nature portfolio

Peer Review File

Epigenetic and metabolic rewiring in metastatic pheochromocytomas and paragangliomas driven by SDHB mutations

Corresponding Author: Dr Maria-Dolores Chiara

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I have had the pleasure of reviewing the manuscript by Cubiella, Alba-Linares et al. which reports a comprehensive analysis of DNA methylation in SDHB-mutated PPGLs.

The study is well-carried out, and the manuscript is well-written. The data presented will be valuable to the PPGL research field and I have no major concerns with the manuscript.

Some minor comments:
1) In figure legend 1f – final sentence, please replace “xxxx” with the number of probes.
2) The authors present a very interesting analysis on how SDHB mutations and the metastatic process interact with regard to the methylome. Unfortunately this analysis is limited by the correlation between SDHB mutations and metastatic status (14/20 metastatic tumors are MUT and 10/14 benign tumors are WT). It is written that “[the] overlap between the two gene sets which was substantially higher than expected by chance”. On the contrary my opinion is that a rather large overlap is expected given the correlation between the two variables analysed.

In the clustering analysis section it is written among other things that “Clusters 1 and 3 included hyper-DMP and hypo-DMPs, respectively, which were present in MT PPGLs and further amplified in MUT PPGLs. This suggests a potential additive effect of SDHB dysfunction on the epigenetic alterations associated with metastasis”. An alternative interpretation of the data is that SDHB mutations drive a hypermethylation phenotype which is noted in both these analyses – but with a diluted signal in the benign vs metastatic analysis.

In my opinion this needs to either be properly discussed in the manuscript, or better yet, the manuscript be amended with additional analyses of mutated and wildtype benign vs metastatic tumors analysed separately (to the extent that the dataset allows given the naturally limited sample numbers).
3) In the discussion: “Collectively, these data suggest that while SDHB mutations initiate a cascade of epigenetic reprogramming that disrupts gene expression patterns, the metastatic process itself introduces additional methylation changes independent of SDHB mutation status. This results in a convergence of two distinct waves of epigenetic deregulation: one driven by SDHB mutations and another arising specifically during metastasis.”
Similar to my comment above, this is an interpretation of the data that the authors make, but is not the only possible interpretation. Moreover I find the idea of two waves of epigenetic changes a bit speculative. It may well be the case but the data presented in the paper does not have the temporal resolution to definitively show this.

Reviewer #2

(Remarks to the Author)
The study by Cubiella et al. analyzed a cohort of metastatic paragangliomas and pheochromocytomas (PPGL) cases harboring succinate dehydrogenases (SDH) mutations. Authors suggest that SDHB mutations initiate a cascade of epigenetic reprogramming that disrupts specific gene expression patterns. They identify a subset of hypomethylated genes

involved in fructose metabolism as potential involved in tumor aggressiveness. Fructose supplementation was able to enhance PPGL-derived cell proliferation in hypoxic conditions. The study is relevant, experiments are appropriate and results support the possibility of targeting fructose metabolism in metastatic PPGL with mutated SDHB.

Specific comments:
1. Is it possible to define if SDHB mutations are associated with inactivation of the enzyme activity? In other words, is it possible to measure succinate content from tumors with mutated SDHB? Succinate acts as an inhibitor of the KDM family, a group of histone demethylase, which can further contribute to epigenetic regulation in SDHB mutated tumors.

2. Lines 347-351: “To identify spatially coordinated DNA methylation alterations that may drive metastatic progression, we conducted a comprehensive bioinformatics analysis to identify differentially methylated regions (DMRs) (FDR < 0.05; $|\Delta\beta| >$ 0.10; see Methods). This analysis uncovered 935 DMRs, of which 883 (94%) exhibited hypermethylation, affecting 647 unique genes (Figure 2a).”
Why authors did not provide the full list of genes?
3. It is important to provide the list of genes belonging to the different identified clusters. This is particularly relevant since gene ontology analysis in clusters 1 evidenced an enrichment in targets of the PRC2/EED-EZH2 polycomb repressive complex. EZH2 activity has been inversely correlated with expression of genes involved in lipid metabolism, glycolysis and ferroptosis (PMID: 35158113, PMID: 40229247). In particular, low EZH2 activity (using selective inhibitors) increases expression of glycolytic genes while decreasing NR5A2 expression (PMID: 40229247). Authors list NR5A2 among TFs from cluster 3, where EZH2 targets are not enriched. A comment in the discussion would support their findings.

Reviewer #3

(Remarks to the Author)
Summary and General Comments:

The article by Cubiella et al. presents an epigenetic characterization of pheochromocytomas and paragangliomas (PPGLs) harboring SDHB mutations, with a particular focus on metastatic cases. The study confirms that SDHB mutation-positive (SDHB-MUT) metastatic PPGLs exhibit widespread DNA hypermethylation, leading to gene silencing—findings that are consistent with previous reports. Notably, the authors identify differential expression of genes involved in fructose transport between SDHB-MUT and SDHB wild-type (SDHB-WT) tumors.

While the study moderately advances the current understanding of epigenetic reprogramming in SDHB-mutated PPGLs, several critical aspects require clarification. The most significant omission is the lack of detailed information regarding the primary cell cultures—referred to as “cell lines”—used to validate gene expression changes and assess functional consequences.
The authors acknowledge the absence of established human PPGL cell lines, a long-standing challenge in the field. Thus, the claim of having generated new PPGL “cell lines” is potentially groundbreaking. However, essential details are missing: the duration these cells can be maintained in vitro, whether they are cultured in 2D or 3D, their ability to be cryopreserved, and basic morphological documentation (e.g., microscopy images). Although the authors state that these cells divide slowly, no growth curves or doubling time data are provided.
The use of synaptophysin immunofluorescence to confirm the neuroendocrine identity of these cells is also problematic. Synaptophysin is typically cytoplasmic, yet the staining shown in Figure 5 appears predominantly nuclear, suggesting suboptimal staining conditions. Moreover, several other neuroendocrine markers should be stained, together with the known hypermethylation marker 5-hmC, which has been well described in SDHx-related tumors.

