Reviewers' comments:

Reviewer #1 (Remarks to the Author); expert in medulloblastoma:

In this manuscript, Zagozeweski et al. show that OTX2 suppresses neuronal differentiation program in part by inhibiting the expression of PAX3. Authors also showed that OTX2-PAX3 axis regulates mTORC1 activities and inhibitors of mTORC1 suppress Group 3 medulloblastoma development in xenograft model. The study reveals an important oncogenic function of OTX2: repressing PAX3-induced differentiation program. Overall the results support conclusion. Addressing following points would strengthen the conclusion.

1. It is unclear how loss of OTX2 or overexpression of PAX3 inhibit mTORC1 activities. Authors only examined the phosphorylation of S6 and 4EBP1, which are downstream targets of mTORC1. Several key regulators and components of mTORC1 are well-known. Are there changes in the levels or activities of key regulators and/or components of mTORC1?
2. The purpose or meaning of figure 5 is unclear. Are there specific cell types, where expression of genes regulating mTORC1 activity or levels are affected by OTX2 KD or PAX3 overexpression? In addition, Fig. 5 an S6 do not support "all four Group 3 MB tumorsphere populations are broadly enriched for a transcription program that represents undifferentiated progenitors (Fig. 5G-I, Figure S6A-F)". What does "broadly enriched" mean? How was this conclusion made? The ribosome and translation/elongation factor genes and mTORC1 genes are house-keeping genes that are expected to be expressed in all cells. Therefore, it is expected that expression of those genes is observed in all cell clusters in Group 3 tumorsperes, thus "broadly enriched" is unfounded.
3. Does inhibition of mTORC1 activity increase neuronal differentiation program in Group 3 tumors?
4. Will it be possible to activate mTORC1 activity in OTX2[-/-] cells or cells overexpressing PAX3 to confirm that OTX2-PAX3 axis suppresses tumorigenicity via reducing mTORC1 activities?
5. A recent study (Wu et al., 2017 Dev Cell) showed that mTORC1 activity is high in SHH and WNT MB but low in Group 3 and Group 4 MB, which is somewhat contradictory to the current study showing strong mTORC1 activity in Group 3 MB. What is the explanation? This may be addressed.
6. In the abstract, authors wrote "OTX2 regulates the repressive chromatin landscape by modulating levels of PRC2 complex genes…" Authors showed that OTX2 KD decrease the protein levels of the EZH2 and SUZ12; however, there is no evidence that this decrease indeed affected repressive chromatin landscape.
7. At the end of Introduction, authors wrote that "PAX3 or PAX6 overexpression inhibits Group 3 MB tumorigenic properties.."; however, only PAX3 overexpression suppressed tumorigenicity.
8. The result section starts with silencing OTX2 in Group 3 tumorspheres. It will be necessary to explain a little more detail about the cell lines, Group 3 tumorspheres, and rational to use it. Also, three different Group 3 tumorspheres were used for different experiments. What is the rational for using certain tumorspheres for certain experiments?
9. Table S3 is cited in the first line on page 8. Isn't it Table S1?
10. "The neuronal differentiation genes βIII tubulin (TUJ1) and EPHB2 were also increased in PAX GOF tumorspheres compared to controls (Fig. 4G,H)." TUJ1 was increased only in PAX6 GOF.

Reviewer #2 (Remarks to the Author); expert in single-cell RNA-seq and transcriptomics:

In the manuscript "An OTX2-PAX3 signaling axis regulates Group 3 medulloblastoma cell fate" Zagozewski et al. study the molecular underpinning of Group 3 medulloblastomas.
The authors link changes in OTX2 abundance to genome-wide changes in H3K4me3 and H3K27me3. They show that transcription factors PAX3 and 6, which are important for neuronal differentiation, are downregulated in Group 3 Medullablastoma. They describe that this downregulation is associated with poor prognosis and might in part be controlled by OTX2.

Remarkably, only PAX3 but not PAX6 overexpression was able to reduce self-renewal and increased survival in a xenograft model. Finally, the authors describe that the proposed OTX2-PAX3 affects mTORC1 signaling in Group 3 medulloblastomas.

This is a potentially interesting study, though the conclusion in establishing the OTX2-PAX3 axis and molecular mechanism of regulating mTORC1 activity appear premature. The authors should address the following points and questions.

