. The authors used some nucleoSides, but not ***deoxycitosine*** *(dC) which would have been the right experiment to do to test replenishment of the intracellular pool. So, the most likely explanation is that the biological rescue in the GEM+dCTP set-up is due to some kind of inhibition of GEM transport by extracellular dCTP, and not by an intracellular effect of dCTP. This is perhaps now secondary because knockdown of HIF1a (i.e. the main player that mediated this whole rewiring) is sufficient to restore GEM sensitivity. So, maybe this should just be toned down.*

Response) Thank you for pointing out this issue. As you pointed out, adding dCTP directly to cell lines was not an appropriate enough method to explain the increase in

dCTP in GR cells, since added extracellular nucleotides would not be transported by nucleoside transporters. In fact, we could not find direct evidence that extracellular dCTP would increase the intracellular dCTP level, resulting in molecular competition with GEM. Thus,to demonstrate clearer results, we further applied the nucleoside agent deoxycytidine (Sigma–Aldrich, Munich, Germany) to reevaluate the experiment shown in Figure 2K/L and Figure EV1c. We measured intracellular dCTP levels in WT and GR cells treated with deoxycytidine (dC) (10 μM) and GEM (1 μM). CE-MS analysis revealed that dCTP levels remained high in GR cells and WT cells treated with GEM in combination with dC (Figure R1a). Moreover, the therapeutic response to 1 μM GEM observed upon treatment with other nucleoside analogs, such as 2-deoxyadenosine monohydrate, 2-deoxyguanosine monohydrate, and thymidine, was similar to that observed upon treatment with dC in WT cells. WT cells treated with dC at concentrations of 10 μM or higher showed GEM resistance, while other nucleoside analogs did not show any molecular competition with GEM (Figure R1-1b). We also confirmed that the cytotoxic efficacy of GEM in WT cells was reduced by the addition of 10 μM dC (Figure R1-1c). As these results showed, increased intracellular dCTP levels occurred after dC treatment, supporting the hypothesis that GR cells may specifically generate dCTP for molecular competition with GEM.

Although we could not find a direct mechanism of biological rescue in cells treated with GEM plus dCTP, we obtained clear results indicating molecular competition with GEM by adding deoxycytidine to the cell lines. However, we also replaced the summary of the sentences to tone down our finding.

We replaced the sentence with "We also measured intracellular dCTP levels in WT and GR cells treated with deoxycytidine (dC) (10 μM) and GEM (1 μM) (Figure 2K). CE-MS analysis revealed that dCTP levels remained high in GR cells and WT cells treated with GEM in combination with dC. Indeed, we confirmed that the cytotoxic efficacy of GEM in WT cells was reduced by the addition of 10 μM dC. (Figure EV1d).

Finally, we compared the therapeutic response to 1 μM GEM observed upon treatment with other nucleoside analogs, such as 2-deoxyadenosine monohydrate, 2-deoxyguanosine monohydrate, and thymidine, with that observed upon treatment with dC in WT cells. WT cells treated with dC at concentrations of 10 μM or higher showed GEM resistance, while other nucleoside analogs did not show any molecular competition with GEM (Figure 2l). These results demonstrated that pyrimidine biosynthesis is increased in GR cells through stimulation of the PPP, which leads to accumulation of dCTP and may cause competitive inhibition of GEM activity.**" in the results section of our manuscript. (Page 7, Lines 23-35)**

We added Figure R1-1a-c as Revised Figure 2K and 2L and Figure EV 1d.

Main concern 2

OK the data are now fine.

Main concern 3

The authors provide a new set of data by using another IDH2 inhibitor, AGI6780, which also inhibits WT IDH2. This is of course very good as it complements previous data based on the AG221 inhibitor. The old data are however now missing from the submission. In my opinion, this should be reintroduced because (1) it is an independent reagent providing coherent results and (2) it may be useful in the future as it pinpoints that also WT IDH2 can produce 2HG (apparently accepted in the literature https://www.liebertpub.com/doi/10.1089/ars.2019.7902) and in sufficient amount to have a biological effect - hence, the AG221 inhibitor could be useful against WT-IDH2 tumors as well, if metabolism is rewired in such a way to promote 2HG production. This might also be important for clinical studies on this compound, as the expected target is ONLY mutant IDH2, while the expectation from these results is that it may benefit also wt IDH2 patients.

