nature research

Peer Review Information

Journal: Nature Genetics
Manuscript Title: Meningioma DNA methylation groups identify biological drivers and therapeutic vulnerabilities
Corresponding author name(s): Dr David Raleigh

Editorial Notes: Redactions – transferred manuscripts (mention of the other journal): This manuscript has been previously reviewed at another journal. This document only contains reviewer comments, rebuttal and decision letters for versions considered at Nature Genetics. Mentions of the other journal have been redacted.

Reviewer Comments & Decisions:

Decision Letter, initial version:

26th Jul 2021

Dear David,

How are you?

I'm sorry that it's taken so long to return this decision to you. Thank you for your patience.

Your Article, "Meningioma DNA methylation grouping reveals biologic drivers and therapeutic vulnerabilities" has now been seen by 3 of your 4 original reviewers. Please note that Reviewer #1 was unable to re-review the paper.

The reviewers acknowledge your extensive revisions and consider the manuscript to be improved. However, some important points are raised. We are interested in the possibility of publishing your study in Nature Genetics, but would like to consider your response to these concerns in the form of a revised manuscript before we make a final decision on publication.

You'll see that Reviewer #2 has requested that you show more evidence of the role of focal CDKN2A/2B deletions in the hypermitotic subgroup. They've also asked you to include additional data

(which you likely already have) and their other concerns require only textual edits. We note Reviewer #3's concerns about novelty and suitability for the journal, but we are willing to overrule on those points. However, Reviewer #3 remain unconvinced by your clustering strategy. We have discussed these concerns extensively as a team and on balance, particularly given that you have garnered support from the remaining reviewers, we believe that we can move forward without any further analyses or experimentation, but we do ask that you go through the text with Reviewer #3's comments in mind and tone down your language wherever possible. I appreciate that you have already done this, but I ask that you do this one last time.

Our aim is to assess your revision in-house, but we will (briefly) return to one or more reviewers if we are unable to do so.

We therefore invite you to revise your manuscript taking into account all reviewer and editor comments. Please highlight all changes in the manuscript text file. At this stage we will need you to upload a copy of the manuscript in MS Word .docx or similar editable format.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

When revising your manuscript:

*1) Include a "Response to referees" document detailing, point-by-point, how you addressed each referee comment. If no action was taken to address a point, you must provide a compelling argument. This response will be sent back to the referees along with the revised manuscript.

*2) If you have not done so already please begin to revise your manuscript so that it conforms to our Article format instructions, available
here.
Refer also to any guidelines provided in this letter.

*3) Include a revised version of any required Reporting Summary:
https://www.nature.com/documents/nr-reporting-summary.pdf
It will be available to referees (and, potentially, statisticians) to aid in their evaluation if the manuscript goes back for peer review.
A revised checklist is essential for re-review of the paper.

Please be aware of our guidelines on digital image standards.

Please use the link below to submit your revised manuscript and related files:

[REDACTED]

Note: This URL links to your confidential home page and associated information about manuscripts you may have submitted, or that you are reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

We hope to receive your revised manuscript within four to eight weeks. If you cannot send it within this time, please let us know.

Please do not hesitate to contact me if you have any questions or would like to discuss these revisions further.

Nature Genetics is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit please visit www.springernature.com/orcid.

We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

Sincerely,

Safia Danovi
Editor
Nature Genetics

Please note that the numbering of reviewers remains unchanged from previous rounds.

Reviewers' Comments:

Reviewer #2:
Remarks to the Author:
Choudhury and colleagues have provided a significantly improved revision of their manuscript. Previous claims pertaining to the impact of DNA methylation array data processing using the SeSAMe pipeline have been appropriately toned down throughout the manuscript, as have previously overstated interpretations related to putative genetic alterations of the HLA and CDKN2A/2B loci. Overall, I commend the authors on their very thorough attention to addressing reviewer concerns raised during the previous rounds of peer review and revision.

There are just a few remaining issues that require attention and clarification:

1. The authors have still failed to demonstrate convincing evidence of focal CDKN2A/2B deletion in meningiomas to support the data shown in Figure 4c, EDF 15, and the over-arching theory related to 'genetic mechanisms' underlying the Hypermitotic subgroup of meningiomas. In my previous review, I wrote:

"It would be straightforward to show the actual copy number alterations at the CDKN2A/2B locus by

meningioma subgroup. Inspection of the composite genome-wide CNV plots shown in Extended Data Figure 4a fails to indicate any appreciable evidence for such recurrent deletions."

However, the authors have yet to provide locus-level CNV data to convince the reader that the alleged focal alterations (i.e., deletions) of CDKN2A/2B are legitimate. The composite plots shown in EDF 4a lack sufficient resolution to indicate focal deletions of the locus, despite an apparently higher frequency of chr9p loss seen in the Hypermitotic subgroup. This contrasts with the described HLA locus alterations, which can be appreciated in EDF 4a – although including locus-level CNV data for both HLA and CDKN2A/2B would be preferred. I would urge the authors to show the focal deletions of CDKN2A/2B (and ideally alterations of HLA) in their dataset (i.e., using IGV or an equivalent browser), as a considerable proportion of their description of the Hypermitotic group (or the Immune-enriched group in the case of HLA) hinges on these alleged cell-cycle associated deletions. If a stronger case cannot be made for these deletions, the authors should once again tone down their interpretation of molecular mechanisms underlying this subgroup.

2. In the section, 'Convergent genetic and epigenetic mechanisms misactivate the cell cycle in meningioma', there is a sentence stating: "Differential expression analysis of enhancers and super-enhancers showed Hypermitotic meningiomas were dominated by…". What is being compared here, gene expression profiles of predicted enhancer and super-enhancer targets? The authors should clarify in the narrative because comparing enhancers/super-enhancers alone will of course not yield gene expression differences.

3. In the sentence, 'There were more CNVs in Hypermitotic meningiomas compared to groups…', presumably the authors meant to write, '…compared to other groups…'?

4. Related to the reclassification of M10G meningioma cells following CDKN2A or CDKN2B suppression (Figure 4e), the authors should include the SVM data that supports the apparent reclassification from the Immune-enriched to Hypermitotic molecular subgroup.

Reviewer #3:
Remarks to the Author:
The authors have gone to great lengths to address my previous criticisms. Did they address them satisfactorily? Unfortunately, my answer is once again no (despite the massive amount of work in responding to my concerns):
First of all, it is clear that the identification of a cell-proliferation subgroup of bad outcome is not dependent on the use of DNAm profiles. This follows from previous papers reporting such associations. The authors contend that their classification is "better", and "novel" as this is shown through a DNAm-driven analysis, or that it could not have been obtained using gene-expression. However, according to Extended Data Fig.16 I observe an absolutely excellent agreement between the DNAm-based and mRNA-expression based classification. For instance, the hypermitotic class is almost identical, and also the immune-enriched and Merlin-intact clusters are very well recapitulated by the mRNA-based clustering. If anything, the mRNA-clustering reveals further substructure to the Merlin-intact and immune-enriched groups. Instead, the authors imply that the mRNA-based classification does not recapitulate well the DNAm-based one, but the authors argument is based on the false and unproven assumption that the DNAm-based classification is the gold-standard or correct one. If the authors were to do the reverse-analysis, one would ,using the authors same argumentation, conclude that the

mRNA-based classification is better.
Having said of all this, I do agree that the novel classification is interesting and of potential clinical value. I also agree that the authors have revealed distinct molecular mechanisms converging on this hypermitotic phenotype, which is interesting. However, these are not really groundbreaking novel discoveries in cancer generally. We already know about convergent molecular mechanisms from a myriad of TCGA papers…Just because the authors have shown this in a new cancer-type (meningioma), this does not in my opinion justify publication in a journal like Nat Genet, but in a clinically-oriented journal, where the clinically relevant questions would undergo better scrutiny. In order to demonstrate that the novel classification is better over others would require more independent datasets (one independent cohort is not enough- this is a key lesson we should have learned from 2 decades of omics research!). I completely understand that it is impossible for the authors to collect another independent cohort, but this is what would be required if one wishes to make statements about a classification being "better".
My other major concern was that the authors were overemphasizing the need to do a CNV-adjusted DNAm analysis to obtain the findings reported here. The author have responded repeatedly by saying that they have "rephrased" everything as "a hypothesis". Having read the new version of the paper, I did not appreciate how this changes the tone or the potentially misleading implications, as also emphasized by the other 3 reviewers. The authors are still very clearly implying that the CNV-adjustment is critical or that this leads to a "better" classification than the one based on minfi. They go to great lengths to argue that the 3-cluster Sesame solution is "better" than the 4-cluster minfi solution. However, all the arguments put forward by the authors are entirely subjective, and not grounded on scientific objectiveness. Let me give one example to illustrate the point: in Response Fig.11, they compare the KM curves for the Sesame and minfi solutions, and claim a "better model" for Sesame. However, the authors are using a very subjective criterion based on "demanding" prognostic separability of the 3 clusters. But how about if we use another criterion: let us argue that the minfi solution is better because clusters C & D (red & orange) exhibit a BIGGER prognostic separability than the Merlin-intact and Hypermitotic groups as shown in Response Fig.11. So, if we were to use this metric, then the minfi 4-cluster solution would be better. Moreover, the more clusters we have less, the less likely it is that all clusters exhibit statistically significant differential prognosis because we have less power… Indeed, the minfi 4-cluster solution displays prognostic separability for 3 clusters, the same number as Sesame….and with one differential analysis being close to statistical significance (P=0.06). It is probably marginal because the authors are still underpowered…In summary, personally, I think it is futile for the authors to present countless subjective and biased arguments to favour one solution over another.
With regard to my technical concerns regarding the PCA, feature selection and optimal number of clusters, here too, I remain unconvinced. It is very clear that the choice of top 3 PCs is suboptimal according to the Ext.Data.Fig.2a. This plot clearly suggests that the number of significant PCs is 7, not 3. Moreover, there is a very strong logical reason why if you do subsequent consensus clustering over the dominant probes of PC1-3, that you are more likely to get 3 optimal clusters out: PCs reflect patterns of non-redundant orthogonal variation in the data that are iteratively maximal, with the probes driving a given PC exhibiting very strong correlations whilst minimizing the correlations with probes in other PCs. Thus, it is in effect a statistical theorem that your solution is more likely to yield an optimal number of clusters that is 3, specially when selecting such a low number of PCs. Once again, I think that it is futile for the authors to argue that their choice of 3 PCs is the "biologically correct" solution and to imply that the CNV-adjustment analysis is somehow critical for this. I sense that the authors are "trapped" in their false belief that the clustering solution they have found is "better".

Reviewer #4:
Remarks to the Author:
In this second revision, the authors have toned down some of their statements and included a number of clarifications. The final manuscript reads well and does the datasets analyzed justice in the description of the results. I am in support of publication.

Author Rebuttal to Initial comments

Editor comments

You'll see that Reviewer #2 has requested that you show more evidence of the role of focal CDKN2A/2B deletions in the hypermitotic subgroup. They've also asked you to include additional data (which you likely already have) and their other concerns require only textual edits. We note Reviewer #3's concerns about novelty and suitability for the journal, but we are willing to overrule on those points. However, Reviewer #3 remain unconvinced by your clustering strategy. We have discussed these concerns extensively as a team and on balance, particularly given that you have garnered support from the remaining reviewers, we believe that we can move forward without any further analyses or experimentation, but we do ask that you go through the text with Reviewer #3's comments in mind and tone down your language wherever possible. I appreciate that you have already done this, but I ask that you do this one last time.

Thank you for the editorial discussion guiding the decision on our study at *Nature Genetics*. As described below, we have made textual edits requested by Reviewer #2, and provided locus-level data for the *CDKN2A/B* and *HLA* loci that were previously summarized in Fig. 3f and Fig. 4c, and provided in Revision Fig. 3 and Revision Fig. 4 at the time of our first revision at *[REDACTED]*. In light of these pre-existing summary statistics and data provided in our response letter at *[REDACTED]*, our interpretation of the new comments from Reviewer #2 is that presentation of locus-level data are important to show to the reader the distribution of focal *CDKN2A/B* or *HLA* deletions across meningioma DNA methylation groups. To that end, we have generated a new table (Extended Data Table 10) for this revision that provides the locus-level data we previously displayed in Revision Fig. 3 and Revision Fig. 4 alongside the DNA methylation group of each meningioma with CNVs targeting *CDKN2A/B* or *HLA*. Of note, the referee suggested that we use IGV to display these data, but IGV can only be used to visualize alignments from exome or transcriptomic data, not inferred segments from DNA methylation profiles.

With respect to Reviewer #3, we have further moderated the tone and strength of our interpretations, and added additional clarification to our figure legends to better explain our methods (and to avoid confusing our readers). We appreciate our prior efforts to moderate the strength of our interpretations were noted by the editorial board, and deemed to be sufficient by Reviewer #2 and Reviewer #4 (and also by Reviewer #1 on our prior revision). In this revision, we have highlighted the sections containing our prior and new moderated statements in yellow, along with the other areas of revision described below. In recognition of Reviewer #3's persistent concerns, we have also articulated that "it is likely other bioinformatic approaches using DNA methylation profiles or other genomic data will reveal new insights into meningioma biology in the

future" in our revised Discussion. We welcome any and all editorial guidance with respect to our tone, or the clarity of our approach, in summarizing our findings and interpretations.

Reviewer #2

Choudhury and colleagues have provided a significantly improved revision of their manuscript. Previous claims pertaining to the impact of DNA methylation array data processing using the SeSAMe pipeline have been appropriately toned down throughout the manuscript, as have previously overstated interpretations related to putative genetic alterations of the HLA and CDKN2A/2B loci. Overall, I commend the authors on their very thorough attention to addressing reviewer concerns raised during the previous rounds of peer review and revision.

Thank you for the positive assessment of our revisions, particularly with respect to the strength of our interpretations and conclusions.

There are just a few remaining issues that require attention and clarification:

1. The authors have still failed to demonstrate convincing evidence of focal CDKN2A/2B deletion in meningiomas to support the data shown in Figure 4c, EDF 15, and the over-arching theory related to 'genetic mechanisms' underlying the Hypermitotic subgroup of meningiomas. In my previous review, I wrote:

"It would be straightforward to show the actual copy number alterations at the CDKN2A/2B locus by meningioma subgroup. Inspection of the composite genome-wide CNV plots shown in Extended Data Figure 4a fails to indicate any appreciable evidence for such recurrent deletions."

However, the authors have yet to provide locus-level CNV data to convince the reader that the alleged focal alterations (i.e., deletions) of CDKN2A/2B are legitimate. The composite plots shown in EDF 4a lack sufficient resolution to indicate focal deletions of the locus, despite an apparently higher frequency of chr9p loss seen in the Hypermitotic subgroup. This contrasts with the described HLA locus alterations, which can be appreciated in EDF 4a – although including locus-level CNV data for both HLA and CDKN2A/2B would be preferred. I would urge the authors to show the focal deletions of CDKN2A/2B (and ideally alterations of HLA) in their dataset (i.e., using IGV or an equivalent browser), as a considerable proportion of their description of the Hypermitotic group (or the Immune-enriched group in the case of HLA) hinges on these alleged cell-cycle associated deletions. If a stronger case cannot be made for these deletions, the authors should once again tone down their interpretation of molecular mechanisms underlying this subgroup.

As IGV can only be used to visualize alignments from exome or transcriptomic data, not inferred segments from DNA methylation profiles, we have generated Extended Data Table 10 for this revision. This new table provides all locus-level *CDKN2A/B* or *HLA* deletions across meningioma DNA methylation groups. Moreover, we have provided numeric breakdowns of all CNVs targeting these loci in our revised Results and Figure Legends.

In the section entitled "*HLA* expression and lymphatic vessels underlie meningioma immune enrichment," we state "CNVs amplifying the polymorphic locus were more frequent in Immune-enriched meningiomas (30%, n=64 of 216) compared to Merlin-intact (17%, n=33 of 192) or Hypermitotic meningiomas (18%, n=28 of 157) (p=0.0033, Chi-squared test). Conversely, CNVs

deleting the polymorphic locus were less frequent in Immune-enriched meningiomas (11%, n=25 of 216) compared to Merlin-intact (15%, n=28 of 192) or Hypermitotic meningiomas (21%, n=33 of 157) (p=0.0412, Chi-squared test).” In the legend displaying these data in Fig. 3f, we state “Meningioma DNA methylation analysis of CNVs containing the *HLA* genes… across Merlin-intact (n=192 meningiomas, 221 losses in 59 meningiomas, 147 gains in 43 meningiomas), Immune-enriched (n=216 meningiomas, 158 losses in 44 meningiomas, 258 gains in 65 meningiomas), and Hypermitotic (n=157 meningiomas, 270 losses in 58 meningiomas, 125 gains in 29 meningiomas) DNA methylation groups (Chi-squared test).”

In the section entitled “Convergent genetic and epigenetic mechanisms misactivate the cell cycle in meningioma”, we state “Focal CNVs deleting *CDKN2A/B* were identified in 7% of meningiomas (n=37 of 565), but were enriched in Hypermitotic meningiomas (15%, n=24 of 157) compared to Merlin-intact (4%, n=8 of 192) or Immune-enriched meningiomas (2%, n=5 of 216)…”. In the legend displaying these data in Fig. 4c, we state “Meningioma DNA methylation analysis of chromosome segment copy number loss containing the *CDKN2A/B* locus across Merlin-intact (n=8 of 192 meningiomas, 4%), Immune-enriched (n=5 of 216 meningiomas, 2%), and Hypermitotic (n=24 of 157 meningiomas, 15%) DNA methylation groups (n=565, Chi-squared test).”

In sum, we are hopeful these granular data will make our findings and analyses clear and well-represented to our readers, but we continue to welcome any and all guidance with respect to the presentation of these findings.

2. In the section, ‘Convergent genetic and epigenetic mechanisms misactivate the cell cycle in meningioma’, there is a sentence stating: “Differential expression analysis of enhancers and super-enhancers showed Hypermitotic meningiomas were dominated by…”. What is being compared here, gene expression profiles of predicted enhancer and super-enhancer targets? The authors should clarify in the narrative because comparing enhancers/super-enhancers alone will of course not yield gene expression differences.

Thank you for bringing this poorly-worded sentence to our attention. The analyses in Extended Data Fig. 14a, b that are described by this sentence were entirely based on our H3K27ac ChIP sequencing experiments (which were integrated in the context of gene expression data in Extended Data Fig. 13, 14c). Thus, we have changed the sentence in question to read “H3K27ac ChIP sequencing analysis of enhancer and super-enhancer availability showed…”. We have also revised the legend corresponding to Extended Data Fig. 14a, b to specifically articulate that enhancer or super-enhancer available was assessed across meningioma DNA methylation groups in these panels.