Major Points:
1) To establish the link between OTX2 and PAX3 and identify mTORC1 genes as downstream targets the authors utilize changes in H3K4me3 and H3K27me3 signal in OTX2 high and low tumor spheres. It would be helpful to evaluate the proposed link, if the authors could add more detail about this part since this is a central claim of the paper. For how many independent tumor spheres were the ChIP-seq experiments for H3K4me3 and H3K27me3 performed and how was the reproducibility between replicates? A number of 8,444 of changed H3K4me3 is a very dramatic change and representing ~40% of all protein coding gene promoters. For evaluation it would be helpful to display a heatmap displaying signal strength for each of the element and an average. This could give an idea of the extend of changes at a given locus. How many of the promoters with differential histone marks where differential expressed? It would be important to show actual mRNA expression changes rather than inference from histone marks alone and furthermore characterize the distal landscape using ChIP-seq for H3K27ac to get a more detailed insight into gene regulatory effects, since these distal elements are shown to be more cell type specific and dynamic. For potential direct link to PAX3 and 6, a genome browser track would be very helpful with indication of differential peaks between high and low OTX2 (as shown in Fig. 2 for different cell lines). According to Table 1, PAX3 only shows significant changes in H3K27me3 whereas PAX6 showed changes in K27 and K4? How do the authors explain these minor differences especially in the light that PAX3 is described as direct downstream target of OTX2? How are the expression levels? Are OTX2 and PAX3 and 6 anti-correlated? Surprisingly, in the browser tracks displayed in Fig 2B the H3K4me3 peaks do not match up with the PAX3 promoter. Do these peaks correspond to other genes or different splice variants/unannotated promoters? Did the authors check for distal elements like enhancers which were explored in Boulay et al. 2017? It would be interesting to see ChIP-seq for H3K27ac since it not only marks active promoters but also active enhancer elements. It would further expand the epigenetic profiling since H3K4me3 not only marks active but also poised and bivalent promoters.
2) Since PAX3/6 are TF with low expression levels it is not very surprising that they were (almost) not captured in scRNA-seq. The scRNA-seq does not indicate many substructures, but I was wondering how the clusters were defined; what do they correspond to, do they represent different stages of differentiation or simply cell cycle stages, how were non-tumor cells defined, what are the differences between OTX2 high and low clusters (according to the dotblots)?
3) Since protein levels for mTORC1 genes are not altered, but the autors detected increased phosporylation, I was wondering if there is increased expression of upstream effectors and if they are regulated by OTX2 and or PAX3.To elucidate the contribution of gene regulation for mTORC1 activation and to explore the claim that PAX3 regulates mTORC1 genes at different regulatory elements, it would be great to perform ChIP-seq for PAX3 or H3K27ac to annotate these regions in OTX2 high and low tumor spheres.Which are the target genes which are upregulated?
4) Why are the effects of the dual mTORC1/2 inhibitor AZD8055 and PQR620 so different? Does AZD8055 inhibit other proteins or is PQR620 under-dosed for most of the experiments?
Minor Points:
1) Please make sure font sizes are adjusted in figures and the figures are not to busy. The figures contain many panels shich makes it difficult get the main message from the figure. E.g. in Fig 5 G-I it might be better to either show a selection of choose a different way for display. For Fig 7 it would be sufficient to show AZD08055 and display the data for PQR620 in the supplment.
2) Please make sure that font sizes and orientation of figure panels are consistent. In several cases makes it hard to even read axis labels or titles of graphs.

Reviewer #3 (Remarks to the Author); expert in epigenetics and medulloblastoma:

Zagozewski and collaborators used multi-omics to unravel the role of OTX2 in Group3 medulloblastoma. OTX2 is overexpressed in all subgroups of medulloblastoma but its specific role in, and the mechanisms by which it maintains Group3 medulloblastoma undifferentiated stem-like fate has not been previously described. The authors show that OTX2 repressed neural differentiation by up-regulating the Polycomb repressive complex PRC2 and suppressing PAX3 and PAX6 expression. Finally, they found that enforced expression of PAX3 but not PAX6 increased survival in vivo and that OTX2 affects mTOR signaling that can be pharmacologically suppress Group3 medulloblastoma progression. This is a well-executed and extensive number of experiments that convincingly demonstrate the role of OTX2 overexpression in enforcing Group3 medulloblastoma stem-like phenotype. Overall, the experiments are well done and merit publication after minor criticisms are addressed.
Criticisms
1.In the introduction I challenge the comment that "there are currently no targeted therapies for the treatment of these devastating tumors". Three recent clinical trials using targeted therapies have been initiated in the last few years SJMB012, SJDAWN and SJELIOT that should be highlighted in the Introduction. At least I would delete the comment that there are no current targeted therapies for medulloblastoma, Group3 or other subgroups.
. OTX2 has been found overexpressed in all subgroups of medulloblastoma, not just Group3 and Group4. In fact, the authors in the Discussion noted that OTX2 is also overexpressed in WNT and it has been found in Sonic Hedgehog tumors as well. I would make that statement in the text.
2. Figure 1B and C, it is unclear that the experiments were done with only one human Group3 medulloblastoma line, D283. I would state which line was used in the text and possibly the figure.
. Figure 1G, I assume that PAX6 is not expressed. I barely see one dot in cluster 9- is this right? If so, I would circle it since it is barely visible in my copy.
3. Figure 2, many of the writing in the panels A, B, C and D cannot be read which makes the analysis of the Figures difficult to evaluate. This will be worse when the figures are reduced. I suggest you increase the fonts.
Figure 2G, protein levels for PAX3 and PAX6 for all three human medulloblastoma cell lines D283, HDMB03 and MB3W1 should be presented.
4. Figure 3. Figure 3C provides pictures of tumorsphere formation after PAX3 and PAX6 overexpression in HDMB03 cells: I do not find them convincing whereas the numbers in Figure 3D are. In the text, the authors state that upon secondary passage, there was a significant reduction in tumorsphere number in PAX3 overexpressed cells. Looking at the numbers in Figure 3D, only in PAX6 overexpressed cells do I see a reduction of the number of spheres. I see no difference in the number of spheres in PAX3 overexpressed HDMB03. The authors should comment.
Figure 3H and 3K, whereas the p value is less than 0.05, the number of animals tested (n=5 or 7 per cohort) is far too low to reach a conclusion. The authors should increase the number of animals. Similarly, the experiments should be repeated minimally with another line, for example D283 since data are presented in Figures 1 and 3.
5. Figure 4, panel C, I would state that the comparison was done in HDMBO3 overexpressing PAX3 and PAX6.
6. Figure 5, same criticism as above: I am not able to read numbers and genes in panels A-C and G-I preventing me from evaluating the data. I assume that PAX3 like PAX6 is not expressed in any of the three lines. Is this true? If the speck for PAX3 I see is real, I would circle it.
7. Figure 6. Figure 6D and 6E, I would indicate the line used possibly on top of the left panels.
8. Figure 7. Whereas I agree that the AZD8055 drug efficiently suppresses pS6 and tumorsphere number in HDMB03 and MB3W1 cells, the required concentrations are high and even greater for PQR620 raising questions as to the validity in using mTORC inhibitor for the treatment of Group 3 medulloblastoma. In addition, the survival curves for mice implanted with HDMB03 treated with PQR620 show no difference in the response using 50 or 100 mg/kg with a relatively small p value. The number of animals per cohort is too small and should be increased. How toxic is the treatment?