Response) Thank you for your critical advice. We are grateful to include our previous results using AGI221 as an IDH2 inhibitor. Since our article mainly focused upon WT IDH2 gain of function, we kindly moved our results with AGI221 to the Appendix Figure S1 as additional results so that we would not confuse the readers with our previous results.

We added the sentence "Furthermore, we also tested the impact of AGI221, another selective IDH2 inhibitor called enasidenib, in both GR cell lines. Similar to the above results, we found a significant cytotoxic effect with the combination of GEM and AGI221 in both in vitro and in vivo experiments (Appendix Figure S1)." **in the results section of our revised manuscript. (Page 13, Lines 7-10)**

We added the previous results obtained with AGI221 to Appendix Figure S1.

Main concern 4

The authors provide direct evidence for 2HG production by WT IDH2. So, it becomes now clear why the cells show a pseudo-hypoxic phenotype like the one observed in mutant IDH2 cancer cells - both conditions display 2HG production which then triggers the phenotype. This is in line with other data (for example https://www.liebertpub.com/doi/10.1089/ars.2019.7902 - where however it was disputed whether the "intermediate" amount of 2HG produced by WT IDH2 would be sufficient to

trigger a phenotype). The authors should cite the main papers where such 2HG production by WT IDH2 has been observed to reinforce their claims.

Response) Thank you for your suggestion. We added this article (Jezek, 2020) as another reference in the manuscript.

We added the sentence, "Although the function of WT IDH2 is still under investigation, many recent studies have compiled the potential mechanisms of WT IDH2, which involve aerobic glycolysis, glutamine-dependent reductive carboxylation, and/or 2-HG production (Li *et al*, 2018; Shi *et al*, 2020, Jezek *et al*, 2020)." **in the discussion section of our revised manuscript. (Page 15, Lines 14-17)**

Main concern 5

The requirement for HIF1a is important, as it now links the observations into a logical model. Whether HIF1a is being truly stabilized by 2HG has not been proven, but the literature in support points in the right direction. The new title is now appropriate.

Response) Thank you for your positive comments. We also think that the new title is clearer than the previous title.

Main concern 6

The requested experiment was done in full.

Main concern 7

The new data now make a fair point on this secondary issue.

Main concern 8

The new experiments answer sufficiently well my doubts. Coupled to the above points, the data now make much more sense.

All minor points were addressed.

 # Referee #2:

I *appreciate that the authors have made such an effort to improve their study. New data provide additional evidence supporting the conclusion that increased IDH2 expression, and subsequent HIF1a stabilisation, contributes to gemcitabine and cisplatin resistance in urothelial cancer models. However, the authors still fail to provide definitive evidence that IDH2/HIF1a-induced changes in dCTP abundance directly contribute to gemcitabine resistance. There is also a significant amount of overlap between the current study and a paper published in Cancer Cell (Shukla, 2017), which elegantly demonstrated that HIF1a upregulates pyrimidine biosynthesis to drive gemcitabine resistance in pancreatic cancer. Unfortunately, the current study provides an incremental advance in knowledge over what has already been published. Arguably the most significant finding in the current study is the association between elevated WT IDH2 expression, metabolic reprogramming and therapy resistance. It is therefore essential that the authors provide some explanation as to why IDH2 expression is increased in gemcitabine-resistant cells. The authors mention IDH2 "gain of function", but it is unclear what they mean by this. Additional concerns are outlined below.*

Response)