Furthermore, only one of the three primary cultures carries an SDHB mutation—the central focus of the study—limiting the interpretability of the functional assays.

Similarly, the “non-tumoral adrenal gland” (AG) cells, reportedly derived from a deceased kidney donor, raise concerns about viability, given the adrenal glands' rapid post-mortem degradation. The authors should clarify the cellular composition of these cultures (e.g., cortical vs. medullary origin) and whether the culture represents one or both adrenal glands from the same patient as they refer to these cells as plural.

Another notable omission is the absence of gene lists used to generate the graphs in Figure 4, which hinders reproducibility and interpretation.

Specific Comments:

1. The manuscript does not specify whether the SDHB mutations in the patients used for epigenetic profiling are germline.
2. Although hundreds of differentially methylated regions (DMRs) are identified between SDHB-MUT and SDHB-WT samples, the authors focus exclusively on one region encompassing PCDHGC3—a gene they have previously studied. Including analysis of at least one novel region would strengthen the manuscript.
3. The eight methylation clusters defined in Figure 3g, stratified by SDHB mutation status and metastatic behavior, require further explanation. In particular, the distinctions among clusters 5, 7, and 8, as well as the relevance of clusters 1 and 3, should be discussed in more depth. A comparison between DMT of MT-SDHB and BN-SDHB should be performed, and gene lists for these datasets should be provided. A heatmap illustrating the clustering should be included. Furthermore, the statistical significance of DMT in the various cluster comparisons is not provided.
4. The rationale behind the specific cluster comparisons in Figure 4 is unclear. The authors should elaborate on the logic

guiding these comparisons, especially where trends in differentially methylated positions (DMPs) are not intuitive (e.g., why compare clusters 1, 5, and 7?). Additionally, experimental validation of the methylation of the genes mentioned in the first paragraph of the “Functional implications of cluster-specific...” section is missing. Is hypermethylation affecting gene transcription? Are the respective proteins affected?
5. The gene list that led the authors to conclude altered fructose metabolism should be provided, and key genes validated by qPCR. Concentrations for glucose and fructose supplementation are not provided. Experimental procedures in the Methods section are also insufficiently described. The study would benefit from comparison with validated tumor models such as RS0 or RS1/2, even though these are not of human origin. The increase in SLC2A5 under hypoxia should be compared to other glucose importers such as GLUT-1 and GLUT-8.
6. As SDHB knockout is claimed to cause SLC2A5 upregulation, validation in other cell model systems is crucial.
7. The analysis of the TCGA dataset shown in Figure 6a is neither described in the main text nor in the Methods section. This analysis should be properly introduced, and its significance explained. What was the rationale for correlating with LDHA, EGLN3, and SLC16A3, given that Pearson’s correlations of 0.2–0.4 are considered weak?
8. The identity of the AG cells is crucial for interpretation, especially since they were used for SDHB knockdown. In addition to basic characterization, succinate levels in the KD cells should be provided.
9. The doubling time of the AG cells is not shown in Supplementary Figure 1. Do these cells proliferate? Since they were infected and selected with puromycin, details on their viability and culture longevity are essential.
10. Supplementary Figure 1 shows highly variable doubling times among patient-derived PPGL primary cultures. The authors should discuss potential reasons for this variability.

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Communications Biology initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I have reviewed the revised manuscript by Cubiella, Alba-Linares, and colleagues. The authors have addressed the comments I raised during the initial review round, and I'm happy to recommend publication of the paper.

Reviewer #2

(Remarks to the Author)
The authors have addressed all comments and made appropriate changes to the manuscript, which is now suitable for publication.

Reviewer #3

(Remarks to the Author)
The authors have satisfactorily addressed the issues previously raised and have consequently improved the manuscript substantially. Although the fact that the patient-derived cell lines will be described elsewhere is not ideal—as it limits the ability to fully assess their relevance—it is nonetheless understandable.

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Communications Biology initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response to Reviewers. Manuscript COMMSBIO-25-3216

Epigenetic and metabolic rewiring in metastatic pheochromocytomas and paragangliomas driven by *SDHB* mutations

Tamara Cubiella, Juan-José Alba-Linares, Jaime San-Juan-Guardado, Alvaro Suarez-4 Priede, Nerea Gómez-Suárez, Maria Tous, Irina Bancos, Carles Villabona, Teresa Serrano, Isabel Tena, Maribel 5 Del Olmo, Lluis Forga, Nuria Valdés, Mario F Fraga, and María-Dolores Chiara

Reviewer #1

We thank the reviewer for his/her positive and encouraging assessment of our work.

Detailed responses:

1) In figure legend 1f – final sentence, please replace "xxxx" with the number of probes.

The Figure legend has been corrected accordingly. The final number of probes is 754,581, which now replaces the placeholder "xxxx" in Figure 1f legend (page 10).

2) The authors present a very interesting analysis on how SDHB mutations and the metastatic process interact with regard to the methylome. Unfortunately this analysis is limited by the correlation between SDHB mutations and metastatic status (14/20 metastatic tumors are MUT and 10/14 benign tumors are WT). It is written that "[the] overlap between the two gene sets which was substantially higher than expected by chance". On the contrary my opinion is that a rather large overlap is expected given the correlation between the two variables analysed.