3. In the sentence, ‘There were more CNVs in Hypermitotic meningiomas compared to groups…’, presumably the authors meant to write, ‘…compared to other groups…’?

Thank you for bringing this error to our attention. Yes, we indeed meant to write “… compared to other groups” in this sentence. We have fixed this error in our revised manuscript.

4. Related to the reclassification of M10G meningioma cells following CDKN2A or CDKN2B suppression (Figure 4e), the authors should include the SVM data that supports the apparent reclassification from the Immune-enriched to Hypermitotic molecular subgroup.

As with many machine learning algorithms and classifiers, SVM does not provide data or details about the classification of individual samples. This approached is described in detail in "An Introduction to Statistical Learning" by Gareth James, Daniela Witten, Trevor Hastie, and Robert Tibshirani (Corrected 7th Printing), a text we now cite in our revised Methods section (Extended Data Reference #14). Based on the aggregate performance of our model across bootstrapped and cross-validated subsets of data from our discovery cohort we calculated the accuracy of our model to be 97.9%, as is additionally described in our Methods. For this revision, we have also provided the performance of our SVM in the second paragraph of our Results in this revision, where we state "A multi-class support vector machine classifier with 97.9% accuracy (95% CI 89.2-99.9%, $p<2.2x10^{-16}$) was constructed...". The accuracy of our SVM is further demonstrated in Extended Data Fig. 2g, where we show sampling distributions of DNA methylation group fractions from our discovery cohort in the context of the observed DNA methylation group fractions from our validation cohort. Extended Data Fig. 2g shows the observed fractions of each DNA methylation group from the validation cohort fall within the sampling distributions from the discovery cohort. Finally, to allow our readers to test and implement this approach independently, we have provided the code for our SVM classifier in our revised Methods, which is:

```
train <- function() {
  library(caret)
  library(e1071)
  library(pROC)

  # construct SVM
  bVals = as.data.frame(t(read.csv("beta_values.csv", row.names=1)))
  clusters = read.csv("groups.csv", row.names=1)

  mlDat = data.frame(clusters = as.character(clusters$x), bVals)
  mlDat$clusters = as.factor(mlDat$clusters)

  # slice data
  set.seed(1234)
  intrain <- createDataPartition(y = mlDat$clusters, p= 0.75, list = FALSE)
  training <- mlDat[intrain,]
  testing <- mlDat[-intrain,]

  # training
  trctrl <- trainControl(method = "repeatedcv", number = 10, repeats = 3)
  svm_Linear <- train(clusters ~., data = training, method = "svmLinear",
                      trControl=trctrl,
                      preProcess = c("center", "scale"),
                      tuneLength = 10)

  # test the model
  test_pred <- predict(svm_Linear, newdata = testing)
  confusionMatrix(test_pred, testing$clusters)
```

```
  saveRDS(svm_Linear, file="svm_linear_classifier.rds")
}

train()
```

Reviewer #3

The authors have gone to great lengths to address my previous criticisms. Did they address them satisfactorily? Unfortunately, my answer is once again no (despite the massive amount of work in responding to my concerns):

First of all, it is clear that the identification of a cell-proliferation subgroup of bad outcome is not dependent on the use of DNAm profiles. This follows from previous papers reporting such associations.

There are no statements or intimations that identifying a group of meningiomas with elevated cell proliferation and/or poor clinical outcomes is dependent on the use of DNA methylation profiling in our revised manuscript. Further, we explicitly acknowledge prior grouping systems (including histologic grading) have identified meningiomas with elevated cell proliferation and poor clinical outcomes in the section entitled "Convergent genetic and epigenetic mechanisms misactivate the cell cycle in meningiomas," where we state "High-grade meningiomas are defined by brisk cell proliferation leading to local recurrence and death in 50-90% of patients[52,53]…" and "FOXM1, a biomarker for meningioma recurrence, drives meningioma cell proliferation, and putative FOXM1 target genes are accessible in the chromatin of meningioma DNA methylation groups with adverse clinical outcomes[6,11,54,55]."

The authors contend that their classification is "better", and "novel" as this is shown through a DNAm-driven analysis, or that it could not have been obtained using gene-expression. However, according to Extended Data Fig.16 I observe an absolutely excellent agreement between the DNAm-based and mRNA-expression based classification. For instance, the hypermitotic class is almost identical, and also the immune-enriched and Merlin-intact clusters are very well recapitulated by the mRNA-based clustering. If anything, the mRNA-clustering reveals further substructure to the Merlin-intact and immune-enriched groups. Instead, the authors imply that the mRNA-based classification does not recapitulate well the DNAm-based one, but the authors argument is based on the false and unproven assumption that the DNAm-based classification is the gold-standard or correct one. If the authors were to do the reverse-analysis, one would, using the authors same argumentation, conclude that the mRNA-based classification is better.

The words "better" and "novel" do not appear in our revised manuscript, and we have also eliminated all statements or intimations that our approach is critical or required for meningioma analyses. Respectfully, the reviewer is mistaken in their interpretation of the heatmap presented in Extended Data Fig. 16a. In this figure, the vertical dendrogram and heatmap are based on RNA sequencing of 200 meningiomas, and the metadata between the vertical dendrogram and the heatmap reflect the DNA methylation group assignments of the same samples. To avoid confusing our readers, we have clarified the organization of these data in the legend corresponding to Extended Data Fig. 16a. As articulated in greater detail in our first response letter at *Nature Genetics*, this figure demonstrates that meningiomas from different DNA

methylation groups are scattered across an unclear optimal number of mRNA-based clusters, which have overlapping clinical outcomes (see Response Fig. 15). In further support of these findings, prior unsupervised meningioma gene expression studies have also only identified groups with redundant, non-unique outcomes (see Response Fig. 16 and PMID 31591222). Thus, it cannot be said that these clustering approaches are identical or recapitulate one another very well. Nevertheless, we have ensured that at no point in our revised manuscript do we claim DNA methylation-based classification of meningiomas is the "gold-standard" or "correct" approach at the exclusion of other bioinformatic strategies.

Having said of all this, I do agree that the novel classification is interesting and of potential clinical value. I also agree that the authors have revealed distinct molecular mechanisms converging on this hypermitotic phenotype, which is interesting. However, these are not really groundbreaking novel discoveries in cancer generally. We already know about convergent molecular mechanisms from a myriad of TCGA papers…Just because the authors have shown this in a new cancer-type (meningioma), this does not in my opinion justify publication in a journal like Nat Genet, but in a clinically-oriented journal, where the clinically relevant questions would undergo better scrutiny. In order to demonstrate that the novel classification is better over others would require more independent datasets (one independent cohort is not enough- this is a key lesson we should have learned from 2 decades of omics research!). I completely understand that it is impossible for the authors to collect another independent cohort, but this is what would be required if one wishes to make statements about a classification being "better".

Thank you for sharing this interpretation of our findings in the context of cancer genomic research. As articulated above, and in our earlier response letters *at [REDACTED]*, we do not contend our approach is "better" than any other -omics strategy. There are 3 instances in our revised manuscript where we have highlight data addressing our central hypothesis. As articulated in the first paragraph of our paper, our central hypothesis is that "controlling for the influence of CNVs on β methylation values, coupled with mechanistic and functional studies, may reveal insights into meningioma biology and inform new treatments for meningioma patients." Below, we articulate the 3 instances in our newly revised manuscript where we have highlighted our data (and moderated interpretations) supporting this hypothesis.

First, at the conclusion of the section entitled "Meningiomas are comprised of 3 DNA methylation groups with distinct clinical outcomes," we state "Thus, controlling for the influence of CNVs on β values may improve meningioma DNA methylation grouping and discrimination of clinical outcomes" and provide bioinformatic and clinical comparisons supporting this hypothesis in Fig. 1, Extended Data Fig. 2, and Extended Data Fig. 5.

Second, at the conclusion of the first paragraph in the section entitled "*HLA* expression and lymphatic vessels underlie meningioma immune enrichment," we state "Differential expression and gene ontology analyses across minfi groups failed to distinguish meningiomas by immune or inflammatory gene expression grams," and provide biologic data supporting this interpretation in Fig. 3 and Extended Data Fig. 7. In further support of these findings, our earlier response letters at *[REDACTED* referenced and described the 4 published meningioma DNA methylation studies that have not controlling for the influence of CNVs on β methylation values, and have not discovered a group of immune enriched meningiomas (much like the minfi analysis in our current study).

Third, in summary of our combined bioinformatic, clinical, and biologic findings across meningioma DNA methylation groups, the second paragraph of our Discussion states "Our study tests the hypothesis that controlling for the influence of CNVs on β methylation values may reveal insights into meningioma biology. In support of this approach, meningioma DNA methylation analysis uncontrolled for the influence of CNVs on β values could not identify an optimal number of meningioma groups (Extended Data Fig. 5b, c), or groups with nonoverlapping differences in clinical outcomes, *NF2* loss, immune enrichment, cell proliferation, and chromosome instability (Extended Data Fig. 5c, 21). Quantifying the signal-to-noise ratio (SNR) of *NF2* loss across meningioma DNA methylation groups, we found an SNR of 5.57 for 3 SeSAMe groups compared to 2.25 for 3 minfi groups."

To further tone down the strength of our interpretations for this revision, and highlight the likely utility of other bioinformatic approaches for understanding meningioma biology, we have modified the ending of the second paragraph of our Discussion to state "Notwithstanding, DNA methylation analyses uncontrolled for the influence of CNVs on β values provides robust classification across brain tumor types[4], and for tumors with minimal chromosome instability, we suspect controlling for the influence of CNVs on β methylation values may provide equivalent results to traditional bioinformatic pipelines. Moreover, it is likely other bioinformatic approaches using DNA methylation profiles or other genomic data will reveal new insights into meningioma biology in the future." We are concerned the myriad mechanistic and functional basic science approaches supporting these bioinformatic findings, which comprise the backbone of our paper's validity across orthogonal techniques, would be unsuitable for a clinical journal.

My other major concern was that the authors were overemphasizing the need to do a CNV-adjusted DNAm analysis to obtain the findings reported here. The author have responded repeatedly by saying that they have "rephrased" everything as "a hypothesis". Having read the new version of the paper, I did not appreciate how this changes the tone or the potentially misleading implications, as also emphasized by the other 3 reviewers.

Respectfully, Reviewer #2 and Reviewer #4 specifically remarked on the satisfactory improvements in the tone of our manuscript in our last revision, and Reviewer #1 did not express concern with this aspect of our study. No other reviewer has expressed concerns that our data or interpretations were misleading at any point during our initial or revised submissions.

The authors are still very clearly implying that the CNV-adjustment is critical or that this leads to a "better" classification than the one based on minfi.

As articulated above, we have ensured the words "better" or "critical" do not appear in our revised manuscript, and we have also eliminated all statements or intimations that our approach is critical or required for meningioma analyses. Rather, we have provided multiple data-driven justifications demonstrating particular aspects of meningioma bioinformatics, clinical outcomes, and biology where controlling for the influence of CNVs on β methylation values may enhance our understanding of meningiomas.

They go to great lengths to argue that the 3-cluster Sesame solution is "better" than the 4-cluster minfi solution. However, all the arguments put forward by the authors are entirely subjective, and not grounded on scientific objectiveness. Let me give one example to illustrate the point: in Response Fig.11, they compare the KM curves for the Sesame and minfi solutions, and claim a

"better model" for Sesame. However, the authors are using a very subjective criterion based on "demanding" prognostic separability of the 3 clusters. But how about if we use another criterion: let us argue that the minfi solution is better because clusters C & D (red & orange) exhibit a BIGGER prognostic separability than the Merlin-intact and Hypermitotic groups as shown in Response Fig.11. So, if we were to use this metric, then the minfi 4-cluster solution would be better. Moreover, the more clusters we have less, the less likely it is that all clusters exhibit statistically significant differential prognosis because we have less power… Indeed, the minfi 4-cluster solution displays prognostic separability for 3 clusters, the same number as Sesame….and with one differential analysis being close to statistical significance (P=0.06). It is probably marginal because the authors are still underpowered…

It is certainly conceivable that DNA methylation groups of meningiomas need not strictly have different clinical outcomes. Indeed, meningiomas DNA methylation groups with overlapping clinical outcomes are all that were previously reported in the only other large-scale meningioma DNA methylation study (PMID 28314689). Respectfully, in the example above the reviewer has focused on a single aspect of our clinical analyses in isolation of the many additional bioinformatic, clinical, and biologic studies we provide and support using orthogonal mechanistic and functional approaches. Moreover, they are incorrect that the separation in local freedom from recurrence between groups C and D in the minfi 4 solution (Extended Data Fig. 5d) is greater than the separation between Merlin-intact and Hypermitotic groups in the SeSAMe solution (Fig. 1c) because the median local freedom from recurrence was not met in either group C or Merlin-intact meningiomas, meaning we do not know what the actual separation is between group C or Merlin-intact meningiomas and group D or Hypermitotic meningiomas, respectively. Even if the differences in clinical outcomes between the selected minfi groups were greater than between SeSAMe groups, this finding would not negate our orthogonal observations that (1) the differences in clinical outcomes between other minfi groups are not statistically significant, and that (2) the biologic differences between minfi groups are not robust (Extended Data Fig. 22).

In summary, personally, I think it is futile for the authors to present countless subjective and biased arguments to favour one solution over another. With regard to my technical concerns regarding the PCA, feature selection and optimal number of clusters, here too, I remain unconvinced. It is very clear that the choice of top 3 PCs is suboptimal according to the Ext.Data.Fig.2a. This plot clearly suggests that the number of significant PCs is 7, not 3. Moreover, there is a very strong logical reason why if you do subsequent consensus clustering over the dominant probes of PC1-3, that you are more likely to get 3 optimal clusters out: PCs reflect patterns of non-redundant orthogonal variation in the data that are iteratively maximal, with the probes driving a given PC exhibiting very strong correlations whilst minimizing the correlations with probes in other PCs. Thus, it is in effect a statistical theorem that your solution is more likely to yield an optimal number of clusters that is 3, specially when selecting such a low number of PCs. Once again, I think that it is futile for the authors to argue that their choice of 3 PCs is the "biologically correct" solution and to imply that the CNV-adjustment analysis is somehow critical for this. I sense that the authors are "trapped" in their false belief that the clustering solution they have found is "better".

Respectfully, the reviewer is mistaken in their interpretation of the elbow plot displayed in Extended Data 2a. The data in this figure demonstrate a large drop in the explained variance among meningiomas from 3 to 4 PCs, suggesting subsequent PCs do not substantially contribute to the variation among tumors in our study. Said another way, we excluded PC4 and subsequent

PCs because they each contributed to less than 5% of the total variance among tumors in our study, and inclusion of these PCs could have introduced noise into subsequent analyses. To avoid confusing our readers, we have clarified these data in the legend corresponding to Extended Data Fig. 2a in our revised manuscript. Further, we have specifically articulated this aspect of our approach in the Methods section of our revised manuscript. In support of our strategy, we found k-means consensus clustering using probes from 4 PCs (or more) resulted in less stable clusters than k-means consensus clustering with probes from 3 PCs (Extended Data Fig. 2b). Although it is certainly possible the number of clusters may match the number of PCs for some biologic entities, the number of PCs selected was independent of the number of clusters identified in our data. A simple demonstration of this interpretation is that 3 PCs were also used for our non-SeSAMe (minfi) analyses, which did not reveal 3 optimal or clean clusters (Extended Data Fig. 5b, c). To show this another way, we repeated our consensus clustering with probes from 2 PCs versus 3 PCs versus 4 PCs, and found that using probes from 2 PCs also generated a solution comprised of 3 meningioma DNA methylation groups that was qualitatively similar to the optimized solution using probes from 3 PCs (Extended Data Fig. 2b). To compare strategies based on 2 PCs versus 3 PCs quantitatively, we generated cumulative distribution functions for 3 meningioma DNA methylation groups comprised of probes from 2 PCs versus 3 PCs, which demonstrated 3PCs produced a superior clustering strategy (Extended Data Fig. 2c). These supporting analyses notwithstanding, we have also ensured the words “better” or “biologically correct” do not appear in our revised manuscript, and we have also eliminated all statements or intimations that our bioinformatic approach is critical or required for meningioma analyses.

Reviewer #4

In this second revision, the authors have toned down some of their statements and included a number of clarifications. The final manuscript reads well and does the datasets analyzed justice in the description of the results. I am in support of publication.

Thank you for the positive assessment of our revisions, and support for the publication of our study.

Decision Letter, first revision:

14th Sep 2021

Dear David,

I hope that you are well

Reviewer #2 has submitted their report and I’m afraid that they’ve flagged some serious problems with the copy number analysis for the CDKN2A/B and HLA loci. As you’ll see from their report and their own analysis of the data (detailed in the attached pdf), they are troubled by the lack of alignment between the data and the text. In their opinion, the genetic evidence for your subgroups is simply insufficient, as they believe the analysis to be seriously flawed.

From our point of view, their report is extremely concerning and as things stand, we are not able to proceed with publication as I'd hoped. As you know, robustness of data is an absolute priority for us and it is not something that we will compromise on. With that said, we are willing to give you a chance to address these serious issues but we would expect all the concerns of Reviewer #2 to be completely addressed, together with a broader reanalysis of the data to ensure the overall integrity of the findings. To manage expectations, we will not pursue the paper any further if the re-analyses significantly dilute the conclusions, or if the revisions do not fully satisfy Reviewer #2.

If you choose to revise the manuscript, please highlight all changes in the manuscript text file. At this stage we will need you to upload a copy of the manuscript in MS Word .docx or similar editable format.

When revising your manuscript:

*1) Include a "Response to referees" document detailing, point-by-point, how you addressed each referee comment. If no action was taken to address a point, you must provide a compelling argument. This response will be sent back to the referees along with the revised manuscript.

*2) If you have not done so already please begin to revise your manuscript so that it conforms to our Article format instructions, available
here.
Refer also to any guidelines provided in this letter.

*3) Include a revised version of any required Reporting Summary:
https://www.nature.com/documents/nr-reporting-summary.pdf
It will be available to referees (and, potentially, statisticians) to aid in their evaluation if the manuscript goes back for peer review.
A revised checklist is essential for re-review of the paper.

Please be aware of our guidelines on digital image standards.