9. Figure 8. Because many different cell lines are using for different assays, I would list the cell line used in the panels of the Figures when possible.

We thank the reviewers for their positive comments and thoughtful suggestions. We have performed an extensive series of experiments to answer the reviewers' questions in a timely manner and are pleased to submit the revised manuscript before the 6-month deadline. While the lab is currently shut down due to the ongoing COVID-19 pandemic, we are fortunate to have been able to complete the vast majority of revisions with only minimal disruption.

As requested by the editors in the original decision letter, we have focused on experiments that:
1) improve our understanding of the molecular mechanisms associated with our novel OTX2 signaling axis.
2) further evaluate mTORC1 regulation and the effect of inhibitors.
3) investigate the association between OTX2/PAX3/mTORC1 and differentiation.

Key highlights for the new data include: 1) western blots for SOX2, EPHB2, DCX, βIII tubulin, RHEB, p-Raptor/total Raptor, and cleaved caspase-3 following OTX2 silencing, PAX3/6 gain of function and/or mTOR inhibitor treatment *in vitro* 2) new *in vivo* studies to assess growth, survival and differentiation 3) additional patient data and 4) more quantitative analyses of our scRNA-seq data. All new data have been highlighted in red in the manuscript.

Reviewer#1; expert in medulloblastoma

In this manuscript, Zagozewski et al. show that OTX2 suppresses neuronal differentiation program in part by inhibiting the expression of PAX3. Authors also showed that OTX2-PAX3 axis regulates mTORC1 activities and inhibitors of mTORC1 suppress Group 3 medulloblastoma development in xenograft model. The study reveals an important oncogenic function of OTX2: repressing PAX3-induced differentiation program. Overall the results support conclusion. Addressing following points would strengthen the conclusion.

1. It is unclear how loss of OTX2 or overexpression of PAX3 inhibit mTORC1 activities. Authors only examined the phosphorylation of S6 and 4EBP1, which are downstream targets of mTORC1. Several key regulators and components of mTORC1 are well-known. Are there changes in the levels or activities of key regulators and/or components of mTORC1?

Response: This is an excellent question. We have evaluated additional key regulators and components of mTORC1 signaling following OTX2 loss as well as PAX3 and PAX6 overexpression. Specifically, a decrease in the upstream mTORC1 regulator, Ras homolog enriched in brain (RHEB) was observed in PAX3 gain of function (GOF) tumorspheres but not PAX6, consistent with p-S6 and p-4E-BP1. Similarly, when OTX2 is silenced in Group 3 medulloblastoma tumorspheres, we observe a decrease in RHEB in both HDMB03 and D283 cells. Interestingly, p-Raptor, a component of the mTORC1 complex, showed either small increases or no change in PAX3 GOF and OTX2 silenced cells. The new data have been added to Fig. 4g-h and Fig. 6d-e as well as pages 15 and 17 of the results section of the revised manuscript. We have also further interrogated our OTX2 ChIP-seq data and have identified OTX2 peaks and binding motifs on the important mTORC1 genes *RPS6*, *EIF4EBP1* and *RPTOR* suggesting that OTX2 can also directly regulate expression of these pathway regulators. Supplementary Table 8 has been updated accordingly.

2. The purpose or meaning of figure 5 is unclear. Are there specific cell types, where expression of genes regulating mTORC1 activity or levels are affected by OTX2 KD or PAX3 overexpression? In addition, Fig. 5 an S6 do not support "all four Group 3 MB tumorsphere populations are broadly enriched for a transcription program that represents undifferentiated progenitors (Fig. 5G-I, Figure S6A-F)". What does "broadly enriched" mean? How was this conclusion made? The ribosome and translation/elongation factor genes and mTORC1 genes are house-keeping genes that are expected to be expressed in all cells. Therefore, it is expected that expression of those genes is observed in all cell clusters in Group 3 tumorspheres, thus "broadly enriched" is unfounded.

Response: These are excellent questions. The reference to “all four Group 3 tumorsphere populations” was a mistake, as we only present data for 3 established/newly derived cell lines. The conclusions were made based on cross-referencing our data with the recent findings by Hovestadt et al., (Nature, 2019) that resolved medulloblastoma patient tumors at a single cell level. Interestingly, in this study, prototypic Group 3 tumors were dominated by the undifferentiated progenitor like program “B” (almost 90% of cells), while the neuronal-differentiated program “C” was dominant in almost all Group 4 tumor cells. As the “B” program was the only one primarily characterized by ribosomal and translation initiation/elongation genes, it appears as though these genes may be associated with the cell stem/progenitor phenotype specifically in Group 3 tumors. Thus, the programs and the genes associated with them appear to be subgroup-specific. While we showed distinct clusters across our tumorsphere models, indeed, the “undifferentiated B” program is expressed across the clusters. Therefore, we will utilize the term “expressed” instead of “broadly enriched.” That being said, we have strengthened our results by providing the following plots and associated quantification: 1. UMAP plots instead of tSNE to be consistent with the patient data. 2. Feature plots showing that clusters exhibiting a high undifferentiated metascore have a low cell cycle metascore. 3. Correlation plots demonstrating the negative correlation between the undifferentiated program and cell cycle as well as the positive correlation between the undifferentiated program and translation initiation. Collectively, our scRNA-seq data demonstrate that protein synthesis pathway activities (GO_Translation_initiation and GO_Ribosome_Biogenesis) are high in the undifferentiated stem/progenitor Group 3 MB cell population. **These findings provide support for the notion that mTORC1 signaling is a major component of a self-renewal program regulating Group 3 MB cell fate**. The new data have been incorporated into Figure 5 and Supplementary Figure 6 as well as the results section on page 16 of the revised manuscript. In this age of large-scale functional genomics studies with patient samples, it was important for us to demonstrate the strength of our tumorsphere models in recapitulating transcription programs observed in Group 3 medulloblastoma patient tumors. This adds strength and validity to our findings and supports the use of our model systems for functional studies both *in vitro* and *in vivo*.