We thank the reviewer for this insightful comment and fully agree that the strong correlation between *SDHB* mutation and metastatic status limits the interpretability of the overlap analysis. Indeed, as the reviewer correctly points out, a substantial overlap between both contrasts would be expected given this correlation, and thus the statistical independence assumed by Fisher's exact test is violated.

Initially, overlap analyses revealing statistically significant associations in both hypermethylation and hypomethylation processes were conducted using Fisher's exact tests. However, as noted above, this approach is not appropriate when the variables under comparison are correlated.

To rigorously assess whether the observed overlap between the two contrasts deviates from expectations given their correlation, we implemented two complementary analytical approaches based on simulated permutations and bootstrap resampling.

Permutation-Based Null Distribution of Overlaps and Odds Ratios: To evaluate the significance of overlapping differential signals between two datasets under the null hypothesis of no coordinated association, we performed a permutation-based simulation approach using multivariate normal sampling. Specifically, we generated 1,000 null datasets using a bivariate normal distribution with zero mean (no true effect) and a specified correlation structure corresponding to the observed Pearson between logFC values from metastasis and *SDHB* mutation comparisons. For each permutation, we sampled random statistics and computed nominal p-values from the absolute simulated Z-scores for each CpG and contrast. Next, we quantified the number of CpGs showing significant associations, stratified by direction of methylation change, for each comparison, as well as the extent of overlap between them. These permutations yielded empirical null distributions for both the overlapping CpG counts and the corresponding odds ratios (OR), against which observed values were compared using joint empirical p values. These empirical p values were calculated separately for hyper- and hypo-methylation alterations using the following formula:

$$p_{\text{empirical, joint}} = \frac{\sum(\text{OR}_{\text{null}} \geq \text{OR}_{\text{obs}}) + 1}{n + 1}$$

where n = 1,000 permutations. The addition of 1 in both terms of the division prevents zero p values and ensures conservative estimates.

Using this approach, we confirmed the reviewer's observation by demonstrating that the observed enrichment was lower than expected ($p_{\text{empirical,hyper}}$ = 1 ; $p_{\text{empirical,hypo}}$ = 1) under a null model that accounts for the correlation between comparisons.

Bootstrapped differential methylation analyses: To address the unbalanced distribution of metastatic and *SDHB*-mutated samples, and to robustly evaluate the significance of overlapping differential methylation signals, we performed 1,000 bootstrap-based differential methylation analyses ensuring an equal distribution of samples across the four groups in each iteration: 7 BN/MUT, 7 BN/WT, 7 MT/MUT, and 7 MT/WT. Hyper- and hypo-DMPs were identified in each bootstrap iteration using the same criteria outlined in the manuscript. Subsequently, given the balanced distribution of groups across comparisons, Fisher's exact tests were performed for each iteration ($p_{\text{fisher}} < 0.05$, OR > 1), and empirical p values were calculated, consistently yielding non-significant over-enrichment for overlapping hypermethylated and hypomethylated DMPs ($p_{\text{empirical,hyper}}$ = 0.964 ; $p_{\text{empirical,hypo}}$ = 0.821).

Once again, the reviewer's observation was confirmed using two complementary and independent methods that explicitly accounted for the correlated structure of the comparisons. A description of the resampling approaches used, along with an appropriate reference, has been incorporated into the Methods section (page 5). We thank the reviewer for his/her insightful comments, which thereby strengthened our findings.

In the clustering analysis section it is written among other things that

"Clusters 1 and 3 included hyper-DMP and hypo-DMPs, respectively, which were present in MT PPGLs and further amplified in MUT PPGLs. This suggests a potential additive effect of SDHB dysfunction on the epigenetic alterations associated with metastasis". An alternative interpretation of the data is that SDHB mutations drive a hypermethylation phenotype which is noted in both these analyses – but with a diluted signal in the benign vs metastatic analysis. In my opinion this needs to either be properly discussed in the manuscript, or better yet, the manuscript be amended with additional analyses of mutated and wildtype benign vs metastatic tumors analysed separately (to the extent that the dataset allows given the naturally limited sample numbers).

We thank the reviewer for this thoughtful comment. We agree that the current data do not support a causal or directional relationship between SDHB dysfunction and metastasis-associated epigenetic changes. To avoid implying such causality, we have revised the sentence to read: "*given the strong correlation between SDHB mutation status and metastatic disease in our cohort, these observations should not be interpreted as evidence of causality or temporal sequence*." (page 14). This revised wording emphasizes the potential interaction between both factors while maintaining a neutral interpretation, as the reviewer rightly suggested.

We also attempted the additional analysis proposed by the reviewer, stratifying tumors by *SDHB* mutation status and comparing benign versus metastatic cases within each subgroup. However, this analysis did not yield statistically significant results (FDR < 0.05), likely due to the limited sample size and the unbalanced distribution of benign and metastatic tumors.

3) In the discussion: "Collectively, these data suggest that while SDHB mutations initiate a cascade of epigenetic reprogramming that disrupts gene expression patterns, the metastatic process itself introduces additional methylation changes independent of SDHB mutation status. This results in a convergence of two distinct waves of epigenetic deregulation: one driven by SDHB mutations and another arising specifically during metastasis."
Similar to my comment above, this is an interpretation of the data that the authors make, but is not the only possible interpretation. Moreover I find the idea of two waves of epigenetic changes a bit speculative. It may well be the case but the data presented in the paper does not have the temporal resolution to definitively show this.