Thank you for your insightful comments and consideration of the core of this topic. Since there was very little evidence regarding the relationship between metabolic rewiring and the mechanism of drug resistance in urothelial carcinoma, we first needed to clarify the metabolic differences in and characteristics of gemcitabine-resistant UC cell lines, and therefore, we considered that it was indispensable to follow the results provided by Shulka (Shukla *et al*, 2017). In addition to the previous article, we found many new insights, such as the driving of reductive glutamine metabolism by WT IDH2, stabilization of Hif1-α due to 2-HG accumulation, and identification of AGI6780 as a new therapeutic agent for drug resensitization in UC cell lines. Therefore, we believe that deeper insights have been obtained to clarify the overall mechanism of metabolic reprogramming by which cancer cells are adapted for further survival.

On the other hand, the exact mechanism by which WT IDH2 increases its expression with long-term GEM exposure is still under investigation. One assumption is that aerobic glycolysis followed by PPP activity may trigger the entire metabolic hub to induce glutamine metabolism, which results in IDH2 overexpression (Barnabas *et al*, 2021). Indeed, our results showed that both glucose and glutamine metabolism were stimulated in GR cells, while WT cells relied on only glucose metabolism, suggesting

that aerobic glycolysis may stimulate secondary glutamine metabolism as another energy source to drive the reductive TCA. Another assumption is that Hif1-α or other glycolytic enzymes (e.g. TKT or CTPS1) might affect the expression of another key transcription factor (e.g. Nrf2) that regulates IDH2 (Dai *et al*, 2022; Rojo de la Vega *et al*, 2018). However, these possibilities need to be further investigated in future studies. The critical finding in the current study is that WT IDH2 plays a key role in metabolic rewiring in UC cells and thus promotes chemoresistance to both GEM and CDDP. Although our study provides the conceptual framework and rationale for future investigation of WT IDH2 inhibitors as potential drugs for the treatment of chemoresistant UC, we are grateful for the opportunity to continue to clarify the mechanism of IDH2 gain of function in another study.

We added the sentence "On the other hand, the exact mechanism by which WT IDH2 increases its expression with long-term GEM exposure is still under investigation. One assumption is that aerobic glycolysis followed by PPP activity may trigger the entire metabolic hub to induce glutamine metabolism, which results in IDH2 overexpression (Barnabas *et al.*, 2021). Indeed, our results showed that both glucose and glutamine metabolism were stimulated in GR cells, while WT cells relied on only glucose metabolism, suggesting that aerobic glycolysis may stimulate secondary glutamine metabolism as another energy source to drive the reductive TCA. Another assumption is that Hif1-α or other glycolytic enzymes (e.g. TKT or CTPS1) might affect the expression of another key transcription factor (e.g., Nrf2) that regulates IDH2 (Dai *et al.*, 2022; Rojo de la Vega *et al.*, 2018). However, these possibilities need to be further investigated in future studies.**" in the discussion section of our manuscript. (Page 15, Lines 25-36).**

It appears that WST-8 is utilized to monitor changes in cell viability throughout the study. Given that this assay measures changes in metabolic activity, an alternative assay should be used to confirm that the various perturbations employed throughout the study do indeed impact cell proliferation or cell survival.

Response)

Thank you for your insight. We agree that the WST-8 assay reflects metabolic activity as the intensity of orange fluorescence, and therefore, cancer cells that showed metabolic rewiring would be affected to some extent. On the other hand, many recent studies discussing the mechanisms of metabolic reprogramming in relation to IDH1/2 also chose the cell counting kit-8 assay (which adopts the WST-8 assay method) for all cell viability/proliferation experiments (Shi *et al.*, 2020; Zeng *et*

_al_, 2022). To support our overall results, we further evaluated ATP levels (ATP detection kit, Abcam, Cambridge, UK), an alternative quantitative method for evaluating cell viability. As the results showed, we found very similar results compared to those found using the WST-8 assay (Figure R2-1a and b), suggesting that the WST-8 assay is valid for the entire study. Since WST-8 tetrazolium salts are metabolized to an orange formazan product evaluated by determining the NAD(P)H concentration and dehydrogenase enzyme activity, our biochemistry department team considered that the overall measurement results would be within the margin of error as long as all control cells were evaluated by the WST-8 assay method.