Please use the link below to submit your revised manuscript and related files:

[READCTED]

Note: This URL links to your confidential home page and associated information about manuscripts you may have submitted, or that you are reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

I appreciate that this news will come as a disappointment but I hope that you can understand my editorial position. I would suggest that you take a few days to consult with your co-authors about next steps. We can discuss things like timelines after that if helpful.

Sincerely,

Safia

Safia Danovi
Senior Editor
Nature Genetics

Reviewers' Comments:

Reviewer #2:
Remarks to the Author:
Choudhury and colleagues have attempted to address my persistent concerns regarding their interpretation of CNVs allegedly targeting the CDKN2A/B locus in the Hypermitotic subgroup and the HLA locus in the Immune-enriched subgroup of meningioma. However, there a numerous significant concerns regarding their rebuttal that I am obligated to summarize below.

For clarification, these concerns do not constitute 'new comments'. I have had significant concerns regarding the authors' interpretation of their CNV data since my original review of this manuscript when it was first under consideration [REDACTED]

'Multiple interpretational issues, including those related to the inference of genetic alterations and their significance are also a source of concern.'

Summary of primary concerns based on the current iteration of this manuscript:

1. In their rebuttal to my request to 'show the focal deletions of CDKN2A/2B (and ideally alterations of HLA) in their dataset (i.e., using IGV or an equivalent browser), as a considerable proportion of their description of the Hypermitotic group (or the Immune-enriched group in the case of HLA) hinges on these alleged cell-cycle associated deletions.', the authors state: 'As IGV can only be used to visualize alignments from exome or transcriptomic data, not inferred segments from DNA methylation profiles, we have generated Extended Data Table 10 for this revision.'

This statement is false. IGV, and many other genome browsers and bioinformatic tools, can be used to visualize CNV data, irrespective of platform. If it were not possible to visualize CNV segments derived from methylation array data, how did the authors prepare the genome-wide CNV plots included in Extended Data Figure 4a? As a reviewer, it is concerning that despite my repeated requests to show the 'real' data here, the authors have repeatedly evaded my request. Instead, they have included Extended Data Table 10 which, according to the authors, 'provides all locus-level CDKN2A/B or HLA deletions across meningioma DNA methylation groups. Moreover, we have provided numeric breakdowns of all CNVs targeting these loci in our revised Results and Figure Legends.'

To show the authors that IGV can be used to visualize CNV segments, I provide IGV output of the CNV segments described in Extended Data Table 10 for CDKN2A/2B in the attachment.

2. Upon quick inspection of the visualized CDKN2A/2B locus, based on the CNV segments provided by the authors in Extended Data Table 10, there appears to be far fewer focal CNV events overlapping

this locus than claimed in the text. Specifically, the authors write: "Focal CNVs deleting CDKN2A/B were identified in 7% of meningiomas (n=37 of 565), but were enriched in Hypermitotic meningiomas (15%, n=24 of 157) compared to Merlin-intact (4%, n=8 of 192) or Immune-enriched meningiomas (2%, n=5 of 216)…". In the legend displaying these data in Fig. 4c, we state "Meningioma DNA methylation analysis of chromosome segment copy number loss containing the CDKN2A/B locus across Merlin-intact (n=8 of 192 meningiomas, 4%), Immune-enriched (n=5 of 216 meningiomas, 2%), and Hypermitotic (n=24 of 157 meningiomas, 15%) DNA methylation groups (n=565, Chi-squared test)."

In Extended Data Table 10 (provided in the CDKN2A/2B tab), there are 31 data rows, corresponding to 22 unique individual sample IDs. Overlapping the provided segments with the CDKN2A/2B locus coordinates (provided in the Key tab), there are 9 Hypermitotic (not 24), 1 Merlin-intact (not 8), and 3 Immune-enriched (not 5) samples with deletions overlapping this locus, and 13 in total (not 37). Beyond these highly discrepant deletion counts between what is quoted and summarized in the manuscript and what is actually provided in Extended Data Figure 10, the fact that several sample IDs were apparently counted multiple times, and most of the provided segments are distal to the CDKN2A/2B locus rather than overlapping it, significantly reduces confidence in the overall data integrity presented here.

3. Considering the issues raised above concerning CNVs at the CDKN2A/2B locus, similar inspection of the HLA deletions and gains was undertaken using the data provided in Extended Data Figure 10. Specifically, the authors write: "CNVs amplifying the polymorphic locus were more frequent in Immune-enriched meningiomas (30%, n=64 of 216) compared to Merlin-intact (17%, n=33 of 192) or Hypermitotic meningiomas (18%, n=28 of 157) (p=0.0033, Chi-squared test). Conversely, CNVs deleting the polymorphic locus were less frequent in Immune-enriched meningiomas (11%, n=25 of 216) compared to Merlin-intact (15%, n=28 of 192) or Hypermitotic meningiomas (21%, n=33 of 157) (p=0.0412, Chi-squared test)." In the legend displaying these data in Fig. 3f, we state "Meningioma DNA methylation analysis of CNVs containing the HLA genes… across Merlin-intact (n=192 meningiomas, 221 losses in 59 meningiomas, 147 gains in 43 meningiomas), Immune-enriched (n=216 meningiomas, 158 losses in 44 meningiomas, 258 gains in 65 meningiomas), and Hypermitotic (n=157 meningiomas, 270 losses in 58 meningiomas, 125 gains in 29 meningiomas) DNA methylation groups (Chi-squared test)."

In Extended Data Table 10 (provided in the HLA gains tab), there are 140 data rows, corresponding to 137 unique sample IDs. According to the numbers provided above, there should be n=125 samples with focal gain of HLA genes (64 Immune-enriched, 33 Merlin-intact, and 28 Hypermitotic). Overlapping the provided segments with the HLA locus coordinates (provided in the Key tab), there are 65 Immune-enriched (not 64), 42 Merlin-intact (not 33), and 26 Hypermitotic (not 28) samples with focal gains, and 133 in total (not 125).

Similarly, in Extended Data Table 10 (provided in the HLA deletions tab), there are 196 data rows, corresponding to 168 unique sample IDs. According to the numbers provided above, there should be n=86 samples with focal deletion of HLA genes (25 Immune-enriched, 28 Merlin-intact, and 33 Hypermitotic). Overlapping the provided segments with the HLA locus coordinates (provided in the Key tab), there are 49 Immune-enriched (not 25), 46 Merlin-intact (not 28), and 38 Hypermitotic (not 33) samples with focal deletions, and 133 in total (not 86).

A summary of these discrepancies as they relate to the reported HLA gains and deletions in the

manuscript text, figure legend, and extended data table is provided in the attachment (p-values calculated using the Chi-squared test).

Overall, the lack of alignment of the reported CNV annotations in the manuscript narrative with the copy number segment data provided in Extended Data Table 10, the latter of which should serve as the basis for the narrative and corresponding data presented throughout the manuscript and supplement, instills considerable concern with regards to the integrity of the data analysis and interpretation of the genetic findings reported in this study. As an example of this concern, the HLA deletions do not appear to be significantly different between the 3 subgroups as reported in the supporting figures and narrative. Thus, it becomes exceedingly difficult for one to maintain confidence in this report.

Author Rebuttal, first revision:

Reviewer #2

Choudhury and colleagues have attempted to address my persistent concerns regarding their interpretation of CNVs allegedly targeting the CDKN2A/B locus in the Hypermitotic subgroup and the HLA locus in the Immune-enriched subgroup of meningioma. However, there a numerous significant concerns regarding their rebuttal that I am obligated to summarize below.

We are simultaneously grateful and embarrassed that the reviewer identified the incongruities between the version of Extended Data Table 10 provided in our previous submission and our analyses of CNVs containing the *CDKN2A/B* or *HLA* loci summarized in the text, figures, and figure legends of our manuscript. We have identified the root cause of these incongruities, and have provided the correct the data in a new version of Extended Data Table 10 (now re-numbered Extended Data Table 2). To begin, we would like to highlight 2 important findings:

1. The version of Extended Data Table 10 we previously provided was generated independent of our CNV analyses across meningioma DNA methylation groups, and we unwisely failed to cross-check our data. Thus, correcting our embarrassing mistake in this table did not dilute the results or the conclusions of our manuscript.

2. Unfortunately, we displayed meningioma CNVs <5 Mb on chromosomes 6 or 9 in the version of Extended Data Table 10 we previously provided, rather than CNVs (focal or broad) containing the *CDKN2A/B* or *HLA* loci. Many meningiomas had more than one CNV on chromosomes 6 or 9 (which is why some samples were duplicated), and many CNVs did not contain the genes of interest (which is why some of the CNVs were non-overlapping with the *CDKN2A/B* or *HLA* loci). We also mistakenly included CNVs from 4 meningiomas (M186, M95, QM367, and QM54) in the HLA_gains tab of Extended Data Table 10 containing chromosome 6 segments that were >5 Mb. Thus, our description of the data in Extended Data Table 10 in our previous response letter ("This new table provides all locus-level *CDKN2A/B* or *HLA* deletions across meningioma DNA methylation groups") was entirely incorrect and horribly misleading.

We are beyond embarrassed by this mistake, and we are forever grateful the reviewer has agreed to consider our manuscript once more. We hope the data provided below and elsewhere in our current submission will confirm that our mistake was one of human error, rather than deception. Nevertheless, to ensure the integrity of our data and the scientific record, we have undertaken a broad re-analysis of our findings for this revision. In addition to providing the correct data in Extended Data Table 2, we have also adjusted the polymorphic *HLA* locus CNV analyses summarized in the text, figures, and figure legends of our manuscript to be consistent with CNV analyses at other loci. Importantly, these adjustments have clarified and standardized our CNV analyses across all loci of interest, and have also not diluted the results or the conclusions of our manuscript.

Broadly, our analyses of CNVs containing *CDKN2A/B, HLA*, or other genes of interest were inclusive of both focal and non-focal CNVs amplifying or deleting entire genes of interest. We recognize definitions of CNV focality lack consensus. Many studies do not define CNV focality with precision, and those that do have definitions ranging from less than 3 Mb (PMID 20593488) to less than 98% of a chromosome arm (PMID 21527027). Considering this confusing landscape, we decided to use a cutoff of 5 Mb to define "focal" deletions in Extended Data Table 10. The CNV analyses summarized in the text, figures, and figure legends of our manuscript were never based on this admittedly arbitrary definition. Moreover, how best to define "focal" CNVs containing single genes of different sizes (*CDKN2A, CDKN2B, NF2,* etc.) versus a polymorphic locus containing 4+ genes (*HLA-DRB5, HLA-DRB1, HLA-DQA1,* and *HLA-DQB1*) is unclear. For meningiomas, non-focal CNVs deleting chromosome 22q segments containing *NF2* are widely recognized as having biologic significance due (at least in part) to deletion of *NF2* despite the fact that these deletions are often broad. Indeed, 350 of the 351 copy number deletions containing *NF2* in our study were larger than 5 Mb. Thus, we decided it would be appropriate to combine and quantify focal plus non-focal CNVs containing other loci of biologic interest across meningioma DNA methylation groups, provided we could orthogonally corroborate the biologic significance of genes of interest encompassed by these loci (an objective we accomplished for *NF2, HLA, CDKN2A/B,* and *USF1* using biochemical, single-cell, mechanistic, and functional approaches). For consistency and to ensure we do not overstate our findings, we do not claim anywhere in the text, figures, or figure legends of our revised manuscript that focal CNVs are uniquely responsible for our findings, and all analyses summarized in the main text involve all CNVs containing all loci of interest in their entirety. We would be happy to revisit these decisions if advisable by the reviewer, or to test any preferred definition(s) of CNV focality across meningioma DNA methylation groups to ensure the integrity of our data. In the interim, we have clarified this approach in the text and methods of our revised manuscript. We have also included a secondary analysis of CNVs <5 Mb containing the *CDKN2A/B* or polymorphic *HLA* loci in our methods, corroborating our broader findings. This secondary analysis is summarized on the last page of this response.

CNVs containing *HLA* genes or *USF1* have not been reported across meningioma DNA methylation groups, but prior observational studies focusing on *CDKN2A/B* deletions in meningiomas (PMID 32642869 and 31729637, references 66 and 70 in our revised manuscript) have not described their methods with precision (Entire locus? Partial locus? Focal deletion? Broad deletion?). Considering the resolution of CNVs derived from Illumina 850k DNA methylation arrays (which were used in our study and are based on probe locations, rather than sequencing reads), we decided the most parsimonious and transparent approach would be to quantify and

report only CNVs containing entire loci of interest. We would also be happy to revisit this decision if advisable by the reviewer, and we apologize for not clarifying this aspect of our approach (a mistake we have fixed in the text and methods of our revised manuscript). As an added sanity check, a prior study found *CDKN2A* deletions in 4.9% of meningiomas (PMID 32642869), and using focal plus non-focal deletions of the entire locus (37 of 565 meningiomas, 6.5%), our study revealed a similar frequency of *CDKN2A/B* deletions in human meningiomas.

For clarification, these concerns do not constitute 'new comments'. I have had significant concerns regarding the authors' interpretation of their CNV data since my original review of this manuscript when it was first under consideration [REDACTED]: 'Multiple interpretational issues, including those related to the inference of genetic alterations and their significance are also a source of concern.'

We agree. These concerns are not new, and we are ashamed they remain unaddressed. We attempted to do so at the time of our initial revision *[REDACTED]* by providing Revision Fig. 3 for the *HLA* locus, and Revision Fig. 4 for the *CDKN2A/B* locus. In retrospect, we realize these figures did not provide sufficiently zoomed-in plots of specific loci like the ones generated by IGV, nor did we indicate the DNA methylation group of individual meningiomas. We again attempted to address these concerns at the time of our first revision at *Nature Genetics* by (1) removing our prior analysis of the entire *HLA* locus, (2) focusing only on *HLA* genes with enriched expression by both single-cell and bulk RNA sequencing in Immune-enriched meningiomas compared to other DNA methylation groups, and (3) providing a breakdown of copy number amplifications or deletions containing individual *HLA* genes, the polymorphic *HLA* locus, or the *CDKN2A/B* locus in the text and figure legends of our manuscript. In re-assessing these analyses on pages 12-15 of our response letter from that time (ChoudhuryNatGenet_Response_v10), we are gratified to confirm our prior analyses were accurate. Subsequently, we were overjoyed to read the reviewer's assessment of our "thorough attention to addressing reviewer concerns raised during the previous rounds of peer review and revision." More recently, we were crestfallen to consider the grievous implications of the wrong data we provided in Extended Data Table 10. We offer our unequivocal apologies for this unacceptable, sloppy error that (now corrected) we hope will not prevent our broader findings from reaching the readership of *Nature Genetics*. That being said, we would not want *any* of our findings to reach press unless every as aspect of our study was watertight and entirely accurate. Thus, we are grateful for the reviewer's careful attention to detail and dedication to peer review. Acknowledging this process has already taken so much of the reviewer's time, we remain steadfast to our commitment to ensure all concerns are completely addressed.

It is our overwhelming hope that our clarifications below, and the correct data provided in Extended Data Table 2, will address these remaining concerns. We agree our errors in Extended Data Table 10 should not instill confidence (as the reviewer fairly articulates below). We deeply regret our missteps. Looking beyond our immediate professional embarrassment, we are immensely grateful the reviewer has so carefully evaluated our data. We are inordinately disappointed to have submitted a manuscript containing incongruous data for peer review, but it is perhaps even more horrifying to consider the scientific ramifications of publishing such a paper. Thank you for helping us work through these problems to ensure the integrity of the scientific record.

1. In their rebuttal to my request to 'show the focal deletions of CDKN2A/2B (and ideally alterations

of HLA) in their dataset (i.e., using IGV or an equivalent browser), as a considerable proportion of their description of the Hypermitotic group (or the Immune-enriched group in the case of HLA) hinges on these alleged cell-cycle associated deletions.', the authors state: 'As IGV can only be used to visualize alignments from exome or transcriptomic data, not inferred segments from DNA methylation profiles, we have generated Extended Data Table 10 for this revision.'

This statement is false. IGV, and many other genome browsers and bioinformatic tools, can be used to visualize CNV data, irrespective of platform. If it were not possible to visualize CNV segments derived from methylation array data, how did the authors prepare the genome-wide CNV plots included in Extended Data Figure 4a? As a reviewer, it is concerning that despite my repeated requests to show the 'real' data here, the authors have repeatedly evaded my request. Instead, they have included Extended Data Table 10 which, according to the authors, 'provides all locus-level CDKN2A/B or HLA deletions across meningioma DNA methylation groups. Moreover, we have provided numeric breakdowns of all CNVs targeting these loci in our revised Results and Figure Legends.'

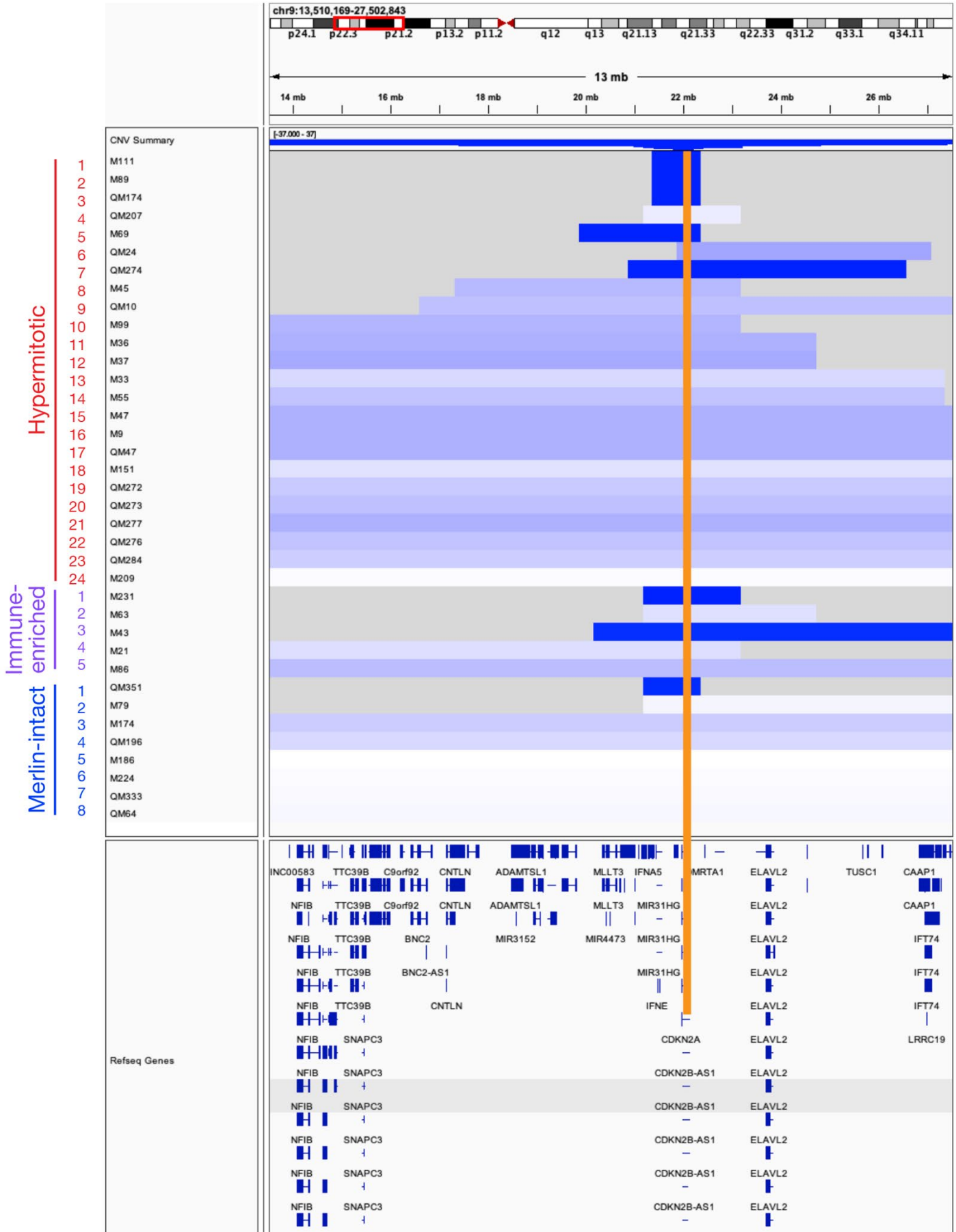
To show the authors that IGV can be used to visualize CNV segments, I provide IGV output of the CNV segments described in Extended Data Table 10 for CDKN2A/2B in the attachment.