3. Does inhibition of mTORC1 activity increase neuronal differentiation program in Group 3 tumors?

Response: This is an excellent question. We have evaluated levels of SOX2 as well as the neuronal differentiation marker βIII Tubulin in our xenograft models following mTORC inhibition and PAX3/PAX6 overexpression by immunohistochemistry. As opposed to our *in vitro* results in which we observe decreases in SOX2 only following PAX3 overexpression and mTOR inhibitor treatment, we observe decreases in SOX2 levels concomitant with increases in βIII tubulin in our animal models.

Interestingly, for PAX3 overexpressing and mTOR inhibitor-treated tumorspheres at sublethal concentrations, there is no change in neuronal differentiation gene levels; however, the drug- treated cells exhibit an increase in apoptosis as depicted by cleaved caspase 3, particularly at the higher concentrations. These results suggest that PAX3, and similarly mTORC signaling, may specifically eliminate the stem cell population *in vitro,* rather than shifting remaining primitive cells towards a neuronal progenitor phenotype. In the more heterogeneous *in vivo* environment, our results demonstrate that differentiation is concomitantly increased. PAX6, on the other hand, does not eliminate SOX2+ cells but clearly induces neuronal differentiation both *in vitro* and *in vivo*. As OTX2 silencing affects both PAX3 and PAX6, it is not surprising that there more global effects on all cell properties including self-renewal, cell cycle and differentiation. The new data are presented in Figure 3k, 4g, 6d-e, 7c and k, as well as Supplementary Figure 4 and 7 of the revised manuscript.

4. Will it be possible to activate mTORC1 activity in OTX2[-/-] cells or cells overexpressing PAX3 to confirm that OTX2-PAX3 axis suppresses tumorigenicity via reducing mTORC1 activities?

Response: While we appreciate the value in a rescue experiment, this may prove difficult as activation of mTORC1 (ie. through IGF activation) would likely be non-specific. Thus, we have chosen to focus on further validation using our gain/loss of function cells.

5. A recent study (Wu et al., 2017 Dev Cell) showed that mTORC1 activity is high in SHH and WNT MB but low in Group 3 and Group 4 MB, which is somewhat contradictory to the current study showing strong mTORC1 activity in Group 3 MB. What is the explanation? This may be addressed.

Response: In the recent Wu et al article, the human tumor data for Group 3 MB (Figure 5A in Wu et al, study) is presented as Non-WNT/non-SHH. Therefore; it is unclear whether the representative image is Group 3 or Group 4 specifically. Importantly, this study only looked at the p-4E-BP1 "arm" of mTORC1 and did not assess the p-S6 "arm" by immunohistochemistry in these patient tumors. Indeed, when we performed our analysis, we did stain SHH tumors for p-4E-BP1 as a positive control for our immunohistochemistry analysis based on the Wu et al paper. While we did not present these data, our results did recapitulate the Wu et al. findings in that p-4E-BP1 was highly expressed in SHH tumors. Moreover, our results (Figure 6g) show that mTORC1 proteins, including p-S6, are more highly expressed in Group 3 tumors, while levels were low in Group 4. Thus, the overall trends BETWEEN subgroups is in accordance with the Wu et al. study. To strengthen our patient data and the connection between Group 3 medulloblastoma and protein synthesis/mTOR pathways, we also performed pathway enrichment analyses using gene expression data from 763 MB patient samples (Cavalli et al., 2017). We observed that rRNA processing, ribosome maturation and activation, translation initiation, translation and post-translational protein targeting were upregulated in Group 3β and 3γ compared to all other MB subtypes. These data have been added to Fig. 6f and on pages 18 of the results section of the revised manuscript.

6. In the abstract, authors wrote "OTX2 regulates the repressive chromatin landscape by modulating levels of PRC2 complex genes…" Authors showed that OTX2 KD decrease the protein levels of the EZH2 and SUZ12; however, there is no evidence that this decrease indeed affected repressive chromatin landscape.

Response: We thank the reviewer for highlighting this. Given the functions of both SUZ12 and EZH2 in chromatin silencing, the effect on the chromatin landscape was inferred based on these results and our co-IP studies in which OTX2 was shown to interact directly with EZH2 and SUZ12. We have revised the statement in the abstract as follows: "*OTX2 silencing modulates the repressive chromatin landscape, decreases levels of PRC2 complex genes and increases the expression of neurodevelopmental transcription factors including PAX3 and PAX6*."

7. At the end of Introduction, authors wrote that "PAX3 or PAX6 overexpression inhibits Group 3 MB tumorigenic properties;" however, only PAX3 overexpression suppressed tumorigenicity.

Response: We apologize for the lack of clarity in this statement. In our *in vitro* studies examining PAX3 and PAX6 overexpression, a reduction of primary tumorsphere numbers and other genes associated with stem cell function was observed with increased PAX6 expression as well. When we mentioned "tumorigenic properties," we were referring to specific cell culture-based changes. We have rephrased this sentence on Page 6 of the revised manuscript as follows: *"Both PAX3 and PAX6 gain of function results in decreased tumorsphere formation and SOX2 levels as well as modulation of Group 3 medulloblastoma gene signatures in vitro. However, only PAX3 overexpression reduces self-renewal and mTORC1 signaling in tumorspheres while also increasing survival in vivo.* Overall, it

seems that PAX6 exhibits a weaker phenotype compared to PAX3 in our cell models, especially when considering our *in vivo* findings.