Thus, we retained all cell viability/proliferation results determined by the WST-8 assay method.

Page 6/Line 16 and Page 25/Line 36 - the authors do not provide any evidence (i.e., stable isotope tracing) demonstrating increased glucose flux through the PPP. These statements should be rephrased.

Response)

Thank you for your suggestions. We changed the subtitle "Increased glucose flux through the pentose phosphate pathway (PPP) and increased pyrimidine biosynthesis generates dCTP for acquiring molecular competition against GEM" (Page 6/Line 16) to "Increased pyrimidine biosynthesis via the pentose phosphate pathway (PPP) generates dCTP for the acquisition of molecular competition with GEM". We also changed the subtitle "Figure 2: Glucose flux through the PPP and pyrimidine biosynthesis generates dCTP in GR cells" (Page 25/Line 36) to "Figure 2: GR cells increase pyrimidine biosynthesis to generate dCTP".

With respect to Figure 2e, the authors should perform a more thorough analysis (either by RT-PCR or RNA-Seq) of differential expression of enzymes involved in both purine and pyrimidine metabolism.

Response)

Thank you for your suggestions. We conducted additional RT–PCR analysis to confirm the expression of enzymes involved in purine and pyrimidine metabolism. To address the representative pyrimidine metabolism enzymes, we confirmed the expression of DHODH (dihydroorotate dehydrogenase), UMPS (uridine monophosphate synthetase), CTPS2 (CTP synthase 2), and TYMS (thymidylate

synthase). To address the representative purine metabolism enzymes, we confirmed the expression of IMPDH1 (inosine monophosphate dehydrogenase 1), ADSL (adenylosuccinate lyase), ATIC (5-aminoimidazole-4-carboxamide ribonucleotide formyl transferase), PFAS (phosphoribosyl formylglycinamidine synthase), and PRPS1 (phosphoribosyl pyrophosphate synthetase 1).

As Figure R2-2a and 2b show, the relative mRNA expression levels of pyrimidine metabolism enzymes were significantly higher in GR cells than in WT cells, while slight differences in the relative mRNA expression levels of purine metabolism enzymes were observed between WT and GR cells. Thus, the additional RT–PCR analysis supports the overall results shown in Figure 2e, which demonstrated that the production of endpoint pyrimidine metabolic compounds was stimulated in GR cells compared to WT cells.

We added the sentence, "Overall, the relative mRNA expression levels of pyrimidine metabolism enzymes were significantly higher in GR cells than in WT cells, while slight differences in the relative mRNA expression levels of purine metabolism enzymes were observed between WT and GR cells (Figure EV1a)**." in the results section of our manuscript. (Page 6, Lines 34-36; Page 7, Lines 1-2) The current result was added as Figure EV1a in the main manuscript.**

Figure 2k clearly demonstrates that supplementing cells with dCTP does not change the intracellular abundance of dCTP. Therefore, elevated dCTP abundance cannot explain the effect of dCTP supplementation on gemcitabine-induced changes in cell viability (Figure 2l). The authors fail to show, other than correlative evidence, that increased intracellular dCTP levels contribute to gemcitabine resistance. The authors should have employed deoxycytidine, which was effectively used by Shukla et al. (Cancer Cell, 2017) to demonstrate that increased levels of dCTP drive gemcitabine resistance in pancreatic cancer.