We thank the reviewer for this observation and fully agree that the phrasing in the original version may have implied a speculative temporal sequence that is not directly supported by our data. As suggested, we have modified this statement to remove the reference to "two waves" of epigenetic deregulation and to adopt a more neutral interpretation, consistent with the revision described above (Discussion section, page 23).

In addition to addressing the reviewer's comments, we have strengthened the manuscript by:

- providing more detailed characterization of the primary cell models,
- analyzing additional carbohydrate transporters to compare their regulation with that of *SLC2A5*, (including analysis of the rat pheochromocytoma cell line, PC12) and,
- generating *SDHB* knockout models in multiple tumor cell lines in addition to adrenal-derived cells.

These new data further support that *SLC2A5* regulation is highly cell-context–dependent and associated with HIF2α accumulation

Reviewer #2:

We sincerely thank the reviewer for his/her positive and encouraging evaluation of our study. We are pleased that they found the work relevant, the experiments appropriate, and the results supportive of the proposed link between *SDHB* mutations, epigenetic reprogramming, and fructose metabolism in metastatic PPGLs.

1. Is it possible to define if SDHB mutations are associated with inactivation of the enzyme activity? In other words, is it possible to measure succinate content from tumors with mutated SDHB? Succinate acts as an inhibitor of the KDM family, a group of histone demethylase, which can further contribute to epigenetic regulation in SDHB mutated tumors.

We thank the reviewer for this insightful comment. While we did not measure succinate levels directly in our tumor samples, our sequencing data clearly established the presence or absence of *SDHx* mutations. In the revised version of the manuscript, we have now expanded the methodological section to include details on the genetic and immunohistochemical characterization of the SDH complex (page 7). Specifically, we analyzed SDHB protein expression by immunohistochemistry, as the loss of SDHB immunoreactivity is a well-recognized surrogate marker of SDH complex inactivation, regardless of which subunit is mutated.

Representative immunohistochemical images are now provided in the new Supplementary Figure S1, illustrating the absence of SDHB staining in tumors harboring *SDHB* mutations, in contrast to those without such alterations. These results are fully consistent with the expected SDH-deficient phenotype, thereby supporting the association between *SDHx* mutations and loss of enzymatic activity at the protein level.

2. Lines 347-351: "To identify spatially coordinated DNA methylation alterations that may drive metastatic progression, we conducted a comprehensive bioinformatics analysis to identify differentially methylated regions (DMRs) (FDR < 0.05; |Δβ| > 0.10; see Methods). This analysis uncovered 935 DMRs, of which 883 (94%) exhibited hypermethylation, affecting 647 unique genes (Figure 2a)." Why authors did not provide the full list of genes?

We thank the reviewer for this helpful comment and fully agree that providing the complete list of genes associated with the identified DMRs adds transparency and value to the study. Accordingly, in the revised version of the manuscript, we now include this information as Supplementary Table S2 (mentioned in page 10), which lists all 647 unique genes identified in our analysis, together with their genomic coordinates and methylation changes.

3. It is important to provide the list of genes belonging to the different identified clusters. This is particularly relevant since gene ontology analysis in clusters 1 evidenced an enrichment in targets of the PRC2/EED-EZH2 polycomb repressive complex. EZH2 activity has been inversely

correlated with expression of genes involved in lipid metabolism, glycolysis and ferroptosis (PMID: 35158113, PMID: 40229247). In particular, low EZH2 activity (using selective inhibitors) increases expression of glycolytic genes while decreasing NR5A2 expression (PMID: 40229247). Authors list NR5A2 among TFs from cluster 3, where EZH2 targets are not enriched. A comment in the discussion would support their findings.

Thank you for this insightful comment. We re-examined the gene lists from each methylation cluster and compared them with the genes reported in the two studies cited by the reviewer (PMID: 35158113 and PMID: 40229247). Consistent with the reviewer's observation, several genes that become upregulated upon EZH2 inhibition in those studies are present within our hypermethylated clusters (clusters 5 and/or 7), including *ACACA, SCD5, ACSL1, ACSL3, LIPE, CPT1A, PNPLA2, GPX4, SCARB1*, and *SLC27A3*. This partial overlap suggests that some metabolic genes that are responsive to EZH2 activity in other models may become stably silenced through DNA hypermethylation in *SDHB*-associated tumors. These findings are compatible with an epigenetic "switch" from reversible repression mediated by EZH2/PRC2 to more stable silencing via DNA methylation.

However, not all EZH2-responsive genes reported in those studies were found in our hypermethylated clusters, and none of them appeared in the hypomethylated cluster 3. This indicates that the epigenetic remodeling associated with SDHB loss does not fully replicate the transcriptional consequences of direct EZH2 inhibition. Conceptually, this is consistent with the different nature of both processes: EZH2 inhibitors induce an acute and direct catalytic blockade, whereas *SDHB* deficiency generates a broader pseudohypoxic epigenetic context integrating metabolic, transcriptional, and chromatin-level effects. Thus, *SDHB*-driven hypermethylation may "lock in" a subset of PRC2/EZH2-regulated genes into a more stable silenced state.

Regarding the reviewer's reference to NR5A2, we verified that the study cited (PMID: 40229247) discusses regulation of NR5A1, not NR5A2. In our dataset, NR5A2 is indeed present in cluster 3, consistent with the absence of enrichment for EZH2 targets in this cluster.

We added this clarification to the Discussion (page 23) and, as requested, we now provide a complete list of genes for each cluster (Supplementary Table S2).