We apologize for our lack of understanding of IGV. We also apologize our prior response and revision were interpreted as an attempt at evasion. This was never our intention, but considering our failed attempts to address these concerns, it is entirely understandable the reviewer had such an impression (an impression we sincerely hope to change). We generated the genome-wide CNV plots in Fig. 1a and Extended Data Fig. 4a using the R package karyoploteR, and we have added this information to the "Copy number analysis" section of our methods. While this package was useful for generating genome-wide plots, to our knowledge it does not have the necessary annotations built-in to generate zoomed-in plots of specific loci, like the ones generated by IGV. Thus, we used the data from Extended Data Table 2 of our current submission to verify our analyses and generate IGV plots, including the figures below showing IGV visualizations of CNVs containing the *CDKN2A/B* or polymorphic *HLA* loci from the meningiomas in our study on pages 4 and 5 of this response. Copy number amplifications in these figures are shown in red. Copy number deletions are shown in blue. The number and DNA methylation group of Merlin-intact, Immune-enriched, and Hypermitotic meningiomas are shown blue, purple, or red, respectively, to the left of the IGV visualizations. The location of genes of interest in relation to the CNVs we analyzed across meningioma DNA methylation groups are superimposed on the IGV visualizations in orange. Some of these CNVs are small/focal and others are broad, but all CNVs contain the entire loci of interest. As described below, these figures (derived from Extended Data Table 2) now accurately reflect the CNVs we quantified and analyzed across meningioma DNA methylation groups. Simply put, Extended Data Table 10 contained the wrong data, but Extended Data Table 2 of our current submission contains the right data. We have revised our methods to state that we verified our CNV analyses using IGV. Of note, *HLA-DQA2* and *HLA-DRB6* (a pseudogene) were also identified in the polymorphic *HLA* locus but were not detected by our meningioma single-cell RNA sequencing. Thus, these genes were excluded from our CNV and expression analyses. The concordance of breakpoints shown in the IGV visualization of CNVs containing the polymorphic *HLA* locus on page 5 of this response is a product of deriving CNVs

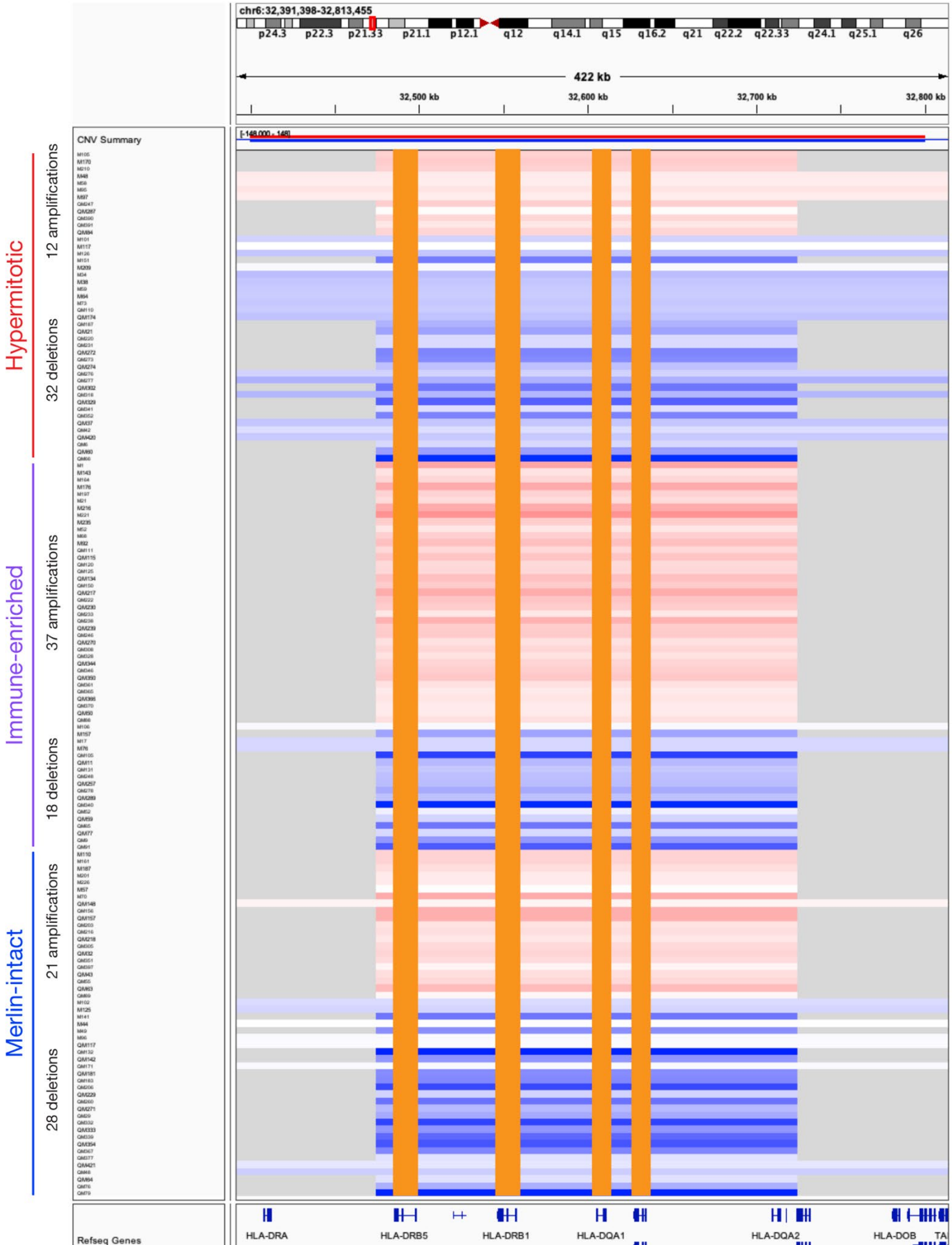
from Illumina 850k DNA methylation arrays, which were used in our study and define breakpoints based on probe locations, rather than sequencing reads.

2. Upon quick inspection of the visualized CDKN2A/2B locus, based on the CNV segments provided by the authors in Extended Data Table 10, there appears to be far fewer focal CNV events overlapping this locus than claimed in the text. Specifically, the authors write: “Focal CNVs deleting CDKN2A/B were identified in 7% of meningiomas (n=37 of 565), but were enriched in Hypermitotic meningiomas (15%, n=24 of 157) compared to Merlin-intact (4%, n=8 of 192) or Immune-enriched meningiomas (2%, n=5 of 216)…”. In the legend displaying these data in Fig. 4c, we state “Meningioma DNA methylation analysis of chromosome segment copy number loss containing the CDKN2A/B locus across Merlin-intact (n=8 of 192 meningiomas, 4%), Immune-enriched (n=5 of 216 meningiomas, 2%), and Hypermitotic (n=24 of 157 meningiomas, 15%) DNA methylation groups (n=565, Chi-squared test).”

nature research



nature research



In Extended Data Table 10 (provided in the CDKN2A/2B tab), there are 31 data rows, corresponding to 22 unique individual sample IDs. Overlapping the provided segments with the CDKN2A/2B locus coordinates (provided in the Key tab), there are 9 Hypermitotic (not 24), 1 Merlin-intact (not 8), and 3 Immune-enriched (not 5) samples with deletions overlapping this locus, and 13 in total (not 37). Beyond these highly discrepant deletion counts between what is quoted and summarized in the manuscript and what is actually provided in Extended Data Figure 10, the fact that several sample IDs were apparently counted multiple times, and most of the provided segments are distal to the CDKN2A/2B locus rather than overlapping it, significantly reduces confidence in the overall data integrity presented here.

As described in the second bullet point on page 1 of this response, we unfortunately displayed meningioma CNVs <5 Mb deleting chromosomes 9 segments in the CDKN2A/B tab of Extended Data Table 10, which is why many of the segments were non-overlapping (and some segments were partially overlapping) with the *CDKN2A/B* locus. Some meningiomas had more than one focal deletion on chromosome 9, which is why only 22 of the 31 data rows corresponded to unique meningiomas.

As described in first bullet point on page 1 of this response, Extended Data Table 10 was generated independent of our prior analyses of CNVs deleting *CDKN2A/B* across meningioma DNA methylation groups. Our analyses (quoted and summarized by the reviewer above) were based on *any* deletions of the *entire CDKN2A/B* locus across the 565 meningiomas included in our study, which is how we arrived at 37 unique meningiomas with deletions of the (entire) *CDKN2A/B* locus that were analyzed in the text, figures, and figure legends of our manuscript (24 hypermitotic, 5 Immune-enriched, and 8 Merlin-intact meningiomas). These 37 unique meningiomas are now shown in Extended Data Table 2 of our current submission, and the correct data from this table were used to generate the IGV visualization of the *CDKN2A/B* locus shown on page 4 of this response. To clarify these analyses, we have deleted our erroneous use of the word "focal" in the sentence of our text describing our analyses of *CDKN2A/B* deletions across meningioma DNA methylation groups (a mistake that was not transmitted to the figure legend describing these analyses, where we stated "…chromosome segment copy number loss containing the *CDKN2A/B* locus…"). Indeed, the word "focal" has been entirely removed the main text and figure legends of our revised manuscript. With respect to our presentation of these data, we have changed the x-axis label in Fig. 4c to read "Chr9p containing *CDKN2A/B*", and we have provided the genomic coordinates of the CNVs we analyzed across meningioma DNA methylation groups in the Key tab of Extended Data Table 2. We have additionally clarified that our analyses were based on CNVs containing the entire *CDKN2A/B* locus (or other entire loci of interest) in the text and figure legends of our revised manuscript. To codify this approach, we have clarified the focal or non-focal nature of CNVs containing loci of interest in the methods of our revised manuscript, where we now state:

> "CNVs of biologic interest across meningioma DNA methylation groups (*NF2*, *HLA, CDKN2A/B,* or *USF1*) were focal (<5 Mb) or non-focal for *HLA* or *CDKN2A/B*, or predominantly non-focal for *NF2* (n=350 of 351 meningiomas) or *USF1* (n=40 of 42 meningiomas). Whether focal or non-focal, CNVs were included for analysis across meningioma DNA methylation groups only when the entire gene or locus of interest was gained or lost."

We are hopeful the corrections described will address the concerns relating to the integrity of our analyses of CNVs containing the *CDKN2A/B* locus across meningioma DNA methylation groups.

3. Considering the issues raised above concerning CNVs at the CDKN2A/2B locus, similar inspection of the HLA deletions and gains was undertaken using the data provided in Extended Data Figure 10. Specifically, the authors write: "CNVs amplifying the polymorphic locus were more frequent in Immune-enriched meningiomas (30%, n=64 of 216) compared to Merlin-intact (17%, n=33 of 192) or Hypermitotic meningiomas (18%, n=28 of 157) (p=0.0033, Chi-squared test). Conversely, CNVs deleting the polymorphic locus were less frequent in Immune-enriched meningiomas (11%, n=25 of 216) compared to Merlin-intact (15%, n=28 of 192) or Hypermitotic meningiomas (21%, n=33 of 157) (p=0.0412, Chi-squared test)." In the legend displaying these data in Fig. 3f, we state "Meningioma DNA methylation analysis of CNVs containing the HLA genes… across Merlin-intact (n=192 meningiomas, 221 losses in 59 meningiomas, 147 gains in 43 meningiomas), Immune-enriched (n=216 meningiomas, 158 losses in 44 meningiomas, 258 gains in 65 meningiomas), and Hypermitotic (n=157 meningiomas, 270 losses in 58 meningiomas, 125 gains in 29 meningiomas) DNA methylation groups (Chi-squared test)."

In Extended Data Table 10 (provided in the HLA gains tab), there are 140 data rows, corresponding to 137 unique sample IDs. According to the numbers provided above, there should be n=125 samples with focal gain of HLA genes (64 Immune-enriched, 33 Merlin-intact, and 28 Hypermitotic). Overlapping the provided segments with the HLA locus coordinates (provided in the Key tab), there are 65 Immune-enriched (not 64), 42 Merlin-intact (not 33), and 26 Hypermitotic (not 28) samples with focal gains, and 133 in total (not 125).

Similarly, in Extended Data Table 10 (provided in the HLA deletions tab), there are 196 data rows, corresponding to 168 unique sample IDs. According to the numbers provided above, there should be n=86 samples with focal deletion of HLA genes (25 Immune-enriched, 28 Merlin-intact, and 33 Hypermitotic). Overlapping the provided segments with the HLA locus coordinates (provided in the Key tab), there are 49 Immune-enriched (not 25), 46 Merlin-intact (not 28), and 38 Hypermitotic (not 33) samples with focal deletions, and 133 in total (not 86).

A summary of these discrepancies as they relate to the reported HLA gains and deletions in the manuscript text, figure legend, and extended data table is provided in the attachment (p-values calculated using the Chi-squared test).

As described in the second bullet point on page 1 of this response, we also unfortunately displayed meningioma CNVs <5 Mb deleting or amplifying chromosomes 6 segments in the HLA tabs of Extended Data Table 10. Some meningiomas in the HLA tabs had more than one focal CNV on Chr6p (which is why duplicate/non-unique samples were included). Conversely, some meningiomas had focal CNVs on Chr6p that did not overlap (or partially overlapped) with *HLA* genes, and some meningiomas with broader CNVs amplifying or deleting the *HLA* locus were entirely excluded from this table (which is why the total number of CNVs containing the *HLA* locus in Extended Data Table 10 was incongruent with the numbers summarized in the text, figures, and figure legends of our manuscript).

As described in first bullet point on page 1 of this response, Extended Data Table 10 was also generated independent of our analyses of CNVs containing the *HLA* locus across meningioma DNA methylation groups. Indeed, these prior analyses were based on *any* amplifications or deletions (focal plus non-focal) containing the entire polymorphic *HLA* locus, or containing entire *HLA* genes of interest revealed by our single-cell RNA sequencing of human meningiomas (Fig. 3e). In the text of our manuscript, we provided an analysis of CNVs containing the polymorphic *HLA* locus that overlapped with 4 of 6 *HLA* genes of interest (*HLA-DRB5, HLA-DRB1, HLA-DQA1,* and *HLA-DQB1*). Unfortunately, in Fig. 3f and in the legend corresponding to Fig. 3f (quoted above) we provided an analysis not of the polymorphic *HLA* locus, but of aggregated CNVs containing any of the 6 individual *HLA* genes of interest revealed by our single-cell RNA sequencing (which is why the Fig. 3f legend we previously provided quoted more *HLA* loses or gains than the total number of meningiomas in each DNA methylation group). We recognize the incongruity between our analyses of the polymorphic *HLA* locus (in the text) and of individual *HLA* genes (in Fig. 3f) was incredibly confusing when juxtaposed, and we have fixed this unfortunate error in our revised manuscript, as described below. The confusion generated by these poorly-explained, parallel analyses was succinctly demonstrated by the table at the end of the reviewer's report. To clarify, the "main text" column of the reviewer's table referenced our analyses of the polymorphic *HLA* locus, the "figure 3f legend" column referenced our analyses of individual *HLA* genes, and the "supplementary table" column referenced the wrong data we provided in Extended Data Table 10. The number of unique meningiomas in the legend corresponding to Fig. 3f was greater than the number of unique meningiomas in the main text because some meningiomas had amplifications or deletions that were large enough to contain entire *HLA* genes, but not large enough to encompass the entire polymorphic *HLA* locus. Moreover, our previous aggregated analysis of CNVs containing any of the 6 individual *HLA* genes of interest mistakenly included *HLA-DMA* and *HLA-DPB1* in Fig. 3f, which are located outside the polymorphic locus and appear to be regulated by changes in DNA methylation (Extended Data Fig. 10d-f), rather than CNVs.

We apologize for these unacceptable errors, which had the same root cause as the aforementioned incongruities in our data that were mistakenly-attributed to *CDKN2A/B* losses in Extended Data Table 10. We also apologize for our confusing presentation of analyses pertaining to the polymorphic *HLA* locus alongside aggregated CNVs containing individual *HLA* genes. We are grateful for the opportunity to fix these mistakes. As part of the broad re-analysis of our findings to ensure the integrity of our data, we discovered some *HLA* CNVs we previously quantified were based on partial overlap with the polymorphic *HLA* locus, rather than gains or losses of the *entire* polymorphic locus (as we did when quantifying CNVs containing the *entire NF2*, *CDKN2A/B*, or *USF1* loci). Although our previous polymorphic *HLA* locus analyses were mathematically correct, they were not consistent with how we performed CNV analyses for other loci of interest elsewhere in our manuscript. Thus, for this revision, we have focused only on CNVs amplifying or deleting the entire polymorphic *HLA* locus to ensure our approach for CNV analysis is consistent across chromosomes. We would be happy to revisit this decision if advisable by the reviewer, but in the interim, we have made sure our approach is clear in our revised methods. To provide the most comprehensive and complete information, the HLA_polymorphic tab of Extended Data Table 2 contains chromosome segment copy number gains or losses of the entire *HLA* polymorphic locus (coordinates provided in the Key tab and described in the methods). In addition, the HLA_DRB5, HLA_DRB1, HLA_DQA1, and HLA_DQB1 tabs contain chromosome segment copy number gains or losses of entire *HLA* genes, which were used to generate

Extended Data Fig. 10c. Of note, the number of unique meningiomas in the HLA_DRB5, HLA_DRB1, HLA_DQA1, and HLA_DQB1 tabs remains greater than the number of unique meningiomas in the HLA_polymorphic tab because some meningiomas had amplifications or deletions that were large enough to contain entire *HLA* genes, but not large enough to encompass the entire polymorphic *HLA* locus.