8. The result section starts with silencing OTX2 in Group 3 tumorspheres. It will be necessary to explain a little more detail about the cell lines, Group 3 tumorspheres, and rational to use it. Also, three different Group 3 tumorspheres were used for different experiments. What is the rational for using certain tumorspheres for certain experiments?

Response: Both HDMB03 and MB3W1 are low passage patient derived cell lines validated as Group 3 medulloblastomas. Therefore, we utilized these two lines for experiments whenever possible along with D283 cells. In the case of our histone ChIP-sequencing, we chose to use D283 as we had previously carried out OTX2 ChIP-sequencing on this cell line. We therefore wanted to utilize the same line for our histone ChIP-seq experiments in order to examine OTX2 binding and the histone landscape in parallel. As the ChIP-seq experiments were meant as a starting point and filter for the rest of the paper, we found that **validations** by ChIP-qPCR, for example, across tumorspheres from 3 independent cell lines with 3 biological replicates for each would be the most rigorous and feasible from our end. The extensive patient data also further strengthen our findings. As for the gain of function experiments, the HDMB03 cells were the most amenable to lentiviral transduction. Of note, we attempted to generate stable gain of function lines with both D283 and MB3W1 with limited success. While we were able to generate control and stable PAX6 overexpressing lines, the PAX3 overexpressing cells senesced and could not be passaged despite successful construct integration. These results further underscore the potent PAX3 phenotype relative to PAX6. For our scRNA-seq data, we were fortunate in that this only required wild-type cells in tumorsphere format; thus, we were able to generate data from tumorspheres derived from all 3 lines.

9. Table S3 is cited in the first line on page 8. Isn't it Table S1?

Response: Yes, thank-you, we made the correction.

10. "The neuronal differentiation genes βIII tubulin (TUJ1) and EPHB2 were also increased in PAX GOF tumorspheres compared to controls (Fig. 4G,H)." TUJ1 was increased only in PAX6 GOF.

Response: We broadly referred to PAX3 and PAX6 changes as PAX gain of function to encompass both genes. Language has been altered to reflect the specific changes observed in the PAX3 vs PAX6 overexpressing tumorspheres similar to Response #7 above.

Reviewer #2: expert in single-cell RNA-seq and transcriptomics

1.To establish the link between OTX2 and PAX3 and identify mTORC1 genes as downstream targets the authors utilize changes in H3K4me3 and H3K27me3 signal in OTX2 high and low tumor spheres. It would be helpful to evaluate the proposed link, if the authors could add more detail about this part since this is a central claim of the paper. For how many independent tumor spheres were the ChIP-seq experiments for H3K4me3 and H3K27me3 performed and how was the reproducibility between replicates? A number of 8,444 of changed H3K4me3 is a very dramatic change and representing

~40% of all protein coding gene promoters. For evaluation it would be helpful to display a heatmap displaying signal strength for each of the element and an average. This could give an idea of the extend of changes at a given locus. How many of the promoters with differential histone marks where differential expressed? It would be important to show actual mRNA expression changes rather than inference from histone marks alone and furthermore characterize the distal landscape using ChIP-seq for H3K27ac to get a more detailed insight into gene regulatory effects, since these distal elements are shown to be more cell type specific and dynamic. For potential direct link to PAX3 and 6, a genome browser track would be very helpful with indication of differential peaks between high and low OTX2 (as shown in Fig. 2 for different cell lines). According to Table 1, PAX3 only shows significant changes in H3K27me3 whereas PAX6 showed changes in K27 and K4? How do the authors explain these minor differences especially in the light that PAX3 is described as direct downstream target of OTX2? How are the expression levels? Are OTX2 and PAX3 and 6 anti-correlated? Surprisingly, in the browser tracks displayed in Fig 2B the H3K4me3 peaks do not match up with the PAX3 promoter. Do these peaks correspond to other genes or different splice variants/unannotated promoters? Did the authors check for distal elements like enhancers which were explored in Boulay et al. 2017? It would be interesting to see ChIP-seq for H3K27ac since it not only marks active promoters but also active enhancer elements. It would further expand the epigenetic profiling since H3K4me3 not only marks active but also poised and bivalent promoters.

Response: We thank the reviewer for these comments and have tried to address them to the best of our abilities considering the time to completion, the directions provided by the editors, the feasibility and the main focus of our manuscript. In the case of our histone ChIP-sequencing, we chose to use D283 tumorspheres only as we had previously carried out OTX2 ChIP-sequencing on this cell line. We therefore wanted to utilize the same line for our histone ChIP-seq experiments in order to examine OTX2 binding and the histone landscape in parallel with the least amount of variability. As the ChIP-seq experiments were only meant as a starting point and filter for the rest of the paper, we found that **validations** by ChIP-qPCR across tumorspheres from 3 independent cell lines with 3 biological replicates for each would be the most rigorous and feasible from our end. While our initial studies in Figure 1 mirror the approaches taken by the Boulay et al., study in terms of epigenetic analyses, this is where the similarities end, as the Boulay study focused almost exclusively on genome-wide chromatin and expression profiling. Instead, we have chosen to focus on validations at the transcript and protein levels in cell lines and patient samples, as well as important functional validations both *in vitro* and *in vivo*. We feel that these approaches have added the most strength, in terms of potential clinical relevance and novelty, to our study. That being said, we have added the following data in response to the reviewer questions/comments:

1. In the 2017 Boulay et al., study, the authors conducted extensive analyses of the distal regulatory elements using a combination of both OTX2 and H3K27ac ChIP sequencing. Thus, we feel that performing these analyses again would be redundant. However, we interrogated the Boulay et al., data to evaluate H3K27ac on the *PAX3* and *PAX6* genes in the Group 3 medulloblastoma patient samples. The results complement our existing findings in Figure 2d showing the broad H3K27me3 enrichment with narrow H3K4me3 peaks at the TSS for *PAX3* and *PAX6*. We have updated the figure and the results section accordingly.