In addition to addressing the reviewer's comments, we have strengthened the manuscript by:

- providing more detailed characterization of the primary cell models,
- analyzing additional carbohydrate transporters to compare their regulation with that of *SLC2A5*, (including analysis of the rat pheochromocytoma cell line, PC12) and,
- generating *SDHB* knockout models in multiple tumor cell lines in addition to adrenal-derived cells.

These new data further support that *SLC2A5* regulation is highly cell-context–dependent and associated with HIF2α accumulation

Reviewer #3:

While the study moderately advances the current understanding of epigenetic reprogramming in SDHB-mutated PPGLs, several critical aspects require clarification. The most significant omission is the lack of detailed information regarding the primary cell cultures—referred to as "cell lines"—used to validate gene expression changes and assess functional consequences.
The authors acknowledge the absence of established human PPGL cell lines, a long-standing challenge in the field. Thus, the claim of having generated new PPGL "cell lines" is potentially groundbreaking. However, essential details are missing: the duration these cells can be maintained in vitro, whether they are cultured in 2D or 3D, their ability to be cryopreserved, and basic morphological documentation (e.g., microscopy images). Although the authors state that these cells divide slowly, no growth curves or doubling time data are provided.
The use of synaptophysin immunofluorescence to confirm the neuroendocrine identity of these cells is also problematic. Synaptophysin is typically cytoplasmic, yet the staining shown in Figure 5 appears predominantly nuclear, suggesting suboptimal staining conditions. Moreover, several other neuroendocrine markers should be stained, together with the known hypermethylation marker 5-hmC, which has been well described in SDHx-related tumors.

We thank the reviewer for these insightful and constructive comments regarding the characterization and maintenance of the primary PPGL-derived cultures. We fully acknowledge the reviewer's concerns regarding the characterization and maintenance of the primary PPGL-derived cultures. Indeed, establishing and maintaining primary cultures from these tumors is technically challenging, an issue our group has been addressing for several years. To date, we have achieved successful cultures in approximately 30% of processed tumor samples. These efforts have required continuous optimization of isolation and culture conditions, and we are currently preparing a dedicated manuscript describing the most relevant methodological advances obtained from this work. Therefore, we kindly ask both the reviewer and the editor to treat the information provided here as confidential and allow us to include in the current manuscript only the data most relevant to demonstrate that these cultures retain the chromaffin lineage identity and are suitable for the functional assays presented.

In response to the reviewer's request, we have now included additional data on primary tumor growth kinetics. Specifically, a new graph showing the tumor doubling time has been added to Figure 5 in the revised manuscript.

We have also expanded the Methods section to include details on the maintenance of these cultures as tumorospheres, a strategy used to preserve their native 3D organization and phenotypic features. For immunofluorescence analyses, spheroids were dissociated and plated on IbiTreat microscopy dishes in medium containing FBS to promote cell adhesion prior to staining.

Regarding synaptophysin immunofluorescence, we fully agree with the reviewer that the observed nuclear localization is atypical. Despite extensive optimization using appropriate positive and negative controls, this pattern consistently persisted, suggesting that it is unlikely to result from a technical artifact but rather reflects a specific feature of these primary cultures. To strengthen the phenotypic characterization, we have complemented these data with an additional marker, tyrosine hydroxylase (TH), which is a well-established marker of chromaffin cells. Co-immunofluorescence analyses show that a subset of cells co-express nestin and TH, supporting the neuroendocrine and chromaffin lineage identity of these cultures. Additionally, given the established role of HIF2α in the pathogenesis of PPGL, we also analysed HIF2α protein expression by immunofluorescence. All three PPGL-derived cultures showed nuclear accumulation of HIF2α under normoxic conditions, despite only one line (PGLb) harboring a pathogenic mutation in an *SDHx* gene (*SDHD*), which is typically associated with HIF2α stabilization. The basis for this finding remains unclear but appears to be specific and align with previous reports in tumor tissues (PMID: 35740651).

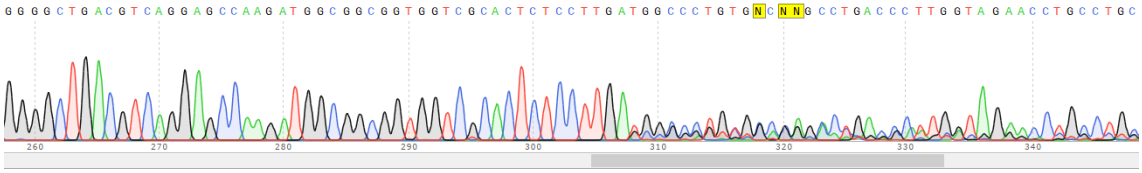
Furthermore, only one of the three primary cultures carries an SDHB mutation -the central focus of the study- limiting the interpretability of the functional assays.

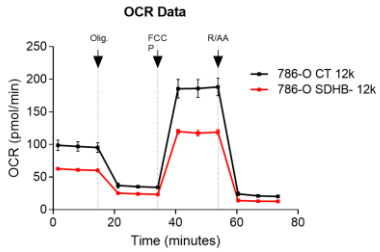
We fully acknowledge that the limited number of primary cultures carrying *SDHB* mutations represents an important limitation of our study. Indeed, among the three primary cultures analyzed, one was derived from a tumor harboring an *SDHD* mutation rather than *SDHB*.