With respect to our re-analysis of the polymorphic *HLA* locus, we used the correct data now provided in Extended Data Table 2 to modify the text of our manuscript as follows:

> "Copy number amplifications containing the entire polymorphic locus were more frequent in Immune-enriched meningiomas (17%, n=37 of 216) compared to Merlin-intact (11%, n=21 of 192) or Hypermitotic meningiomas (12%, n=12 of 157) (p=0.0174, Chi-squared test) (Fig. 3f). Conversely, copy number deletions containing the entire polymorphic locus were less frequent in Immune-enriched meningiomas (8%, n=18 of 216) compared to Merlin-intact (15%, n=28 of 192) or Hypermitotic meningiomas (20%, n=32 of 157) (p=0.0036, Chi-squared test) (Fig. 3f)."

To avoid the confusion previously created by our parallel analyses of the polymorphic *HLA* locus alongside aggregated CNVs containing individual *HLA* genes, we now show our analysis of CNVs containing the entire polymorphic *HLA* locus summarized in the preceding paragraph in Fig. 3f. Importantly, these corrections have not diluted the results or conclusions of our manuscript, and statistical significance was maintained across all comparisons. The Fig. 3f legend now reads:

> "Meningioma DNA methylation analysis of chromosome segment CNVs containing the entire polymorphic *HLA* locus encompassing *HLA-DRB5, HLA-DRB1, HLA-DQA1,* and *HLA-DQB1* across Merlin-intact (n=192 meningiomas, 21 gains, 28 losses), Immune-enriched (n=216 meningiomas, 37 gains, 18 losses), and Hypermitotic (n=157 meningiomas, 12 gains, 32 losses) DNA methylation groups (Chi-squared test)."

Thus, the Fig. 3f legend is now consistent with the data presented in the text of our revised manuscript, and also with the data presented in Extended Data Table 2.

The reviewer previously stated "to establish any putative association between HLA genomic status and HLA expression, the authors should evaluate HLA expression by subgroup and account for underlying HLA copy number status." To do so at the time of our first revision at *Nature Genetic*, we provided plots of *HLA-DRB5, HLA-DRB1, HLA-DQA1,* or *HLA-DQB1* expression across DNA methylation groups from RNA sequencing of 200 meningiomas according to whether these individual *HLA* genes were gained, lost, or neutral. This data is shown in Extended Data Fig. 10c. In the text of our manuscript, we describe these data by stating:

> "Expression of *HLA-DRB5, HLA-DRB1, HLA-DQA1,* and *HLA-DQB1* correlated with CNVs amplifying or deleting these genes, and the expression of *HLA-DRB5, HLA-DRB1, HLA-DQA1,* and *HLA-DQB1* was enriched in Immune-enriched meningiomas compared to other groups (Extended Data Fig. 10c)."

For *HLA* genes located outside the polymorphic locus that were nevertheless detected as differentially enriched in single cells from Immune-enriched meningiomas compared to single cells from other meningioma DNA methylation groups (Fig. 3e), we now state:

> *"HLA-DMA* and *HLA-DPB1*, located outside the polymorphic locus on chromosome 6p but also with enriched expression in single-cell transcriptomes from Immune-enriched meningiomas (Fig. 3e), were hypomethylated and had increased expression in Immune-enriched meningiomas compared to other groups (Extended Data Fig. 10d-f)."

Our interpretation from previous rounds of review and revision is that our analyses in Extended Data Fig. 10c-f were well received, underscoring the importance of pairing our analyses of the polymorphic *HLA* locus in its entirety alongside complementary analyses of individual *HLA* genes. Thus, we have retained our prior DNA methylation/expression analyses of *HLA-DMA* and *HLA-DPB1* in Extended Data Fig. 10d-f, and also our CNV/expression analyses in Extended Data Fig. 10c, both of which were already performed according to whether entire *HLA-DRB5, HLA-DRB1, HLA-DQA1,* or *HLA-DQB1* loci were gained or lost in individual meningiomas across DNA methylation groups. Of note, the number of tumors shown in Extended Data Fig. 10c is different from the number of tumors shown in the HLA_DRB5, HLA_DRB1, HLA_DQA1, and HLA_DQB1 tabs of Extended Data Table 2 because the analyses in Extended Data Fig. 10c required integration of CNV data from Extended Data Table 2 (inclusive of meningiomas from both UCSF [M#] and HKU [QM#]) with RNA sequencing data that was only available on meningiomas from UCSF (M#).

Overall, the lack of alignment of the reported CNV annotations in the manuscript narrative with the copy number segment data provided in Extended Data Table 10, the latter of which should serve as the basis for the narrative and corresponding data presented throughout the manuscript and supplement, instills considerable concern with regards to the integrity of the data analysis and interpretation of the genetic findings reported in this study. As an example of this concern, the HLA deletions do not appear to be significantly different between the 3 subgroups as reported in the supporting figures and narrative. Thus, it becomes exceedingly difficult for one to maintain confidence in this report.

This summary is fair, humbling, and embarrassing to receive. We are hopeful our clarifications provided above, and correct data provided in Extended Data Table 2, will address these concerns and re-instill the confidence in our work that we overwhelmingly hope to achieve. We are grateful the reviewer has stuck with us for so long, and we remain steadfast in our commitment to ensure all concerns are completely addressed. Thus, if our analyses remain unclear or suboptimal, we welcome any and all feedback to improve the data analysis and presentation. We hope our analyses (and the data from which our analyses were derived) are now transparent in the text, figures, figure legends, and Extended Data Table 2 of our current submission. Acknowledging the reviewer has requested we particularly "show the focal deletions of CDKN2A/2B (and ideally alterations of HLA) in [our] dataset," we incorporated the following secondary analyses and edits into the "Copy number analysis" section of our methods, restricted to focal CNVs:

> "As an additional test of CNV specificity across meningioma DNA methylation groups, focal deletions <5 Mb of the entire *CDKN2A/B* locus were more common in Hypermitotic meningiomas compared to other DNA methylation groups (5 *CDKN2A/B* deletions in 157 Hypermitotic meningiomas compared to 3 deletions in 408 non-Hypermitotic meningiomas, p=0.0413, Fisher's exact test). Focal amplifications <5 Mb of the entire polymorphic *HLA* locus were more common in Immune-enriched meningiomas compared to other DNA methylation groups (37 polymorphic *HLA* amplifications in 216

Immune-enriched meningiomas compared to 20 amplifications in 192 Merlin-intact meningiomas and 8 amplifications in 157 Hypermitotic meningiomas, p=0.0013, Chi-squared test)."

Given the small numbers of tumors with focal deletions of the entire *CDKN2A/B* locus, we were unable to run a Chi-squared test for this part of our secondary analyses because >20% of the contingency table had less than 5 counts. Thus, we combined non-Hypermitotic meningiomas into one group and assessed statistical significance compared to Hypermitotic meningiomas using Fisher's exact test. More broadly, considering (1) the correlation of *HLA* gene expression with focal plus non-focal CNVs containing entire *HLA* genes in the polymorphic *HLA* locus (Extended Data Fig. 10c), (2) the finding that these genes are enriched in single Immune-enriched meningioma cells compared to single cells from other DNA methylation groups (Fig. 3e), and (3) the aforementioned analyses of the entire *CDKN2A/B* locus, we decided it was appropriate to retain our broader analyses of *HLA* and *CDKN2A/B* copy number status (inclusive of both focal and non-focal amplifications or deletions containing entire loci of interest) in the main text, figures, and figure legends of our manuscript. Conversely, considering the lack of consensus regarding focal CNV definitions described on page 1 of this response, we decided it would be inappropriate and disingenuous to adjust how we define the size of "focal" CNVs in a manner that may bias the data in favor of particularly findings. As stated above, we would be happy to revisit these decisions if advisable by the reviewer, or to test any preferred definition(s) of CNV focality across meningioma DNA methylation groups to ensure the integrity of our data.

In conclusion, we again apologize for the wrong data we provided in Extended Data Table 10, and also for our confusing juxtaposed analyses of CNVs containing the polymorphic *HLA* locus and individual *HLA* genes in the text, figures, and figure legends of our previous submission. As part of our broader re-analysis in ensure the integrity of our data, we have also included CNVs containing the *NF2* or *USF1* loci in Extended Data Table 2 for transparency and completeness. Like *CDKN2A/B* and *HLA*, we have verified our *NF2* and *USF1* locus-level data are consistent with the findings summarized in the text, figures, and figure legends of our manuscript using IGV, and we have highlighted these areas in yellow (as we also did for our verified and clarified CNV analyses of the *CDKN2A/B* and *HLA* loci). We hope our careful inspection and re-analysis of the data will re-instill confidence in our interpretation of CNVs and the broader relevance of our study for understanding meningioma biology.

Decision Letter, second revision:

2nd Nov 2021

Dear Dr Raleigh,

Your Article entitled "Meningioma DNA methylation grouping reveals biologic drivers and therapeutic vulnerabilities" has now been seen once again by Reviewer #2, whose comments are attached. In the light of their advice we have decided that we cannot offer to publish your manuscript in Nature Genetics.

As you know from previous emails and conversations, we agreed to return your revised manuscript to this reviewer with the proviso that we would only move forward with their full support. As you'll see from their report, they continue to have concerns about the data and, in their opinion, your conclusions are not sufficiently supported by genetic data. As such, we will not be pursuing the paper any further. Please note that this decision is final and we will not consider an appeal.

I am sorry that we have reached this point, but I hope that you can understand our editorial position. I hope that you will find the referee's comments helpful when preparing your paper for submission elsewhere.

Sincerely,

Safia

Safia Danovi
Senior Editor
Nature Genetics

Reviewers' Comments:

Reviewer #2:
Remarks to the Author:
In their rebuttal accompanying their revision, Choudury and colleagues have provided a detailed, yet confounding explanation pertaining to the sources of discrepancy and inconsistency related to CDKN2A/2B deletions they have summarized in their meningioma DNA methylation array dataset. As this revision process has demanded considerable attention and time-consuming efforts on my part, I have primarily focused this review on the topic of genetic evidence implicating CDKN2A/2B deletions in meningioma, as claimed to contribute to the hypermitotic subgroup of meningioma by the study co-authors. In the rebuttal letter, the authors state the following source of error related to Extended Data Table 10 that was provided in their previous submission:

• The version of Extended Data Table 10 we previously provided was generated independent of our CNV analyses across meningioma DNA methylation groups, and we unwisely failed to cross-check our data.
• Thus, correcting our embarrassing mistake in this table did not dilute the results or the conclusions of our manuscript.

I have multiple issues with these 2 statements. First, previous EDT10 did in fact include meningioma methylation subgroup annotations, and therefore it is difficult to appreciate how their provided explanation holds any validity. Moreover, sample-level CNV calls, as annotated in EDT10, should be completely agnostic of any subgroup membership, as the table simply lists samples that the authors claim to harbor CDKN2A/2B deletions.

The second issue I have relates to the second sentence above. The previous version of CDKN2A/2B deletions reported in EDT10 does not match the current version provided in EDT2. In EDT10, the authors previously included only focal deletions (defined as <5Mb) anywhere on chr9, and the current annotation in EDT2 includes all deletions (broad or focal) overlapping the entire CDKN2A gene. However, despite these varying definitions as to what the authors consider deletion of CDKN2A/2B, the Figure related to this data (Figure 4c) has never changed over the entire course of peer review. This is indeed troubling, because the narrative pertaining to the genetic evidence supporting CDKN2A/2B deletion in meningioma has changed considerably.

For instance, looking back at early versions of this manuscript while under consideration [REDACTED] (spring 2021), the authors initially claimed that 'CNVs deleting the CDKN2A/2B locus were enriched in Hypermitotic meningiomas (62%), and were associated with worse LFFR.'

In a previous version at Nature Genetics (summer 2021), the authors stated 'Focal CNVs deleting CDKN2A/B were identified in 7% of meningiomas (n=37 of 565), but were enriched in Hypermitotic meningiomas (15%, n=24 of 157) compared to Merlin-intact (4%, n=8 of 192) or Immune-enriched meningiomas (2%, n=5 of 216) (Fig. 4c and Extended Data Table 10), and were associated with worse LFFR.'

In the current version at Nature Genetics (fall 2021), the authors state 'Copy number deletions containing the entire CDKN2A/B locus were identified in 6.5% of meningiomas (n=37 of 565), but were enriched in Hypermitotic meningiomas (15%, n=24 of 157) compared to Merlin-intact (4%, n=8 of 192) or Immune-enriched meningiomas (2%, n=5 of 216) (Fig. 4c and Extended Data Table 2), and were associated with worse LFFR (Extended Data Fig. 15a).'

Based on the prior discrepancies associated with EDT10 summarized in detail during my last review, I once again revisited this data in comparison to the data now provided in EDT2. Filtering EDT10 down to focal deletions (<5Mb) overlapping CDKN2A, there were 13 events. Performing the same analysis for EDT2, identifies 8 events (5 hypermitotic, 2 immune-enriched, 1 Merlin-intact). It is not clear why previous deletions summarized in EDT10 have been removed in EDT2, specifically those annotated for the following sample IDs: QM175, QM283, QM60, QM61, M20.

To summarize, early iterations of this manuscript described CDKN2A deletions (presumed to be focal) in 62% of hypermitotic meningiomas. Subsequent iterations, including the current manuscript reduced this frequency to a broader definition of deletions overlapping the locus (focal plus broad events) and report a deletion frequency of 15% in the hypermitotic group. Taking the data in EDT2 as ground truth, the actual frequency of focal deletion at this locus is 5/157 = 3.2%. This 3.2% is a long way from the initial 62% originally described by the co-authors, which is very troubling to this reviewer. Had my initial concerns regarding these deletions not been raised, and this study published as is, the field would now believe that >60% of hypermitotic meningiomas harbor focal CDKN2A/2B deletions.

It has been a long journey to trace the proposed deletions of CDKN2A/2B deletion in hypermitotic meningioma, as defined by the co-authors of this study. For a cancer genetics-oriented study targeting a top tier journal in the field of Genetics, one would expect that the associated genetic data identified and summarized in the study would be robust, consistent, and featured prominently in the manuscript to substantiate the study narrative and conclusions. Disappointingly, this has not been the case for this effort. The genetics reported in this study have lacked consistency and have been bluntly

misleading, for the most part hidden in supplemental tables or deceptively summarized in bar graphs. When cancer genetics data is solid, the actual genetic events are shown as is and featured, not buried in an Extended Data Table. Furthermore, generalizing chromosomal and chromosome arm deletions as causative events linked to a single gene (i.e., chr9p deletion = CDKN2A/2B deletion; chr1q gain = USF1 amplification) is an oversimplification employed in this study. Although the authors have toned down some of their conclusions over the course of their manuscript evolution, this culminates in genetic data that is relatively weak and unconvincing.

In conclusion, based on the persistence of discrepancies related to the reported deletions of CDKN2A/2B, and the reality that the genetics data presented in this study does not strongly support the implications of the manuscript narrative, I cannot endorse this study for publication in Nature Genetics. Considering the time and effort invested, I am hopeful that the authors of this study will take my constructive comments and suggestions to heart and attempt to analyze and contextualize their genetics data such that they can confidently present their findings in the future more rigorously.

Author Rebuttal, second revision:

Reviewer #2

In their rebuttal accompanying their revision, Choudury and colleagues have provided a detailed, yet confounding explanation pertaining to the sources of discrepancy and inconsistency related to CDKN2A/2B deletions they have summarized in their meningioma DNA methylation array dataset. As this revision process has demanded considerable attention and time-consuming efforts on my part, I have primarily focused this review on the topic of genetic evidence implicating CDKN2A/2B deletions in meningioma, as claimed to contribute to the hypermitotic subgroup of meningioma by the study co-authors. In the rebuttal letter, the authors state the following source of error related to Extended Data Table 10 that was provided in their previous submission:

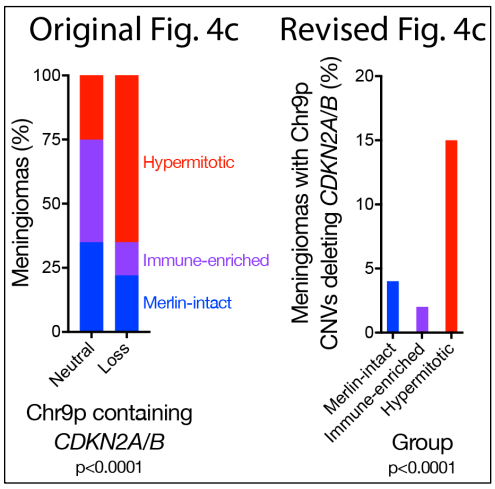
We apologize our previous explanations were insufficient. In response, we have revised the text, methods, figures, and figure legends of our manuscript to ensure the genetic data supporting our report of Hypermitotic and Immune-enriched meningiomas are robust, accessible, discussed with full transparency, and aligned with our conclusions. Our approach to quantifying CNVs targeting *CDKN2A/B* was the same as our approach to quantifying other CNVs of biologic interest (*HLA, NF2*, etc.). *NF2* CNVs have not been a source of concern during the course of peer review, and we have focused on *CDKN2A/B* and *HLA* CNVs in this letter.

• The version of Extended Data Table 10 we previously provided was generated independent of our CNV analyses across meningioma DNA methylation groups, and we unwisely failed to cross-check our data.
• Thus, correcting our embarrassing mistake in this table did not dilute the results or the conclusions of our manuscript.

I have multiple issues with these 2 statements. First, previous EDT10 did in fact include meningioma methylation subgroup annotations, and therefore it is difficult to appreciate how their provided explanation holds any validity. Moreover, sample-level CNV calls, as annotated in EDT10, should be completely agnostic of any subgroup membership, as the table simply lists samples that the authors claim to harbor CDKN2A/2B deletions.