Response)

Thank you for pointing out this crucial issue. To demonstrate clearer results, we further applied the nucleoside agent deoxycytidine (Sigma–Aldrich, Munich, Germany) to reevaluate the experiment shown in Figure 2K/L and Figure EV1c. We measured intracellular dCTP levels in WT and GR cells treated with deoxycytidine (dC) (10 μM) and GEM (1 μM). CE-MS analysis revealed that dCTP levels remained high in GR cells and WT cells treated with GEM in combination with dC (Figure R2-3a). Moreover, the therapeutic response to 1 μM GEM observed upon treatment with other nucleoside analogs, such as 2-deoxyadenosine monohydrate, 2-deoxyguanosine

monohydrate, and thymidine, was similar to that observed upon treatment with dC in WT cells. WT cells treated with dC at concentrations of 10 μM or higher showed GEM resistance, while other nucleoside analogs did not show any molecular competition with GEM (Figure R2-3b). We also confirmed that the cytotoxic efficacy of GEM in WT cells was reduced by the addition of 10 μM dC (Figure R2-3c). As these results showed, increased intracellular dCTP levels occurred after dC treatment, supporting the hypothesis that GR cells specifically generate dCTP for molecular competition with GEM.

We replaced the sentence with "

We also measured intracellular dCTP levels in WT and GR cells treated with deoxycytidine (dC) (10 μM) and GEM (1 μM) (Figure 2K). CE-MS analysis revealed that dCTP levels remained high in GR cells and WT cells treated with GEM in combination with dC. Indeed, we confirmed that the cytotoxic efficacy of GEM in WT cells was reduced by the addition of 10 μM dC. (Figure EV1d).

Finally, we compared the therapeutic response to 1 μM GEM observed upon treatment with other nucleoside analogs, such as 2-deoxyadenosine monohydrate, 2-deoxyguanosine monohydrate, and thymidine, with that observed upon treatment with dC in WT cells. WT cells treated with dC at concentrations of 10 μM or higher showed GEM resistance, while other nucleoside analogs did not show any molecular competition with GEM (Figure 2l). These results demonstrated that pyrimidine biosynthesis is increased in GR cells through stimulation of the PPP, which leads to accumulation of dCTP and may cause competitive inhibition of GEM activity.**" in the results section of our manuscript. (Page 7, Lines 23-35)**

We added Figure R2-3a-c as Revised Figure 2K and 2L and Figure EV 1d.

The authors must also demonstrate that deoxycytidine supplementation abrogates the changes in cellular sensitivity to gemcitabine observed following HIF1a/IDH2 knockdown and IDH2 inhibition.

Response)

Thank you for your suggestions. We further compared the changes in cellular sensitivity to gemcitabine observed following HIF1a/IDH2 knockdown and IDH2 inhibition.

As Figure R2-4a shows, we compared the viability of T24GR cells transfected with siHIF1A#1 and #2 and treated with GEM alone or with GEM in combination with deoxycytidine (dC) supplementation. dC strongly reduced GEM sensitivity in T24GR

cells transfected with siHIF1A#1 and #2. Likewise, deoxycytidine (dC) supplementation also reduced GEM sensitivity in T24GR cells transfected with siIDH2#1 and #2. Similar results were obtained in UMUC3GR cells transfected with siHIF1A#1/#2 and siIDH2#1/#2.
Moreover, we compared the viability of T24GR cells treated with GEM plus AGI6780 alone and in combination with dC (Figure R2-4c). As we expected, dC also showed molecular competition with GEM even under AGI6780 treatment.
These results suggest that GEM-resistant cells acquire drug resistance due to a dCTP increase under the influence of IDH2/HIF1A-mediated metabolic rewiring.

In Figure 5c, it appears that purine AND pyrimidine species are increased in J82 IDHox cells relative to parental J82 cells. For this reason, the abundance of dATP and dTTP should be determined in addition to dCTP (Figure 5j). The authors should also determine the impact of both leflunomide and mizoribine on the sensitivity of J82 IDHox cells to gemcitabine.