To address this limitation and better reproduce the metabolic and epigenetic consequences associated with *SDHB* loss, we had performed experiments designed to mimic the metabolic environment of SDH-deficient tumors. Specifically, we exposed the cultures to hypoxia or treated them with cell-permeable methyl-succinate to mimic SDH inactivation. Although these experimental models cannot fully substitute for *SDHB*-mutant cells, they provided valuable complementary evidence suggesting that the observed effects are strongly influenced by both the cellular context and the activation of the hypoxia pathway. Notably, hypoxia-induced *SLC2A5* upregulation was more pronounced in the culture derived from the *SDHD*-mutated tumor, supporting the idea that alterations in *SDH*-related metabolism, irrespective of the specific subunit affected, contribute to the transcriptional and metabolic changes reported in our study.

In parallel, we generated several *SDHB* knockout cell models and observed that the cellular response to *SDHB* loss was not uniform across different cell types. For instance, HeLa and MCF7 cells did not exhibit increased *SLC2A5* expression upon *SDHB* knockout, whereas 786-O cells showed significant induction. This difference is particularly relevant because 786-O cells harbor deletions in *VHL* and *HIF1A*, resulting in constitutive HIF2α stabilization even under normoxic conditions. These findings suggest that HIF2α, rather than HIF1α, may mediate *SLC2A5* regulation in the context of SDH deficiency.

We also confirmed the efficiency of our CRISPR/Cas9-mediated *SDHB* knockout targeting *SDHB* through genome sequencing of the targeted region in AG *SDHB*-KO cells, confirming precise editing at the expected locus. Furthermore, we have previously demonstrated that this knockout induces succinate accumulation in cells (see reference PMID: 31216007). In addition, independent Seahorse analyses (unrelated to this manuscript) show that *SDHB* knockout leads to a marked reduction in mitochondrial metabolic activity, further supporting the functional efficacy of the gene editing. These data are shown here for completeness but are not included in the current manuscript, as they form part of a separate study on renal cancer currently in preparation.





Similarly, the "non-tumoral adrenal gland" (AG) cells, reportedly derived from a deceased kidney donor, raise concerns about viability, given the adrenal glands' rapid post-mortem degradation. The authors should clarify the cellular composition of these cultures (e.g., cortical vs. medullary origin) and whether the culture represents one or both adrenal glands from the same patient as they refer to these cells as plural.

We thank the reviewer for this important comment and apologize for the lack of methodological detail regarding the origin and preparation of the adrenal gland (AG) primary cultures. These samples were derived from brain-dead organ donors undergoing multiorgan procurement surgery, during which systemic circulation is maintained until organ retrieval. The adrenal glands were transferred to our laboratory immediately after removal, thereby preserving tissue integrity and viability. As a result, the success rate of AG primary cultures is considerably higher than that of tumor-derived cultures, which require prior macroscopic examination by pathology services before processing.

Regarding the cellular composition, the adrenal medulla was macroscopically dissected from the gland prior to processing to enrich the cultures for chromaffin cells. However, due to the anatomical proximity between the cortical and medullary regions, we cannot fully exclude minor contamination by cortical cells within the culture.
Finally, we clarify that the AG primary culture used in this study was derived from a single adrenal gland.

Another notable omission is the absence of gene lists used to generate the graphs in Figure 4, which hinders reproducibility and interpretation.

We thank the reviewer for this thoughtful and constructive comment. We fully agree that the list of genes belonging to each identified cluster should be provided. Accordingly, in the revised version of the manuscript, we now include this information as Supplementary Table S2.

Specific comments:

1. The manuscript does not specify whether the SDHB mutations in the patients used for epigenetic profiling are germline.

We thank the reviewer for this pertinent comment. We confirm that all *SDHB* mutations identified in the patients included in the epigenetic profiling were germline variants. This information has now been explicitly stated in the revised version of the manuscript (page 4).

In addition, whenever tumor tissue is available, we systematically perform SDHB immunohistochemistry to assess protein expression. The loss of SDHB immunoreactivity serves as a well-established and reliable surrogate marker of SDH complex inactivation, thereby confirming the functional impact of the underlying germline *SDHx* mutations. This information is included in page 7 and 8 as well as in Supplementary Figure S1.

2. Although hundreds of differentially methylated regions (DMRs) are identified between SDHB-MUT and SDHB-WT samples, the authors focus exclusively on one region encompassing PCDHGC3—a gene they have previously studied. Including analysis of at least one novel region would strengthen the manuscript.

We appreciate the reviewer's insightful comment and fully agree that including an additional example beyond *PCDHGC3* strengthens the manuscript. Accordingly, we selected *SATB2*, a gene whose reduced expression has been consistently associated with poor prognosis and/or metastatic behavior in colorectal cancer, renal carcinoma, and neuroendocrine neoplasms. In our dataset, we identified three hypermethylated DMRs partially or fully overlapping regulatory elements of this gene. Based on these findings, we have now incorporated description of the data on page 11 and a new panel in Figure 2 in the revised manuscript illustrating the potential epigenetic repression of *SATB2* in our cohort.

3. The eight methylation clusters defined in Figure 3g, stratified by SDHB mutation status and metastatic behavior, require further explanation. In particular, the distinctions among clusters 5, 7, and 8, as well as the relevance of clusters 1 and 3, should be discussed in more depth.

We thank the reviewer for this valuable comment and fully agree that a more detailed interpretation of the methylation clusters was needed. In the revised version of the manuscript, we have substantially expanded the description of

these clusters in the Results section (page 14) to clarify their distinct methylation patterns and biological implications.

Specifically, we now provide a more detailed explanation of clusters 1 and 3, which display predominant hyper- and hypomethylation, respectively, in metastatic PPGLs that become further accentuated in *SDHB*-mutant tumors. This pattern suggests potential additive or synergistic interactions between SDHB dysfunction and metastasis-associated epigenetic alterations, although the effect of metastasis appears more pronounced.