The second issue I have relates to the second sentence above. The previous version of CDKN2A/2B deletions reported in EDT10 does not match the current version provided in EDT2. In EDT10, the authors previously included only focal deletions (defined as <5Mb) anywhere on chr9, and the current annotation in EDT2 includes all deletions (broad or focal) overlapping the entire CDKN2A gene. However, despite these varying definitions as to what the authors consider deletion of CDKN2A/2B, the Figure related to this data (Figure 4c) has never changed over the entire course of peer review. This is indeed troubling, because the narrative pertaining to the genetic evidence supporting CDKN2A/2B deletion in meningioma has changed considerably.

We apologize for the misunderstanding. As now described in the *Copy number analysis* section of our Methods, and further clarified in this revision, our CNV calls were indeed performed agnostic of meningioma DNA methylation groups. The DNA methylation groups of samples were unblinded only after CNVs were called. After unblinding, we analyzed the number and percentage of meningiomas in each DNA methylation group with copy number losses (focal or broad) including the *CDKN2A/B* locus. This definition identified 37 losses of the *CDKN2A/B* locus, and those losses provided the basis for our CNV analyses in the text, figures, and figures legends of our manuscript. This analysis has not changed over the past 13 months of peer review, but we have supported this approach with new analyses of focal versus broad CNVs, and complete versus partial CNVs, as described below. Unfortunately, when compiling the supplemental data for this manuscript, the table we generated (previous version of Extended Data Table 10) listed all focal deletions on the chromosome 9p arm, instead of all deletions (focal or broad) including the *CDKN2A/B* locus. Importantly, Fig. 4c was never based on the erroneous data we mistakenly provided in Extended Data Table 10. The current version of Extended Data Table 2 includes the data supporting Fig. 4c, and is consistent with our conclusions.



Given the reviewer's persistent concern, we have changed the formatting of Fig. 4c, which remains consistent with Extended Data Table 2 and shows the percentage of CNVs deleting the *CDKN2A/B* locus in each meningioma DNA methylation group. We used similar formatting to present CNVs deleting the *NF2* locus in Fig. 2a, which has not been a source of concern since our initial submission to *[REDACTED]* in the fall of 2020. To ensure our genetic data are presented consistently, we have also revised our presentation of CNVs amplifying or deleting the *HLA* locus in Fig. 3f, or CNVs amplifying chr1q segments containing

USF1 in Fig. 4f, to be consistent with Fig. 2a and revised Fig. 4c. Previously, Fig. 4c showed the distribution of meningioma DNA methylation groups with CNVs deleting chr9p segments containing *CDKN2A/B*, with the total number of CNVs summed to 100% (n=37). The majority of these CNVs were found in Hypermitotic meningiomas (n=24), but our prior formatting of Fig. 4c could be taken to imply that the majority of Hypermitotic meningiomas themselves had CNVs deleting *CDKN2A/B*. Our data do not support this interpretation, and we apologize for our confusing prior presentations of these genetic data.

For instance, looking back at early versions of this manuscript while under consideration at [REDACTED] (spring 2021), the authors initially claimed that 'CNVs deleting the CDKN2A/2B locus were enriched in Hypermitotic meningiomas (62%), and were associated with worse LFFR.'

The same sentence from our initial submission to *[REDACTED]* in the fall of 2020 read "62% of *CDKN2A/B* losses occurred in Hypermitotic meningiomas." Neither of these iterations stated 62% (or any majority) of Hypermitotic meningiomas encoded *CDKN2A/B* losses. Rather, as quoted by the reviewer in the sentences below, our intention was to convey that *CDKN2A/B* deletions were themselves more common in Hypermitotic meningiomas than other DNA methylation groups. We acknowledged our initial phrasings of this sentence were suboptimal on prior resubmissions, and have further optimized this sentence and others like it, as described below.

In a previous version at Nature Genetics (summer 2021), the authors stated 'Focal CNVs deleting CDKN2A/B were identified in 7% of meningiomas (n=37 of 565), but were enriched in Hypermitotic meningiomas (15%, n=24 of 157) compared to Merlin-intact (4%, n=8 of 192) or Immune-enriched meningiomas (2%, n=5 of 216) (Fig. 4c and Extended Data Table 10), and were associated with worse LFFR.'

We enormously regret our erroneous and solitary use of the word "focal" in this sentence from our summer 2021 resubmission. We did not use this word in our previous submissions to *Nature* in the fall of 2020 or the spring of 2021 (or at any time in our figures or figure legends). To ensure our genetic data sufficiently align with our conclusions, we do not claim anywhere in our revised text, methods, figures, or figure legends that focal CNVs are uniquely responsible for any of our findings. We would be happy to test any preferred definition(s) of CNV focality across meningioma DNA methylation groups to ensure the integrity of our data.

In the current version at Nature Genetics (fall 2021), the authors state 'Copy number deletions containing the entire CDKN2A/B locus were identified in 6.5% of meningiomas (n=37 of 565), but were enriched in Hypermitotic meningiomas (15%, n=24 of 157) compared to Merlin-intact (4%, n=8 of 192) or Immune-enriched meningiomas (2%, n=5 of 216) (Fig. 4c and Extended Data Table 2), and were associated with worse LFFR (Extended Data Fig. 15a).'

This sentence accurately summarizes the number and distribution of CNVs deleting *CDKN2A/B* in the meningiomas from our study. We overwhelmingly regret the length of time it has taken to optimize this sentence. Moreover, given the persistent concern related to the nature of CNVs we analyzed across meningioma DNA methylation groups, we have further revised this sentence in our main text to now read:

> Copy number deletions of any size containing the entire *CDKN2A/B* locus were identified in 6.5% of meningiomas (n=37 of 565), but were enriched in Hypermitotic meningiomas (15%, n=24 of 157) compared to Merlin-intact (4%, n=8 of 192) or Immune-enriched meningiomas (2%, n=5 of 216) (Fig. 4c and Extended Data Table 2), and were associated with worse LFFR (Extended Data Fig. 15a).

We have also revised the title of Extended Data Table 2 and the sentences in our main text and figure legends specifying the number and distribution of CNVs deleting chr22q segments containing the *NF2* locus, amplifying or deleting chr6p segments containing the *HLA* locus, or amplifying chr1q segments containing the *USF1* locus. In each, we specifically state that CNVs of any size encompassing entire loci were analyzed. We would be happy to revisit this approach if advisable, but a prior study found *CDKN2A* deletions in 4.9% of meningiomas (PMID 32642869), and our study revealed a similar frequency of *CDKN2A/B* deletions in meningiomas (37 of 565 meningiomas, 6.5%), externally validating our approach. Our rational for this approach is articulated in the *Copy number analysis* section of our Methods, and is also provided here for ease of evaluation.

First, we recognize definitions of CNV focality lack consensus. Many studies do not define CNV focality with precision, and those that do have definitions ranging from less than 3 Mb (PMID 20593488) to less than 98% of a chromosome arm (PMID 21527027). Moreover, how best to define "focal" CNVs containing single genes of different sizes (*CDKN2A, CDKN2B, NF2,* etc.) versus polymorphic loci containing multiple genes (*HLA-DRB5, HLA-DRB1, HLA-DQA1,* and *HLA-DQB1*) is unclear. For meningiomas, non-focal CNVs deleting chromosome 22q segments containing *NF2* are widely recognized as having biologic significance due (at least in part) to deletion of *NF2* despite the fact that these deletions are often broad. Indeed, 350 of the 351 copy number

deletions containing *NF2* in our study were larger than 5 Mb. Thus, we decided it would be appropriate to combine and quantify focal and non-focal CNVs containing other genes of interest across meningioma DNA methylation groups, provided we could orthogonally corroborate the biologic significance of these genes (an objective we accomplished for *NF2, HLA, CDKN2A/B,* and *USF1* using clinical, histological, genetic, biochemical, single-cell, mechanistic, and functional approaches).

Second, considering the resolution of CNVs derived from Illumina 850k DNA methylation arrays (which were used in our study and are based on probe locations, rather than sequencing reads), we decided the most parsimonious and transparent approach would be to quantify and report only CNVs containing entire loci of interest. To ensure our analyses are robust and legitimate, we have also provided secondary analyses of CNVs partially overlapping loci of interest, or restricted to focal CNVs, as described in our next response.

Based on the prior discrepancies associated with EDT10 summarized in detail during my last review, I once again revisited this data in comparison to the data now provided in EDT2. Filtering EDT10 down to focal deletions (<5Mb) overlapping CDKN2A, there were 13 events. Performing the same analysis for EDT2, identifies 8 events (5 hypermitotic, 2 immune-enriched, 1 Merlin-intact). It is not clear why previous deletions summarized in EDT10 have been removed in EDT2, specifically those annotated for the following sample IDs: QM175, QM283, QM60, QM61, M20.

Regarding the 8 meningiomas with focal CNVs deleting the *CDKN2A/B* locus in Extended Data Table 2, it appears the reviewer may have missed our analyses of focal plus broad CNVs across DNA methylation groups described in our previous response letters and in the *Copy number analysis* section of our Methods. Although it is true 8 of the 37 meningiomas in Extended Data Table 2 encode CNVs <5 Mb that delete the *CDKN2A/B* locus, the remaining 29 meningiomas in Extended Data Table also encode CNVs that delete the *CDKN2A/B* locus. We present genetic data from these 37 meningiomas in our main text and Fig 4c. For further clarity, as described above, we have revised Fig. 3f, Fig. 4c, and Fig. 4f to be consistent with Fig. 2a, and have also revised our main text and figure legends. We are hopeful these revisions will ensure our experimental approach is accessible and discussed with full transparency.

Nevertheless, when restricting our analyses to the 8 meningiomas with CNVs <5 Mb deleting the *CDKN2A/B* locus, our data still demonstrate that such CNVs are more common in Hypermitotic meningiomas compared to other DNA methylation groups. It appears the reviewer may have missed this analysis on the final page of our most recent response letter (and in the *Copy number analysis* section of our Methods), where we stated:

> As an additional test of CNV specificity across meningioma DNA methylation groups, focal deletions <5 Mb of the entire *CDKN2A/B* locus were more common in Hypermitotic meningiomas compared to other DNA methylation groups (5 *CDKN2A/B* deletions in 157 Hypermitotic meningiomas compared to 3 deletions in 408 non-Hypermitotic meningiomas, p=0.0413, Fisher's exact test).

The same held true when we restricted our analysis of the Immune-enriched group to meningiomas with CNVs <5 Mb amplifying the polymorphic *HLA* locus. In our most recent response letter and methods we stated:

> Focal amplifications <5 Mb of the entire polymorphic *HLA* locus were more common in Immune-enriched meningiomas compared to other DNA methylation groups (37 polymorphic *HLA* amplifications in 216 Immune-enriched meningiomas compared to 20 amplifications in 192 Merlin-intact meningiomas and 8 amplifications in 157 Hypermitotic meningiomas, p=0.0013, Chi-squared test).

The 5 meningiomas mentioned above that were previously displayed in Extended Data Table 10 but not displayed in Extended Data Table 2 only partially overlapped with the *CDKN2A/B* locus, as shown in the reviewer's IGV visualization from page 2 of their review of our summer 2021 resubmission to *Nature Genetics*. As described in our response letters, main text, and methods, we analyzed CNVs encompassing entire loci of interest due to technical limitations associated with precisely defining CNV locations from Illumina 850k DNA methylation arrays. The rationale for this approach has been expanded in the revised *Copy number analysis* section of our Methods (as described above). Moreover, the 5 partial deletions of the *CDKN2A/B* locus were found in 1 Immune-enriched meningioma (M20) and 4 Hypermitotic meningiomas (QM175, QM283, QM60, QM61). Thus, the trends and statistical significance across DNA methylation groups were preserved even when we analyzed meningiomas with CNVs of any size deleting the entire (n=37) or partial (n=5) *CDKN2A/B* locus (n=28 of 157 Hypermitotic meningiomas, n=8 of 192 Merlin-intact meningiomas, n=6 of 216 Immune-enriched meningiomas, p<0.0001, Chi-squared test). The same held true to re-analyses of CNVs including the *HLA* locus. These secondary analyses incorporating partial-locus CNVs with entire-locus CNVs have been added to the *Copy number analysis* section of our Methods.

To summarize, early iterations of this manuscript described CDKN2A deletions (presumed to be focal) in 62% of hypermitotic meningiomas. Subsequent iterations, including the current manuscript reduced this frequency to a

broader definition of deletions overlapping the locus (focal plus broad events) and report a deletion frequency of 15% in the hypermitotic group. Taking the data in EDT2 as ground truth, the actual frequency of focal deletion at this locus is 5/157 = 3.2%. This 3.2% is a long way from the initial 62% originally described by the co-authors, which is very troubling to this reviewer. Had my initial concerns regarding these deletions not been raised, and this study published as is, the field would now believe that >60% of hypermitotic meningiomas harbor focal CDKN2A/2B deletions.

We apologize for the confusion created by our previous phrasing of these data. As described in our responses above, we never stated a majority of Hypermitotic meningiomas encoded CNVs deleting *CDKN2A/B*, but we recognized our initial phrasings of these data could be improved and have revised our main text, methods, figures, and figure legends to address these concerns. Moreover, in the *Convergent genetic and epigenetic mechanisms misactivate the cell cycle in meningioma* section of our main text from our previous (and current) resubmissions, we state "Loss of the endogenous CDK4/6 inhibitor *CDKN2A/B* on chromosome 9p is a rare biomarker for meningioma recurrence," and "Most Hypermitotic meningiomas did not have CNVs deleting *CDKN2A/B* (Fig. 4c)." These statements highlight the importance of other cell cycle drivers that we also report in Hypermitotic meningiomas, such as FOXM1 and USF1. To highlight that *CDKN2A/B* loss is but one of several mechanisms misactivating the cell cycle in Hypermitotic meningiomas, we generated a new integrated analysis of genetic and epigenetic features in Hypermitotic meningiomas that is presented in our results and Extended Data Table 12 for this revision:

> To define the distribution of genetic and epigenetic mechanisms misactivating the cell cycle in Hypermitotic meningiomas, *CDKN2A/B* methylation (Fig. 4d) and *FOXM1* expression (Extended Data Fig. 11, 13) were analyzed alongside CNVs of any size deleting the entire *CDKN2A/B* locus (Fig. 4c) or amplifying the entire *USF1* locus (Fig. 4f). *CDKN2A/B* β methylation values in the top quartile of the 565 meningiomas in our study were defined as hypermethylated. *FOXM1* expression in the top quartile of the 200 meningiomas in the discovery cohort with available RNA sequencing (Extended Data Table 8) were defined as increased. Among the 63 Hypermitotic meningiomas in the discovery cohort (Extended Data Table 1), there were 13 tumors with CNVs deleting *CDKN2A/B*, 14 tumors with CNVs amplifying *USF1*, 37 tumors with *CDKN2A* hypermethylation, 32 tumors with *CDKN2B* hypermethylation, and 26 tumors with increased *FOXM1* expression (Extended Data Table 12). Removing duplicates, 52 of 63 Hypermitotic meningiomas in the discovery cohort had *CDKN2A/B* deletion, *USF1* amplification, *CDKN2A/B* hypermethylation, or increased expression of *FOXM1* (83%). Multiple genetic or epigenetic mechanisms misactivating the cell cycle were identified in 40 of 63 Hypermitotic meningiomas (63%).

To ensure our results are robust, accessible, fully transparent, and aligned with our conclusions, we generalized this approach to an integrated analysis of genetic and epigenetic features in Immune-enriched meningiomas that is presented in our discussion and Extended Data Table 13 for this revision:

> Integrating epigenetic drivers alongside genetic alterations may be important for understanding the biology of meningioma DNA methylation groups. We identified CNVs of any size deleting the entire *CDKN2A/B* locus or amplifying the entire *USF1* locus in 54 of 157 Hypermitotic meningiomas (35%) (Extended Data Table 2), but when integrated with *CDKN2A/B* hypermethylation and *FOXM1* expression, the epigenetic and genetic alterations underlying meningioma cell proliferation we report were found in 83% of Hypermitotic tumors (Extended Data Table 12). We used the same approach to integrate CNVs of any size amplifying the entire polymorphic *HLA* locus with hypomethylation of *HLA-DMA*, *HLA-DPB1*, or meningeal lymphatic genes (*LYVE1*, *CCL21*, *CD3E*), defined by β methylation values in the bottom quartile of the 565 meningiomas in our study (Extended Data Table 13). Removing duplicates, the epigenetic and genetic alterations underlying meningioma immune infiltration we report were found in 166 of 216 Immune-enriched meningiomas (77%). Multiple genetic or epigenetic mechanisms misactivating underlying immune infiltration were identified in 157 of 216 Immune-enriched meningiomas (73%).

It has been a long journey to trace the proposed deletions of CDKN2A/2B deletion in hypermitotic meningioma, as defined by the co-authors of this study. For a cancer genetics-oriented study targeting a top tier journal in the field of Genetics, one would expect that the associated genetic data identified and summarized in the study would be robust, consistent, and featured prominently in the manuscript to substantiate the study narrative and conclusions. Disappointingly, this has not been the case for this effort. The genetics reported in this study have lacked consistency and have been bluntly misleading, for the most part hidden in supplemental tables or deceptively summarized in bar graphs. When cancer genetics data is solid, the actual genetic events are shown as is and featured, not buried in an Extended Data Table. Furthermore, generalizing chromosomal and chromosome arm deletions as causative events linked to a single gene (i.e., chr9p deletion = CDKN2A/2B deletion; chr1q gain = USF1 amplification) is an oversimplification employed in this study. Although the authors have toned down some of their conclusions over the course of their manuscript evolution, this culminates in genetic data that is relatively weak and unconvincing. In conclusion, based on the persistence of discrepancies related to the reported deletions of CDKN2A/2B, and the reality that the genetics data presented in this study does not strongly support the implications of the manuscript narrative, I cannot endorse this study for publication

in Nature Genetics. Considering the time and effort invested, I am hopeful that the authors of this study will take my constructive comments and suggestions to heart and attempt to analyze and contextualize their genetics data such that they can confidently present their findings in the future more rigorously.