2. Regarding expression levels and their relationship to changes in histone marks, we agree that this is very important. Thus, in keeping in line with looking at our specific signaling axis, we have assessed transcript levels of *PAX3* and *PAX6* in tumorspheres from 3 independent cell lines following OTX2 silencing in Figure 2g. Now, we have also assessed protein levels for PAX3 and PAX6 in OTX2 silenced cells by western blot. The new results have been incorporated into Figure 2h of the revised manuscript for both D283 and HDMB03. Unfortunately, we were not able to perform the experiment for MB3W1 cells before the lab shutdown due to the current COVID-19 pandemic. I hope this will suffice. Of note, despite significant increases in PAX3 and PAX6 transcript levels following OTX2 silencing across all 3 cell lines, only PAX3 sustained this increase at the protein level. These results underscore

the importance of following up epigenetic studies with both protein data and functional assays. Our new data further validate PAX3 as the lead target in our manuscript. We believe that additional studies surrounding global changes in promoters at the transcript levels as well as follow-up ChIP sequencing studies to further evaluate the distal regulatory landscape, while very important, are beyond the scope of the current manuscript.

3. In Supplementary Table 8, we show that there are several OTX2 binding peaks and motifs on additional mTORC1 pathway genes such as *RPS6*, *EIF4EBP1* and *RPTOR*, but not on *RHEB*. These results suggest that OTX2 not only regulates mTORC1 activity output but also the expression of genes associated with the pathway.

4. Regarding whether OTX2 and PAX3/PAX6 are "anticorrelated," while we did try to evaluate this across patient samples, OTX2 is expressed in virtually all Group 3 medulloblastoma cells; thus, we could not define an inverse correlation.

2) Since PAX3/6 are TF with low expression levels it is not very surprising that they were (almost) not captured in scRNA-seq. The scRNA-seq does not indicate many substructures, but I was wondering how the clusters were defined; what do they correspond to, do they represent different stages of differentiation or simply cell cycle stages, how were non-tumor cells defined, what are the differences between OTX2 high and low clusters (according to the dotblots)?

Response: These are excellent questions. Regarding our tumorsphere scRNA-seq data (Figure 5), the R package Seurat (v3.0.0) was used for analysis and filtering to remove cells with high mitochondrial content and low RNA counts (<200 features). Differences between the G2M and S phase scores were regressed out to maintain signals separating non-cycling and cycling cells, as this is relevant to potentially distinguishing between stem and/or progenitor cells. These details are in the methods section of the manuscript. It should be noted, however, that this dataset represents tumorspheres from 3 different medulloblastoma cell lines. Thus, there are no "non-tumor" cells. We have strengthened our results by providing UMAP plots, feature plots and correlation plots to further demonstrate the relationship between the undifferentiated program and genes associated with protein synthesis as detailed in Response #2 to Reviewer #1.

Regarding the Group 3 medulloblastoma patient data in Figure 1, single cell RNAseq (scRNAseq) clustering was performed using the Seurat FindClusters built-in function which uses the Shared Nearest Neighbor (SNN) modularity optimization-based clustering algorithm with a resolution of 0.4. Group 3 medulloblastoma tumors contain a mixed population of malignant cells with divergent differentiation along the glutamatergic cerebellar lineages (Vladoiu et al., 2019), consistent with a cerebellar stem cell of origin. We filtered out non-tumor cells based on known Group 3 medulloblastoma oncogene expression (OTX2/MYC) as well as markers for microenvironment such as microglia (CD74), pericytes (BCAN) and endothelial cells (Eng) (Figure 1A-B below). Following these steps, we excluded non-tumor cells (cluster 5, 10 and 11) from the analysis.

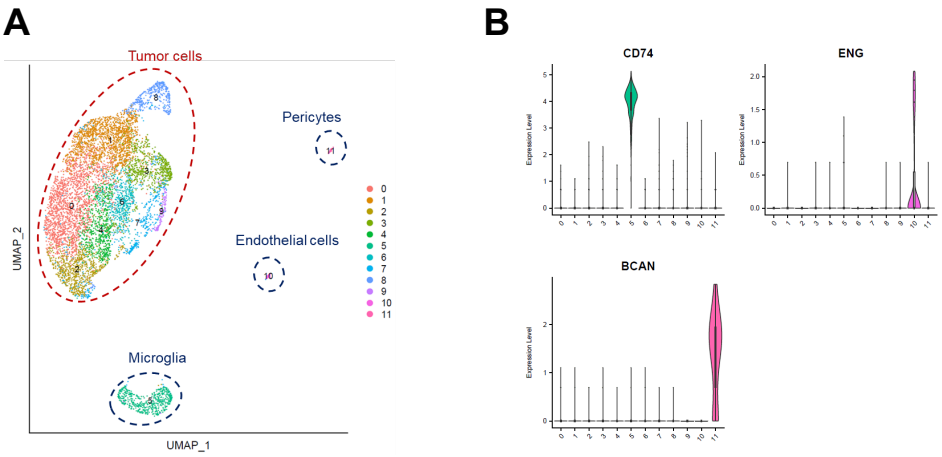


Figure 1: A. UMAP showing heterogeneity of human Group 3 medulloblastoma. B. Expression of microenvironment markers CD74, ENG and BCAN in Group 3 medulloblastoma tumors.