We have also elaborated on the distinctions among clusters 5, 7, and 8, highlighting that these clusters represent different degrees of *SDHB*-associated hypermethylation: cluster 5 shows a stronger mutation-driven effect than metastasis, cluster 7 reaches maximal methylation levels irrespective of tumor behavior, and cluster 8 includes loci methylated exclusively in *SDHB*-mutant PPGLs. Together, these revisions provide a clearer interpretation of how *SDHB* loss and metastatic progression differentially shape the PPGL methylome.

A comparison between DMT of MT-SDHB and BN-SDHB should be performed, and gene lists for these datasets should be provided.

We thank the reviewer for this comment and fully understand the relevance of comparing methylation profiles between metastatic (MT) and benign (BN) tumors within the *SDHB*-mutated subgroup. However, this analysis is severely limited by the small number of *SDHB*-mutated benign tumors in our cohort, resulting in an unbalanced sample distribution (14/20 metastatic vs. 4/14 benign) and consequently low statistical power. Under these conditions, the comparison between MT-*SDHB* and BN-*SDHB* groups did not yield statistically significant differentially methylated positions (DMPs) at an FDR < 0.05.

A heatmap illustrating the clustering should be included. Furthermore, the statistical significance of DMT in the various cluster comparisons is not provided.

In the revised version of the manuscript, we have now included a heatmap illustrating the clustering of methylation patterns across the PPGL groups, organized according to the previously defined clusters. This visualization highlights both the methylation dynamics and the degree of relatedness among clusters.

We would also like to clarify that the clustering analysis was performed using 9,872 differentially methylated positions (DMPs), comprising all significant DMPs identified in the MT versus BN comparison (4,936) and the 4,936 most significant DMPs detected in the MUT versus WT comparison. These DMPs were selected based on statistical significance (FDR < 0.05) and effect size criteria as described in the Methods section. Therefore, all features used in the clustering analysis already met rigorous statistical thresholds, and no additional testing was required for this step.

4. The rationale behind the specific cluster comparisons in Figure 4 is

unclear. The authors should elaborate on the logic guiding these comparisons, especially where trends in differentially methylated positions (DMPs) are not intuitive (e.g., why compare clusters 1, 5, and 7?). Additionally, experimental validation of the methylation of the genes mentioned in the first paragraph of the "Functional implications of cluster-specific..." section is missing. Is hypermethylation affecting gene transcription? Are the respective proteins affected?

We thank the reviewer for this thoughtful comment. We agree that clarifying the rationale for the cluster comparisons in Figure 4 improves the readability of the manuscript, and we have now expanded this explanation in the revised text (page 13-14). Briefly:

1. Clusters 1, 5, and 7 were compared because they are among the clusters with the highest number of CpG sites and all of them show hypermethylation associated with metastatic status and/or *SDHB* mutation. These clusters therefore represent the major *SDHB*/metastasis-associated hypermethylated signatures in our dataset and are expected to converge on related biological programs.
2. Clusters 1, 2, and 4 were analyzed together because they comprise the most closely related groups of hypermethylated CpGs across metastasis and/or *SDHB* mutation (as shown in the cluster map and corresponding heatmap). Comparing these clusters allowed us to identify pathways that are consistently affected by hypermethylation across different clinical/genetic contexts.
3. The comparison including clusters 1, 2, and 3 was designed to contrast hypermethylated (clusters 1 and 2) versus hypomethylated (cluster 3) loci. This analysis highlights that hyper- and hypomethylation events are linked to distinct functional programs, as would be biologically expected, and as we show in the manuscript.
4. Finally, the joint analysis of clusters 1, 2, 4, and 5 was used to determine whether the two major groups of hypermethylated clusters (those primarily associated with metastasis/*SDHB* co-occurrence versus those predominantly driven by *SDHB* mutation) converge on similar pathways. Indeed, these clusters show enrichment in overlapping molecular processes, supporting the idea that SDHB dysfunction and metastatic progression ultimately affect related regulatory networks.

Regarding the question of whether **hypermethylation affects gene transcription? Are the respective proteins affected?,** we must indicate that, unfortunately, we do not have paired multi-omic data (methylation and transcriptome/proteome from the same samples) that would allow us to directly assess whether hypermethylation affects gene transcription or protein expression. We consider these analyses to be beyond the scope of the present study, which was specifically focused on defining the global methylation landscape and identifying cluster-specific epigenetic alterations in PPGLs. Nevertheless, we have performed functional studies on one of the genes highlighted in our methylation analysis, *SLC2A5*. These experiments demonstrated that *SLC2A5* is transcriptionally regulated in response to metabolic alterations linked to *SDH* deficiency, supporting its functional relevance in the context of our findings. We believe that comprehensive methylation–expression–

protein correlations, although highly valuable, fall beyond the scope of the present study, which focuses on defining the global methylation architecture of PPGLs.

5. The gene list that led the authors to conclude altered fructose metabolism should be provided, and key genes validated by qPCR. Concentrations for glucose and fructose supplementation are not provided. Experimental procedures in the Methods section are also insufficiently described. The study would benefit from comparison with validated tumor models such as RS0 or RS1/2, even though these are not of human origin. The increase in SLC2A5 under hypoxia should be compared to other glucose importers such as GLUT-1 and GLUT-8(SLC2A8).

We thank the reviewer for this insightful and constructive comment. In the revised version of the manuscript, we have removed the term *"fructose metabolism"* to better reflect the scope of our study, which specifically focuses on the role of *SLC2A5* (GLUT5) as a fructose transporter, rather than on a comprehensive analysis of fructose metabolic pathways.