We agree that focusing only on CNVs deleting *CDKN2A/B* provides a weak genetic basis to explain the biologic differences across the meningioma DNA methylation groups, but we provide myriad genetic data supporting the existence of Merlin-intact, Immune-enriched, and Hypermitotic meningiomas that have not been challenged. We were embarrassed but also grateful the reviewer caught the short-lived incongruity between Extended Data Table 10 and our analyses of CNVs deleting *CDKN2A/B*. We have corrected this error and ensured that all our data and analyses are water tight. Thus, we respectfully contend the validity, impact, and conceptual advance of our study cannot be reduced to the relative rarity of *CDKN2A/B* deletions in meningiomas. Indeed, we have pointed out this solitary genetic event cannot explain the elevated cell proliferation we observe across all Hypermitotic meningiomas since our initial submission to *[REDACTED]* in the fall of 2020 (reference 53 at that time). To explain this experimentally, we analyze *CDKN2A/B* deletions (Fig. 4c, 4e and Extended Data Fig. 15, 18b), *CDKN2A/B* hypermethylation (Fig. 4d), misactivation of the FOXM1 gene expression program (Fig. 4b and Extended Data Fig. 11-13), and CNVs amplifying *USF1* (Fig. 4f-l and Extended Data Fig. 17, 18c) using mechanistic and functional studies in meningioma cells, organoids, and mouse models. We have modified our discussion to ensure our findings are discussed with full transparency, including stating that additional drivers of meningioma cell proliferation must exist.

We agree our study includes many supplemental figures and tables due to our scope and journal formatting restrictions. Nevertheless, our very first main figure (Fig. 1a) shows meningioma CNVs across the 565 tumors in our study. We also show sample-level DNA methylation, RNA sequencing, CNV, and protein expression data across meningioma DNA methylation groups in many other main figures (Fig. 1b, Fig. 2b, Fig. 2c, Fig. 2i, Fig. 3a, Fig. 3g, Fig. 3h, Fig. 4a, and Fig. 4d). To further highlight our genetic data supporting Merlin-intact, Immune-enriched, and Hypermitotic meningiomas for this revision, we have revised the heatmap in Fig. 1b to also include sample-level CNV metadata.

We agree that boiling chromosomal arm variations down to single genes is an oversimplification. Like most human cancers, we and other investigators show the majority of CNVs in meningiomas are broad, but understanding the biologic relevance of recurrently altered genes is an important step toward understanding the biologic and genetic diversity of cancer. In our main text, the section entitled *Convergent genetic and epigenetic mechanisms misactivate the cell cycle in meningioma* states “Although other genes on chromosome 1q may also contribute to Hypermitotic meningioma recurrence, USF1 was the only transcription factor from chromosome 1q with an enriched binding motif at the *CDK6* promoter (Extended Data Table 11).” More broadly, we support the biologic relevance of the genes we report across meningioma DNA methylation groups using orthogonal clinical, histological, genetic, biochemical, single-cell, mechanistic, and functional approaches for (i) *USF1* in Fig. 4g-i and Extended Data Fig. 17, 18c; (ii) *NF2* in Fig. 2b-j, Extended Data Fig. 6, and Extended Data Table 3-7; (iii) *HLA* in Fig. 3a-e, 3g-l, and Extended Data Fig. 7-10; and (iv) *CDKN2A/B* in Fig. 4c-e and Extended Data Fig. 15, 18b. To ensure the potential contributions of other genes are discussed with full transparency (and that our results are aligned with our conclusions), we have added the following paragraph to our discussion:

> Our study sheds light on biologic contributions of individual genes amplified, deleted, differentially methylated, or differentially expressed across meningioma DNA methylation groups. Nevertheless, it is unlikely *NF2*, *NR3C1*, *HLA*, *LYVE-1*, *FOXM1*, *CDKN2A/B*, *USF1*, or the other genes we report comprise the full spectrum of biologic drivers or therapeutic vulnerabilities in meningiomas. Our study and others show the majority of CNVs in meningiomas are broad (Fig. 1a and Extended Data Fig. 4a), and the biologic contributions of broad CNVs cannot be reduced to individual genes. Thus, future studies may reveal contributions from other genes recurrently deleted or amplified in meningiomas.

Decision Letter, third revision:

IMPORTANT: Please note the reference number: NG-A57863R2-Z Raleigh. This number must be quoted whenever you communicate with us regarding this paper.

6th Dec 2021

Dear David,

Thank you for your message of 6th Dec 2021, asking us to reconsider our decision on your manuscript "Meningioma DNA methylation grouping reveals biologic drivers and therapeutic vulnerabilities". I'm sorry that it's taken so long to return this initial decision to you but, as you know, we're in a busy period and appeals take second priority over new submissions.

I have now discussed the points of your letter with my colleagues, and we have agreed to consider your appeal. Before we do so however, we would like you to provide a summary Oncoprint heatmap showing all of your tumours, as intimated by Reviewer #2 in their last report. This should include epi/genetic/transcriptomic changes and proposed driver events for all subtypes. Once we have this, we plan to approach Reviewer #4. We have not yet spoken to them about the appeal, so we might have to revise the plan if they are unable to re-review the paper but we'll cross that bridge if and when we get to it.

We therefore invite you to re-submit the revised manuscript

When preparing a revision, please ensure that it fully complies with our editorial requirements for format and style; details can be found in the Guide to Authors on our website (http://www.nature.com/ng/).

Please be sure that your manuscript is accompanied by a separate letter detailing the changes you have made and your response to the points raised. At this stage we will need you to upload:
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Please use the link below to be taken directly to the site and view and revise your manuscript:

[REDACTED]

With kind wishes,

Safia

Safia Danovi
Senior Editor
Nature Genetics

Author Rebuttal, third revision:

Reviewer #2

In their rebuttal accompanying their revision, Choudury and colleagues have provided a detailed, yet confounding explanation pertaining to the sources of discrepancy and inconsistency related to CDKN2A/2B deletions they have summarized in their meningioma DNA methylation array dataset. As this revision process has demanded considerable attention and time-consuming efforts on my part, I have primarily focused this review on the topic of genetic evidence implicating CDKN2A/2B deletions in meningioma, as claimed to contribute to the hypermitotic subgroup of meningioma by the study co-authors. In the rebuttal letter, the authors state the following source of error related to Extended Data Table 10 that was provided in their previous submission:

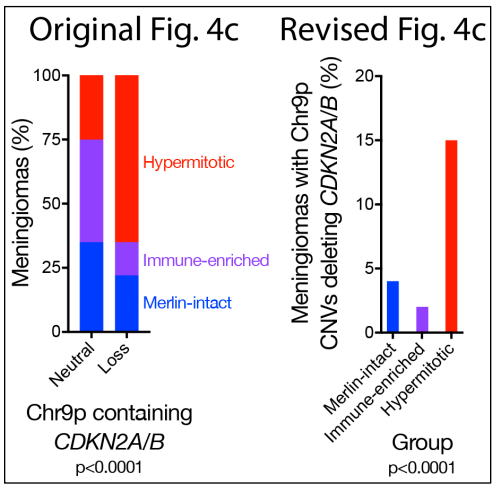
We apologize our previous explanations were insufficient. In response, we have revised the text, methods, figures, and figure legends of our manuscript to ensure the genetic data supporting our report of Hypermitotic and Immune-enriched meningiomas are robust, accessible, discussed with full transparency, and aligned with our conclusions. Our approach to quantifying CNVs targeting *CDKN2A/B* was the same as our approach to quantifying other CNVs of biologic interest (*HLA, NF2*, etc.). *NF2* CNVs have not been a source of concern during the course of peer review. Thus, as requested, we have focused on CNVs targeting *CDKN2A/B* or *HLA* in this letter. Revisions to our manuscript or extended data are highlighted in yellow for ease of evaluation.

• The version of Extended Data Table 10 we previously provided was generated independent of our CNV analyses across meningioma DNA methylation groups, and we unwisely failed to cross-check our data.
• Thus, correcting our embarrassing mistake in this table did not dilute the results or the conclusions of our manuscript.

I have multiple issues with these 2 statements. First, previous EDT10 did in fact include meningioma methylation subgroup annotations, and therefore it is difficult to appreciate how their provided explanation holds any validity. Moreover, sample-level CNV calls, as annotated in EDT10, should be completely agnostic of any subgroup membership, as the table simply lists samples that the authors claim to harbor CDKN2A/2B deletions.

The second issue I have relates to the second sentence above. The previous version of CDKN2A/2B deletions reported in EDT10 does not match the current version provided in EDT2. In EDT10, the authors previously included only focal deletions (defined as <5Mb) anywhere on chr9, and the current annotation in EDT2 includes all deletions (broad or focal) overlapping the entire CDKN2A gene. However, despite these varying definitions as to what the authors consider deletion of CDKN2A/2B, the Figure related to this data (Figure 4c) has never changed over the entire course of peer review. This is indeed troubling, because the narrative pertaining to the genetic evidence supporting CDKN2A/2B deletion in meningioma has changed considerably.

We apologize for the misunderstanding. As now described in the *Copy number analysis* section of our Methods, and further clarified in this letter, our CNV calls were indeed performed agnostic of meningioma DNA methylation groups. The DNA methylation groups of samples were unblinded only after CNVs were called. After unblinding, we analyzed the number and percentage of meningiomas in each DNA methylation group with copy number losses (focal or broad) including the *CDKN2A/B* locus. This definition identified 37 losses of the *CDKN2A/B* locus, and those losses provided the basis for our CNV analyses in the text, figures, and figures legends of our manuscript. This analysis has not changed over the course of peer review, but we have supported this approach with new analyses of focal versus broad CNVs, and complete versus partial CNVs, as described below. Unfortunately, when compiling the supplemental data for this manuscript at the time of our second revision at *Nature Genetics*, the table we generated (previously Extended Data Table 10) listed all focal deletions on the chromosome 9p arm, instead of all deletions (focal or broad) including the *CDKN2A/B* locus. Importantly, Fig. 4c was never based on the erroneous data we mistakenly provided in Extended Data Table 10. The current version of Extended Data Table 2 includes the data supporting Fig. 4c, and is consistent with our conclusions.



Given the reviewer's persistent concern, we have changed the formatting of Fig. 4c, which remains consistent with Extended Data Table 2 and shows the percentage of CNVs deleting the *CDKN2A/B* locus in each meningioma DNA methylation group. We used similar formatting to present CNVs deleting the *NF2* locus in Fig. 2a, which has not been a source of concern since our initial submission to *[REDACTED]* in the fall of 2020. To ensure our genetic data are presented consistently,

we have also revised our presentation of CNVs amplifying or deleting the *HLA* locus in Fig. 3f, or CNVs amplifying chr1q segments containing USF1 in Fig. 4f, to be consistent with Fig. 2a and revised Fig. 4c. Previously, Fig. 4c showed the distribution of meningioma DNA methylation groups with CNVs deleting chr9p segments containing *CDKN2A/B*, with the total number of CNVs summed to 100% (n=37). The majority of these CNVs were found in Hypermitotic meningiomas (n=24), but our prior formatting of Fig. 4c could be taken to imply that the majority of Hypermitotic meningiomas themselves had CNVs deleting *CDKN2A/B*. Our data do not support this interpretation, and we apologize for our confusing prior presentations of these genetic data. We hope our new formatting, shown above for ease of evaluation, will clarify these important data.

For instance, looking back at early versions of this manuscript while under consideration at [REDACTED] (spring 2021), the authors initially claimed that 'CNVs deleting the CDKN2A/2B locus were enriched in Hypermitotic meningiomas (62%), and were associated with worse LFFR.'

The same sentence from our initial submission to *[REDACTED]* in the fall of 2020 read "62% of *CDKN2A/B* losses occurred in Hypermitotic meningiomas." Neither of these iterations stated 62% (or any majority) of Hypermitotic meningiomas encoded *CDKN2A/B* losses. Rather, as quoted by the reviewer in the sentences below, our intention was to convey that *CDKN2A/B* deletions were themselves more common in Hypermitotic meningiomas than other DNA methylation groups. We acknowledged our initial phrasings of this sentence were suboptimal on prior resubmissions, and have further optimized this sentence and others like it, as described below.

In a previous version at Nature Genetics (summer 2021), the authors stated 'Focal CNVs deleting CDKN2A/B were identified in 7% of meningiomas (n=37 of 565), but were enriched in Hypermitotic meningiomas (15%, n=24 of 157) compared to Merlin-intact (4%, n=8 of 192) or Immune-enriched meningiomas (2%, n=5 of 216) (Fig. 4c and Extended Data Table 10), and were associated with worse LFFR.'

We enormously regret our erroneous and solitary use of the word "focal" in this sentence from our summer 2021 resubmission. We did not use this word in our previous submissions to *[REDACTED]* in the fall of 2020 or the spring of 2021 (or at any time in our figures or figure legends). To ensure our genetic data sufficiently align with our conclusions, we do not claim anywhere in our revised text, methods, figures, or figure legends that focal CNVs are uniquely responsible for any of our findings. We would be happy to test any preferred definition(s) of CNV focality across meningioma DNA methylation groups to ensure the integrity of our data.

In the current version at Nature Genetics (fall 2021), the authors state 'Copy number deletions containing the entire CDKN2A/B locus were identified in 6.5% of meningiomas (n=37 of 565), but were enriched in Hypermitotic meningiomas (15%, n=24 of 157) compared to Merlin-intact (4%, n=8 of 192) or Immune-enriched meningiomas (2%, n=5 of 216) (Fig. 4c and Extended Data Table 2), and were associated with worse LFFR (Extended Data Fig. 15a).'

This sentence accurately summarizes the number and distribution of CNVs deleting *CDKN2A/B* in the meningiomas from our study. We overwhelmingly regret the length of time it has taken to optimize this sentence. Moreover, given the persistent concern related to the nature of CNVs we analyzed across meningioma DNA methylation groups, we have further revised this sentence in our main text to now read:

> Copy number deletions of any size containing the entire *CDKN2A/B* locus were identified in 6.5% of meningiomas (n=37 of 565), but were enriched in Hypermitotic meningiomas (15%, n=24 of 157) compared to Merlin-intact (4%, n=8 of 192) or Immune-enriched meningiomas (2%, n=5 of 216) (Fig. 4c and Extended Data Table 2), and were associated with worse LFFR (Extended Data Fig. 15a).

We have also revised the title of Extended Data Table 2 and the sentences in our main text and figure legends specifying the number and distribution of CNVs deleting chr22q segments containing the *NF2* locus, amplifying or deleting chr6p segments containing the *HLA* locus, or amplifying chr1q segments containing the *USF1* locus. In each, we specifically state that CNVs of any size encompassing entire loci were analyzed. A prior study found *CDKN2A* deletions in 4.9% of meningiomas (PMID 32642869), and our study revealed a similar frequency of *CDKN2A/B* deletions in meningiomas (37 of 565 meningiomas, 6.5%), suggesting our approach is externally valid. Our rational for this approach is presented in the *Copy number analysis* section of our Methods, and is also summarized in the next 2 paragraphs of this letter for ease of evaluation.

First, we recognize definitions of CNV focality lack consensus. Many studies do not define CNV focality with precision, and those that do have definitions ranging from less than 3 Mb (PMID 20593488) to less than 98% of a chromosome arm (PMID 21527027). Moreover, how best to define "focal" CNVs containing single genes of different sizes (*CDKN2A, CDKN2B, NF2,* etc.) versus polymorphic loci containing multiple genes (*HLA-DRB5, HLA-DRB1, HLA-DQA1,* and *HLA-DQB1*) is unclear. For meningiomas, non-focal CNVs deleting chromosome

22q segments containing *NF2* are widely recognized as having biologic significance due (at least in part) to deletion of *NF2* despite the fact that these deletions are often broad. Indeed, 350 of the 351 copy number deletions containing *NF2* in our study were larger than 5 Mb. Thus, we decided it would be appropriate to combine and quantify focal and non-focal CNVs containing other genes of interest across meningioma DNA methylation groups, provided we could orthogonally corroborate the biologic significance of these genes (an objective we accomplished for *NF2, HLA, CDKN2A/B,* and *USF1* using clinical, histological, genetic, biochemical, single-cell, mechanistic, and functional approaches).

Second, considering the resolution of CNVs derived from Illumina 850k DNA methylation arrays (which were used in our study and are based on probe locations, rather than sequencing reads), we decided the most parsimonious and transparent approach would be to quantify and report only CNVs containing entire loci of interest. To ensure our analyses are robust and legitimate, we have also provided secondary analyses of CNVs partially overlapping loci of interest, or restricted to focal CNVs, as described in the next response of this letter.

Based on the prior discrepancies associated with EDT10 summarized in detail during my last review, I once again revisited this data in comparison to the data now provided in EDT2. Filtering EDT10 down to focal deletions (<5Mb) overlapping CDKN2A, there were 13 events. Performing the same analysis for EDT2, identifies 8 events (5 hypermitotic, 2 immune-enriched, 1 Merlin-intact). It is not clear why previous deletions summarized in EDT10 have been removed in EDT2, specifically those annotated for the following sample IDs: QM175, QM283, QM60, QM61, M20.

Regarding the 8 meningiomas with focal CNVs deleting the *CDKN2A/B* locus in Extended Data Table 2, it appears the reviewer may have missed our analyses of focal plus broad CNVs across DNA methylation groups described in our previous response letters and in the *Copy number analysis* section of our Methods. Although it is true 8 of the 37 meningiomas in Extended Data Table 2 encode CNVs <5 Mb that delete the *CDKN2A/B* locus, the remaining 29 meningiomas in Extended Data Table also encode CNVs that delete the *CDKN2A/B* locus. We present genetic data from these 37 meningiomas in our main text and Fig 4c. For further clarity, as described above, we have revised Fig. 3f, Fig. 4c, and Fig. 4f to be consistent with Fig. 2a, and have also revised our main text and figure legends. We are hopeful these revisions will ensure our experimental approach is accessible and discussed with full transparency.

Nevertheless, when restricting our analyses to the 8 meningiomas with CNVs <5 Mb deleting the *CDKN2A/B* locus, our data still demonstrate that such CNVs are more common in Hypermitotic meningiomas compared to other DNA methylation groups. It appears the reviewer may have missed this analysis on the final page of our most recent response letter (and in the *Copy number analysis* section of our Methods), where we stated:

> As an additional test of CNV specificity across meningioma DNA methylation groups, focal deletions <5 Mb of the entire *CDKN2A/B* locus were more common in Hypermitotic meningiomas compared to other DNA methylation groups (5 *CDKN2A/B* deletions in 157 Hypermitotic meningiomas compared to 3 deletions in 408 non-Hypermitotic meningiomas, p=0.0413, Fisher's exact test).