Interestingly, *OTX2* expression is higher in clusters 9, 3 and 8. Clusters 9 and 3 correspond to clusters of cycling cells (Figure 2A below) while Cluster 8 consists of tumor cells expressing photoreceptor genes such as *CRX* and *RCVRN* (Figure 2B below). These data have been added to the results section on pages 8-9 of the revised manuscript.

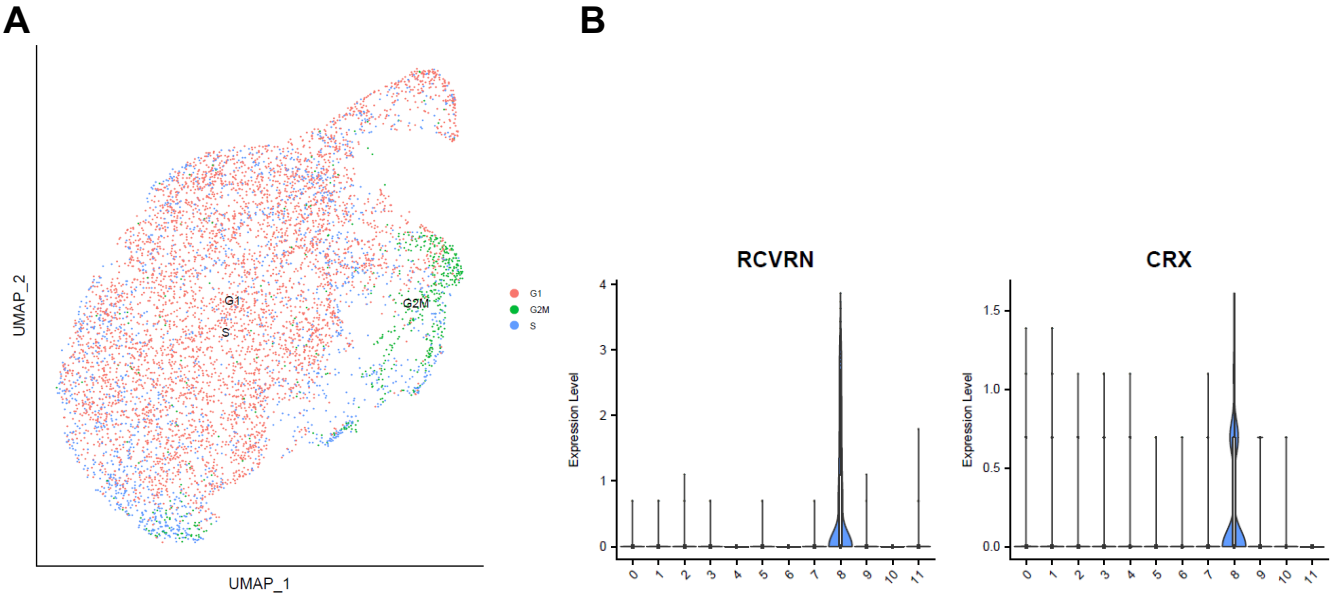
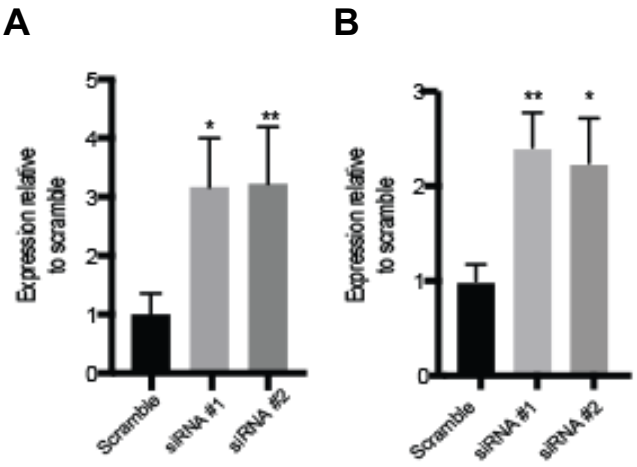


Figure 2: A. UMAP representing cell cycle phase according to Cell-cycle Scoring from Seurat software. B. Expression of photoreceptor-specific genes *RCVRN* and *CRX* in cluster 8 of Group 3 medulloblastoma tumors.

3) Since protein levels for mTORC1 genes are not altered, but the authors detected increased phosphorylation, I was wondering if there is increased expression of upstream effectors and if they are regulated by OTX2 and or PAX3.To elucidate the contribution of gene regulation for mTORC1 activation and to explore the claim that PAX3 regulates mTORC1 genes at different regulatory elements, it would be great to perform ChIP-seq for PAX3 or H3K27ac to annotate these regions in OTX2 high and low tumor spheres. Which are the target genes which are upregulated?

Response: As stated above in response to Reviewer #1, comment #1, we have evaluated additional key regulators and components of mTORC1 signaling following OTX2 loss and/or PAX3 overexpression. In particular, we looked at RHEB, an important upstream regulator of mTORC1 signaling, and the patterns are consistent with p-S6 and p-4E-BP1 changes following PAX3 GOF and OTX2 silencing. Specifically, RHEB levels are decreased. Interestingly, p-Raptor, a component of the mTORC1 complex, showed either small increases or no change in PAX3 GOF and OTX2 silenced cells. Of note, we also show in Supplementary Table 8 that there are several OTX2 binding peaks and motifs on mTORC1 pathway genes such as *RPS6*, *EIF4EBP1* and *RPTOR*, but not on *RHEB*. These results suggest that OTX2 regulates mTORC1 activity output AND the expression of genes associated with the pathway. For example, *DDIT4*, a known negative regulator of mTORC1 signaling, is on the list in Supplementary Table 8. As proof of principle, we evaluated expression levels of *DDIT4* following OTX2 silencing and in accordance with an overall decrease in mTORC1 activity, we observe an increase in *DDIT4* expression when OTX2 is knocked down in HDMB03 (Figure 3A below) and D283 (Figure 3B below) tumorspheres. These data are not provided in the manuscript but are presented here for clarity.