The concentrations of glucose and fructose used in the supplementation assays were already provided in the Materials and Methods section; we have now also added them to the corresponding Figure legend for clarity.

Regarding the reviewer's suggestion to compare our findings with validated tumor models such as RS0 or RS1/2, we unfortunately do not have access to these non-human systems. Instead, we have incorporated data from rat pheochromocytoma PC12 cells, for which RNA-seq analyses under different hypoxic time points (12, 24, and 48 h) were already available in our laboratory. These data have now been included as a new Supplementary Figure (Fig. S2) and show that *SLC2A5* is significantly upregulated under hypoxia, supporting its transcriptional activation by HIF signaling.

As further suggested, we also examined the expression of other glucose transporters, *SLC2A1* (GLUT1) and *SLC2A8* (GLUT8). As expected, *SLC2A1* was strongly upregulated by hypoxia in all cell types, consistent with its role as a canonical HIFα target. In contrast, *SLC2A8* was induced by hypoxia only in HeLa and MCF7, but not in PPGL-derived cells where we observed downregulation in PGLa and PGLb but not in PCC cells. Upon succinate treatment, *SLC2A1* expression decreased, while *SLC2A8* remained unchanged in PGLa and showed induction in PCC cells.

Overall, among the transporters analyzed, *SLC2A5* exhibited the most consistent and robust regulation under hypoxia and pseudohypoxia, distinguishing it from canonical glucose transporters and supporting a distinct metabolic role in *SDHB*-deficient tumors. The comparative data have been incorporated into the revised manuscript (Figure 6 and Supplementary Figure S3), and the Results section has been updated accordingly.

6. As SDHB knockout is claimed to cause SLC2A5 upregulation, validation in other cell model systems is crucial.

We agree that validation in additional models is important. In our manuscript, we showed that *SDHB* knockout (KO) in non-tumoral adrenal cells induces *SLC2A5* expression. In response to this comment, and as detailed above, we examined *SLC2A5* expression in an independent pheochromocytoma cell line, where hypoxia led to a time-dependent increase in *SLC2A5*. Furthermore, we generated *SDHB*-knockout (KO) models in HeLa, MCF7, and 786-O cells. Among these, *SLC2A5* upregulation was observed exclusively in the 786-O *SDHB*-KO model, which exhibits constitutive HIF-2α stabilization. Importantly, *SLC2A5* induction in 786-O cells was also recapitulated by hypoxia, further supporting a mechanistic link between SDH dysfunction, pseudohypoxia, and HIF signaling. In contrast, *SDHB* KO HeLa and MCF7 cells did not show *SLC2A5* upregulation. Together, these results demonstrate that the effect is not universal across cell types and support that *SLC2A5* regulation displays a cell-type–specific dependency on *SDHB* loss and HIF-2α signaling. These new results have been included in Figure 6 and are described in the Results section (page 12-13) of the revised manuscript. Accordingly, we have revised the Discussion section to incorporate this point.

7. The analysis of the TCGA dataset shown in Figure 6a is neither described in the main text nor in the Methods section. This analysis should be properly introduced, and its significance explained. What was the rationale for correlating with LDHA, EGLN3, and SLC16A3, given that Pearson's correlations of 0.2–0.4 are considered weak?

We thank the reviewer for noting this issue. The panel referred to as Figure 6a was inadvertently included in the submitted version and should not have been part of that Figure. These correlations were part of preliminary exploratory analyses not directly relevant to the main findings of the study. We have now removed this panel from the revised Figure to maintain consistency and focus on the validated results.

We also appreciate the reviewer's comment regarding the low Pearson's correlation coefficients. Indeed, the observed correlations ($r = 0.2–0.4$) are weak and would not support meaningful biological conclusions in this context, further justifying the removal of these data from the final version of the manuscript.

8. The identity of the AG cells is crucial for interpretation, especially since they were used for SDHB knockdown. In addition to basic characterization, succinate levels in the KD cells should be provided.

We thank the reviewer for this important comment. In the revised manuscript, we have now included additional characterization data for the adrenal gland (AG) cells, including their growth kinetics (doubling time). As mentioned above, we also confirmed the efficiency of our CRISPR/Cas9-mediated *SDHB* knockout by sequencing the targeted genomic region, verifying precise editing at the expected locus.

As mentioned in our response to a previous comment, we have already validated the metabolic consequences of *SDHB* loss, including succinate accumulation and reduced mitochondrial activity, confirming the functional impact of the knockout.

9. The doubling time of the AG cells is not shown in Supplementary Figure 1. Do these cells proliferate? Since they were infected and selected with puromycin, details on their viability and culture longevity are essential.

We confirm that the AG cells do proliferate, albeit at a very slow rate comparable to that of the non-infected cultures. As mentioned above, we are preparing a separate manuscript specifically focused on the establishment and characterization of these primary adrenal cultures and therefore prefer not to include all methodological details here. Nevertheless, we confirm that the cells remain viable over extended culture periods, can be cryopreserved and successfully recovered after thawing. Although viability is slightly reduced post-thaw, they remain suitable for experimental use.

10. Supplementary Figure 1 shows highly variable doubling times among patient-derived PPGL primary cultures. The authors should discuss potential reasons

We acknowledge the variability in doubling times observed among the patient-derived PPGL primary cultures. Although we cannot pinpoint a definitive explanation, such variability is likely multifactorial and may reflect intrinsic differences in tumor genotype, degree of differentiation, or proliferative potential among individual samples. These features are commonly observed in primary cultures derived from patient tumors and are consistent with the well-known biological heterogeneity of tumors.