The same held true when we restricted our analysis of the Immune-enriched group to meningiomas with CNVs <5 Mb amplifying the polymorphic *HLA* locus. In our most recent response letter and methods we stated:

> Focal amplifications <5 Mb of the entire polymorphic *HLA* locus were more common in Immune-enriched meningiomas compared to other DNA methylation groups (37 polymorphic *HLA* amplifications in 216 Immune-enriched meningiomas compared to 20 amplifications in 192 Merlin-intact meningiomas and 8 amplifications in 157 Hypermitotic meningiomas, p=0.0013, Chi-squared test).

The 5 meningiomas mentioned above that were previously displayed in Extended Data Table 10 but not displayed in Extended Data Table 2 only partially overlapped with the *CDKN2A/B* locus, as shown in the reviewer's IGV visualization from page 2 of their review of our summer 2021 resubmission to *Nature Genetics*. As described in our response letters, main text, and methods, we analyzed CNVs encompassing entire loci of interest due to technical limitations associated with precisely defining CNV locations from Illumina 850k DNA methylation arrays. The rationale for this approach has been expanded in the revised *Copy number analysis* section of our Methods (as described above). Moreover, the 5 partial deletions of the *CDKN2A/B* locus were found in 1 Immune-enriched meningioma (M20) and 4 Hypermitotic meningiomas (QM175, QM283, QM60, QM61). Thus, the trends and statistical significance across DNA methylation groups were preserved when we analyzed meningiomas with CNVs of any size deleting the entire (n=37) or partial (n=5) *CDKN2A/B* locus (n=28 of 157 Hypermitotic meningiomas, n=8 of 192 Merlin-intact meningiomas, n=6 of 216 Immune-enriched meningiomas, p<0.0001, Chi-squared test). The same held true to re-analyses of CNVs including the *HLA* locus. These secondary analyses incorporating partial-locus CNVs along side entire-locus CNVs have been added to the *Copy number analysis* section of our Methods.

To summarize, early iterations of this manuscript described CDKN2A deletions (presumed to be focal) in 62% of hypermitotic meningiomas. Subsequent iterations, including the current manuscript reduced this frequency to a broader definition of deletions overlapping the locus (focal plus broad events) and report a deletion frequency of 15% in the hypermitotic group. Taking the data in EDT2 as ground truth, the actual frequency of focal deletion at this locus is 5/157 = 3.2%. This 3.2% is a long way from the initial 62% originally described by the co-authors, which is very troubling to this reviewer. Had my initial concerns regarding these deletions not been raised, and this study published as is, the field would now believe that >60% of hypermitotic meningiomas harbor focal CDKN2A/2B deletions.

We apologize for the confusion created by our previous phrasing of these data. As described in our responses above, we never stated a majority of Hypermitotic meningiomas encoded CNVs deleting *CDKN2A/B* but we recognized our initial phrasings of these data could be improved and have revised our main text, methods, figures, and figure legends to address these concerns. Moreover, in the *Convergent genetic and epigenetic mechanisms misactivate the cell cycle in meningioma* section of our main text from our previous (and current) resubmissions, we state "Loss of the endogenous CDK4/6 inhibitor *CDKN2A/B* on chromosome 9p is a rare biomarker for meningioma recurrence," and "Most Hypermitotic meningiomas did not have CNVs deleting *CDKN2A/B* (Fig. 4c)." These statements highlight the importance of other cell cycle drivers that we also report in Hypermitotic meningiomas, such as FOXM1 and USF1. To highlight that *CDKN2A/B* loss is but one of several mechanisms misactivating the cell cycle in Hypermitotic meningiomas, we generated a new integrated analysis of genetic and epigenetic features in Hypermitotic meningiomas that is presented in our results and Extended Data Table 12 for this revision:

> To define the distribution of genetic and epigenetic mechanisms misactivating the cell cycle in Hypermitotic meningiomas, *CDKN2A/B* methylation (Fig. 4d) and *FOXM1* expression (Extended Data Fig. 11, 13) were analyzed alongside CNVs of any size deleting the entire *CDKN2A/B* locus (Fig. 4c) or amplifying the entire *USF1* locus (Fig. 4f). *CDKN2A/B* β methylation values in the top quartile of the 565 meningiomas in our study were defined as hypermethylated. *FOXM1* expression in the top quartile of the 200 meningiomas in the discovery cohort with available RNA sequencing (Extended Data Table 8) were defined as increased. Among the 63 Hypermitotic meningiomas in the discovery cohort (Extended Data Table 1), there were 13 tumors with CNVs deleting *CDKN2A/B*, 14 tumors with CNVs amplifying *USF1*, 37 tumors with *CDKN2A* hypermethylation, 32 tumors with *CDKN2B* hypermethylation, and 26 tumors with increased *FOXM1* expression (Extended Data Table 12). Removing duplicates, 52 of 63 Hypermitotic meningiomas in the discovery cohort had *CDKN2A/B* deletion, *USF1* amplification, *CDKN2A/B* hypermethylation, or increased expression of *FOXM1* (83%). Multiple genetic, epigenetic, or transcriptomic mechanisms misactivating the cell cycle were identified in 40 of 63 Hypermitotic meningiomas (63%).

To ensure our results are robust, accessible, fully transparent, and aligned with our conclusions, we generalized this approach to an integrated analysis of genetic and epigenetic features in Immune-enriched meningiomas that is presented in our discussion and Extended Data Table 13 for this revision:

> Integrating epigenetic drivers alongside genetic alterations may be important for understanding the biology of meningioma DNA methylation groups. We identified CNVs of any size deleting the entire *CDKN2A/B* locus or amplifying the entire *USF1* locus in 54 of 157 Hypermitotic meningiomas (35%) (Extended Data Table 2), but when integrated with *CDKN2A/B* hypermethylation and *FOXM1* expression, the epigenetic and genetic alterations underlying meningioma cell proliferation we report were found in 83% of Hypermitotic tumors (Extended Data Table 12). We used the same approach to integrate CNVs of any size amplifying the entire polymorphic *HLA* locus with hypomethylation of *HLA-DMA*, *HLA-DPB1*, or meningeal lymphatic genes (*LYVE1*, *CCL21*, *CD3E*), defined by β methylation values in the bottom quartile of the 565 meningiomas in our study (Extended Data Table 13). Removing duplicates, the epigenetic and genetic alterations underlying meningioma immune infiltration we report were found in 166 of 216 Immune-enriched meningiomas (77%). Multiple genetic or epigenetic mechanisms underlying immune infiltration were identified in 157 of 216 Immune-enriched meningiomas (73%).

It has been a long journey to trace the proposed deletions of CDKN2A/2B deletion in hypermitotic meningioma, as defined by the co-authors of this study. For a cancer genetics-oriented study targeting a top tier journal in the field of Genetics, one would expect that the associated genetic data identified and summarized in the study would be robust, consistent, and featured prominently in the manuscript to substantiate the study narrative and conclusions. Disappointingly, this has not been the case for this effort. The genetics reported in this study have lacked consistency and have been bluntly misleading, for the most part hidden in supplemental tables or deceptively summarized in bar graphs. When cancer genetics data is solid, the actual genetic events are shown as is and featured, not buried in an Extended Data Table. Furthermore, generalizing chromosomal and chromosome arm deletions as causative events linked to a single gene (i.e., chr9p deletion = CDKN2A/2B deletion; chr1q gain = USF1 amplification) is an oversimplification employed in this study. Although the authors have toned down some of their conclusions over the course of their manuscript evolution, this culminates in genetic data that is relatively weak and unconvincing. In conclusion, based on the persistence of discrepancies

related to the reported deletions of CDKN2A/2B, and the reality that the genetics data presented in this study does not strongly support the implications of the manuscript narrative, I cannot endorse this study for publication in Nature Genetics. Considering the time and effort invested, I am hopeful that the authors of this study will take my constructive comments and suggestions to heart and attempt to analyze and contextualize their genetics data such that they can confidently present their findings in the future more rigorously.

We agree that focusing only on CNVs deleting *CDKN2A/B* provides a weak genetic basis to explain the biologic differences across the meningioma DNA methylation groups, but we provide myriad genetic data supporting the existence of Merlin-intact, Immune-enriched, and Hypermitotic meningiomas that have not been challenged. We were embarrassed but also grateful the reviewer caught the short-lived incongruity between Extended Data Table 10 and our analyses of CNVs deleting *CDKN2A/B*. We have corrected this error and ensured that all our data and analyses are water tight. Thus, we respectfully contend the validity, impact, and conceptual advance of our study cannot be reduced to the relative rarity of *CDKN2A/B* deletions in meningiomas. Indeed, we have pointed out this solitary genetic event cannot explain the elevated cell proliferation we observe across all Hypermitotic meningiomas since our initial submission to *[REDACTED]* in the fall of 2020 (reference 53 at that time). To explain this experimentally, we analyze *CDKN2A/B* deletions (Fig. 4c, 4e and Extended Data Fig. 15, 18b), *CDKN2A/B* hypermethylation (Fig. 4d), misactivation of the FOXM1 gene expression program (Fig. 4b and Extended Data Fig. 11-13), and CNVs amplifying *USF1* (Fig. 4f-l and Extended Data Fig. 17, 18c) using mechanistic and functional studies in meningioma cells, organoids, and mouse models. We have modified our discussion to ensure our findings are discussed with full transparency, including stating that additional drivers of meningioma cell proliferation must exist.

We agree our study includes many supplemental figures and tables due to our scope and journal formatting restrictions. Nevertheless, our very first main figure (Fig. 1a) shows meningioma CNVs across the 565 tumors in our study. We also show sample-level DNA methylation, RNA sequencing, CNV, and protein expression data across meningioma DNA methylation groups in many other main figures (Fig. 1b, Fig. 2b, Fig. 2c, Fig. 2i, Fig. 3a, Fig. 3g, Fig. 3h, Fig. 4a, and Fig. 4d). To further highlight our genetic, epigenetic, and transcriptomic data supporting Merlin-intact, Immune-enriched, and Hypermitotic meningiomas in the main figures of this revision, we generated a new oncoprint heatmap in Fig. 5a that displays sample level CNV, DNA methylation, and gene expression data. This new figure is also shown below in this letter for ease of evaluation.

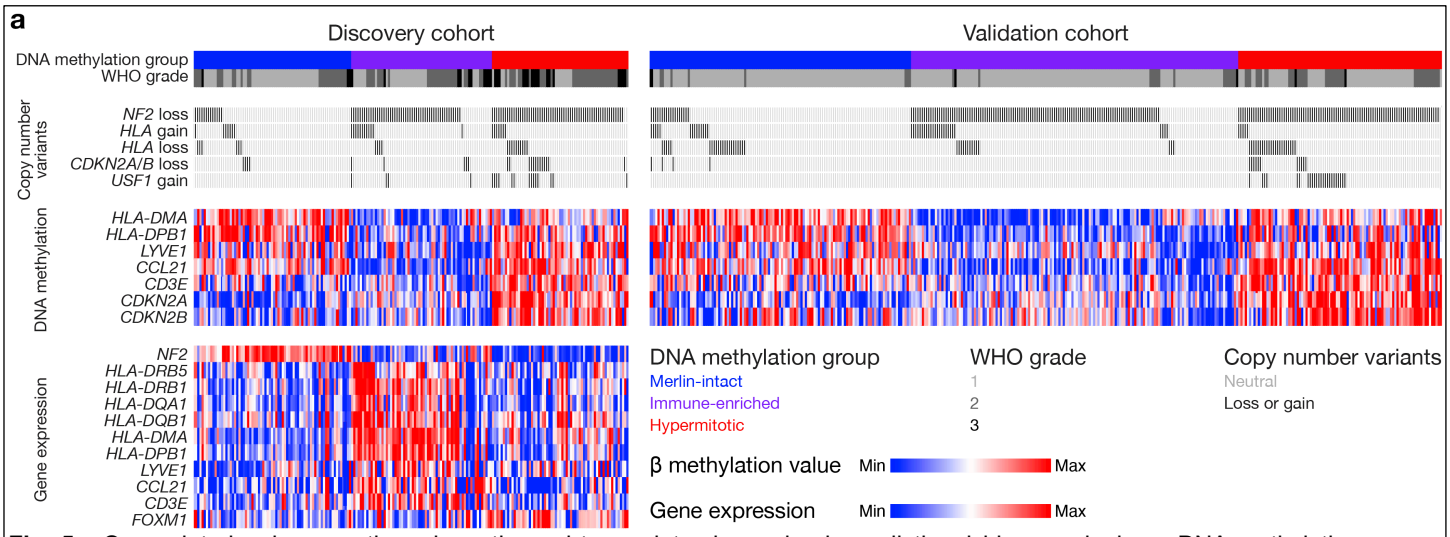


Fig. 5a. Oncoprint showing genetic, epigenetic, and transcriptomic mechanisms distinguishing meningioma DNA methylation groups across the 565 tumors in this study. CNVs of any size deleting or amplifying entire genes, scaled β methylation values, and scaled transcripts per million (TPM) are shown. The focal versus broad nature of CNVs are described in the *Copy number analysis* section of the Methods. *HLA* gain/loss shows the polymorphic locus. β methylation values and TPM are scaled from the bottom 10th percentile to the top 90th percentile of each row. RNA sequencing was performed on the discovery cohort (n=200) but not the validation cohort (n=365).

We agree that boiling chromosomal arm variations down to single genes is an oversimplification. Like most human cancers, we and other investigators show the majority of CNVs in meningiomas are broad, but understanding the biologic relevance of recurrently altered genes in CNVs is an important step toward understanding the biologic and genetic diversity of cancer. Moreover, we support our focus and subsequent mechanistic and functional studies of *NF2, HLA, CDKN2A/B,* and *USF1* with orthogonal bioinformatic approaches. For instance, in our main text, the section entitled *Convergent genetic and epigenetic mechanisms misactivate the cell cycle in meningioma* states “Although other genes on chromosome 1q may also contribute

to Hypermitotic meningioma recurrence, USF1 was the only transcription factor from chromosome 1q with an enriched binding motif at the *CDK6* promoter (Extended Data Table 11)." More broadly, we support the biologic relevance of the genes we report across meningioma DNA methylation groups using orthogonal clinical, histological, genetic, biochemical, single-cell, mechanistic, and functional approaches for (i) *USF1* in Fig. 4g-i and Extended Data Fig. 17, 18c; (ii) *NF2* in Fig. 2b-j, Extended Data Fig. 6, and Extended Data Table 3-7; (iii) *HLA* in Fig. 3a-e, 3g-l, and Extended Data Fig. 7-10; and (iv) *CDKN2A/B* in Fig. 4c-e and Extended Data Fig. 15, 18b. To ensure the potential contributions of other genes are discussed with full transparency (and that our results are aligned with our conclusions), we have added the following paragraph to our discussion:

> Our study sheds light on biologic contributions of individual genes amplified, deleted, differentially methylated, or differentially expressed across meningioma DNA methylation groups. Nevertheless, it is unlikely *NF2*, *NR3C1*, *HLA*, *LYVE-1*, *FOXM1*, *CDKN2A/B*, *USF1*, or the other genes we report comprise the full spectrum of biologic drivers or therapeutic vulnerabilities in meningiomas. Our study and others show the majority of CNVs in meningiomas are broad (Fig. 1a and Extended Data Fig. 4a), and the biologic contributions of broad CNVs cannot be reduced to individual genes. Thus, future studies may reveal contributions from other genes recurrently deleted or amplified in meningiomas.

Decision Letter, fourth revision:

Our ref: NG-A57863R3

6th Jan 2022

Dear Dr. Raleigh,

Thank you for submitting your revised manuscript "Meningioma DNA methylation grouping reveals biologic drivers and therapeutic vulnerabilities" (NG-A57863R3). It has now been seen again by Reviewer #4 and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Genetics, pending minor revisions to satisfy Reviewer #4's final requests, and to comply with our editorial and formatting guidelines.

If the current version of your manuscript is in a PDF format, please email us a copy of the file in an editable format (Microsoft Word or LaTex)-- we can not proceed with PDFs at this stage.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements soon. Please do not upload the final materials and make any revisions until you receive this additional information from us.

Thank you again for your interest in Nature Genetics Please do not hesitate to contact me if you have any questions.

Sincerely,

Safia Danovi
Editor
Nature Genetics

Reviewer #4 (Remarks to the Author):

The current revision of the meningioma paper by Choudhury et al reads well. The purpose of my review was to evaluate whether the classification proposed is justified, and whether the biological interpretation of the subtypes is sufficiently substantiated by the data.
1. The three DNA methylation profiling based groups (Merlin-intact, immune-enriched hypermitotic) are shown in two separate cohorts which argues that are robust.
2. The Merlin-intact group is defined by the significantly lower rate of NF2 loss (16% vs respectively 76%/95%), corroborated by mRNA and protein expression data showing transcription and translation of NF2 into the Merlin protein. Well justified.
3. The immune-enriched group contains significantly higher fractions of non-meningioma cells, as demonstrated by deconvolution of DNA methylation profiles as well as RNA profiles into cell types

using well established methods (Fig 3A/3D). Note that an immune-cell enriched cluster can be detected across many solid cancer types, i.e. PMID 17683518 and many others.
4. A cluster of samples associated with increased cell cycle activity can also be found in many cancers. See for example the classical Perou breast cancer paper which describes 'proliferative' breast cancers (PMID 10963602). Here, this cluster is labeled 'hypermitotic' whereas it is often referred as the Proliferative subtype (see PMID 21720365 for another example). FOXM1 has been previously reported as a master regulator of a proliferative phenotype, for example in ovarian cancer (also PMID 21720365). Visually, there appears to be enrichment of CDKN2A loss (chr9) and USF1 loss (chr1) in the hypermutation group per Supplementary Figure 4a. The authors do not use these observations as the main justification for labeling this group hypermitotic and they acknowledge that only a subset of the hypermitotic cases is characterized by either of those deletions. I agree with the authors that this is a minor point. The main justification for labeling this group as hypermitotic is the Ki67 staining in Fig 4A, as well as the frequent observation of similar phenotypes in other cancer types.
I am in support of publishing this manuscript. The authors have made their datasets available via GEO but the accession is not yet public. In this reviewer's opinion, data availability should be a prerequisite for acceptance.

Final Decision Letter:

In reply please quote: NG-A57863R4 Raleigh

22nd Mar 2022

Dear Dr. Raleigh,

I am delighted to say that your manuscript "Meningioma DNA methylation groups identify biological drivers and therapeutic vulnerabilities" has been accepted for publication in an upcoming issue of Nature Genetics.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Genetics style. Once your paper is typeset, you will receive an email with a link to choose the appropriate publishing options for your paper and our Author Services team will be in touch regarding any additional information that may be required.

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Sincerely,

Safia Danovi
Editor
Nature Genetics