Response)
Thank you for your suggestion. As you pointed out, the levels of dATP and dTTP were also increased with WT-IDH2 overexpression to some extent (dATP was 2.86 times higher in J82 IDH2ox cells and dTTP was 3.63 times higher in J82 IDH2ox cells compared to J82 WT cells). We added these results to Figure 5j (revised Figure 5j). Furthermore, we used both leflunomide and mizoribine to assess the sensitivity of J82 IDH2ox cells to gemcitabine. The results are shown in Figure R2-5. We confirmed that leflunomide reversed the cytotoxic effect of GEM on J82 cells with IDH2 overexpression, while only slight differences in cytotoxicity were observed with the combination of mizoribine and GEM. These results also support the hypothesis that leflunomide inhibits the molecular competition of dCTP and therefore increases the cytotoxic effect of GEM.
We added Figure R2-5a as Revised Figure 5j and Figure R2-5b as Figure EV5a.

The authors should determine whether or not genetic (siRNA) or pharmacological (AGI6780) inhibition of IDH2 sensitizes WT T24 or UMUC3 cells to gemcitabine/cisplatin.

Response)
Thank you for your suggestion. In accordance with your recommendation, we compared the relative viability of WT T24 and UMUC3 cells transfected with siNTC and siIDH2 (siIDH2#1 and #2) treated with GEM/CDDP. The results are shown in

Figure R2-6a-c. As the results showed, no significant differences were observed among T24WT cells transfected with siNTC, siIDH2#1, and siIDH2#2 upon GEM/CDDP treatment. Likewise, UMUC3WT cells transfected with siNTC, siIDH2#1, and siIDH2#2 did not show differences in cytotoxicity after treatment with GEM/CDDP (Figure R2-6a and 6b). Furthermore, the combination of AGI6780 and GEM/CDDP did not show any significant differences in cytotoxicity compared to GEM/CDDP alone in either WT cell line (Figure R2-6c and 6d). These results probably suggest that wild-type T24 and UMUC3 cells do not rely on reductive glutamine metabolism since no metabolic reprogramming has occurred in these cell lines.

What is the relative viability/proliferation rate of T24GR/UMUC3GR siIDH2 cells compared to T24GR/UMUC3GR siNTC cells?

Response)

Thank you for your suggestion. We cultured T24GR and UMUC3GR cells transfected with NTC and siIDH2#1 and siIDH2#2. We compared the three types of cell lines (siNTC, siIDH2#1, and siIDH2#2) after 24 h and 48 h (Figure R2-6e). We confirmed that significant changes in the proliferation rate were largely not observed among the siNTC, siIDH2#1, and siIDH2#2 groups. Thus, we found that IDH2 downregulation itself does not affect the viability/proliferation rate of GR cells.

Referee #3:

The authors have performed extensive additional experiments and satisfactorily addressed

my concerns. The results provide interesting insights into a novel form of metabolic dysregulation through wild type IDH2.

Response) Thank you for your positive comments. We appreciate your help in improving our manuscript to reach such a concrete conclusion.

We again thank the reviewers for their helpful suggestions. We hope that this manuscript now meets the standards for publication in The EMBO Journal.

Best regards,

Takeo Kosaka, MD, PhD
Department of Urology, Keio University School of Medicine,
35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan.
Phone: 81-3-5363-3824, Fax: 81-3-3225-1985,
Email: takemduro@gmail.com

Figures for reviewers removed

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3rd Revision - Editorial Decision **29th Nov 2022**

Dear Dr Kosaka,

Thank you for submitting the revised version of your manuscript. I have now evaluated your amended manuscript and concluded that the remaining minor concerns have been sufficiently addressed.

Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. I would thus like to ask for your consent on keeping the additional referee figures included in this file.

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Kind regards,

Daniel Klimmeck

Daniel Klimmeck, PhD
Senior Editor
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Corresponding Author Name: Takeo Kosaka (Leading Contact), Masanori Hasegawa , Mototsugu Oya
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Manuscript Number: EMBOJ-2022-110620

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This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.

Please note that a copy of this checklist will be published alongside your article.

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The data shown in figures should satisfy the following conditions:

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- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
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Each figure caption should contain the following information, for each panel where they are relevant:

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- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
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- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
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