.
Figure 3: *DDIT4* expression in HDMB03 (A) and D283 (B) tumorspheres following OTX2 silencing using 2 siRNAs.

Regarding the PAX3 ChIP sequencing, we have previously considered this experiment in the lab. Unfortunately, the 2 ChIP-grade PAX3 antibodies that were utilized in a handful of studies (ie. Nat Commun. 2019; 10: 2316; PLoS Biol. 2019 Feb 26;17(2):e3000153), has now been discontinued by Santa Cruz and is no longer available. To our knowledge, there are currently no ChIP-grade antibodies that could be used in this experiment. Of note, all the mTORC1 genes had multiple PAX3 motifs in their promoter regions, but given that motif and the promoter size, one or more is expected. Without additional evidence (ie. PAX3 peaks), the motifs may not be biologically relevant (Wasserman WW, Sandelin A. Applied bioinformatics for the identification of regulatory elements, Nat Rev Genet, 2004, vol. 5 (pg. 276-87). Thus, we have not included these data in the revised manuscript.

4) Why are the effects of the dual mTORC1/2 inhibitor AZD8055 and PQR620 so different? Does AZD8055 inhibit other proteins or is PQR620 under-dosed for most of the experiments?

Response: The biological effects on the cells are the same, and in line with PAX3 overexpression. For example, both drugs significantly inhibit tumorsphere formation, cell viability and SOX2 levels. Interestingly, and similar to PAX3 GOF cells, at the lower concentrations in which cell viability is unaffected, we observe decreases in SOX2 without concomitant increases in neuronal differentiation markers like βIII tubulin. These results suggest that like PAX3 GOF cells, mTOR inhibitors may eliminate the stem cell population through cell senescence, or at higher concentrations, cell death

without an overall increase in neuronal differentiation. Regarding dose, indeed, AZD8055 is much more potent, with concentrations in the low nM range significantly inhibiting tumorigenic properties. While PQR620 is much less potent *in vitro*, it is blood brain barrier penetrant, which is why we utilized this drug for our *in vivo* studies. However, to strengthen our *in vivo* findings, and thus the impact of our work, we repeated our PQR620 drug experiment in Figure 7. Now, the lower concentration of 50 mg/kg is also significant (N=10, $p<0.001$***) and the 100 mg/kg has been strengthened (N=9 at $p<0.01$**). In our original submission, only the 100 mg/kg was significant at $p<0.05$ *. In addition, we performed an additional 14-day timed experiment to assess growth differences and stem/differentiation markers by IHC in our xenografts, an area that all the reviewers and editors have asked us to focus on. We found that similar to PAX3 GOF cells, mTOR inhibitor treatment results in decreased SOX2 and increased βIII tubulin levels. The new results have been incorporated into Figure 7 and Supplementary Figure 7 as well as described on page 19 in the results section of the revised manuscript.

Minor Points:
1) Please make sure font sizes are adjusted in figures and the figures are not too busy. The figures contain many panels which makes it difficult get the main message from the figure. E.g. in Fig 5 G-I it might be better to either show a selection of choose a different way for display. For Fig 7 it would be sufficient to show AZD08055 and display the data for PQR620 in the supplement.

2) Please make sure that font sizes and orientation of figure panels are consistent. In several cases makes it hard to even read axis labels or titles of graphs.

Response: We have updated the figures and the font sizes as suggested by the reviewer.

Reviewer #3; expert in epigenetics and medulloblastoma

Zagozewski and collaborators used multi-omics to unravel the role of OTX2 in Group3 medulloblastoma. OTX2 is overexpressed in all subgroups of medulloblastoma but its specific role in, and the mechanisms by which it maintains Group3 medulloblastoma undifferentiated stem-like fate has not been previously described. The authors show that OTX2 repressed neural differentiation by up-regulating the Polycomb repressive complex PRC2 and suppressing PAX3 and PAX6 expression. Finally, they found that enforced expression of PAX3 but not PAX6 increased survival in vivo and that OTX2 affects mTOR signaling that can be pharmacologically suppress Group3 medulloblastoma progression. This is a well-executed and extensive number of experiments that convincingly demonstrate the role of OTX2 overexpression in enforcing Group3 medulloblastoma stem-like phenotype. Overall, the experiments are well done and merit publication after minor criticisms are addressed.

Criticisms
1.In the introduction I challenge the comment that "there are currently no targeted therapies for the treatment of these devastating tumors". Three recent clinical trials using targeted therapies have been initiated in the last few years SJMB012, SJDAWN and SJELIOT that should be highlighted in the Introduction. At least I would delete the comment that there are no current targeted therapies for medulloblastoma, Group3 or other subgroups.

Response: Thank-you for pointing this out, as the statement is unclear. We have adjusted it to include the recent trials and the changes are highlighted on page 4 of the revised manuscript.

2. OTX2 has been found overexpressed in all subgroups of medulloblastoma, not just Group3 and Group4. In fact, the authors in the Discussion noted that OTX2 is also overexpressed in WNT and it has been found in Sonic Hedgehog tumors as well. I would make that statement in the text.

Response: Indeed, high OTX2 expression is observed in the WNT, Group 3 and Group 4 subgroups. However, OTX2 levels are very low or undetectable in SHH medulloblastoma samples as supported by our recent publication (Liang et al., Cancer Research, 2018) where we analyzed OTX2 expression across 763 subgrouped tumors. In accordance with other datasets, while rare, *OTX2* is indeed expresed in < 10% of SHH primary samples (Liang et al., 2018). Therefore, we updated the statement in the discussion on page 23 as suggested by the reviewer.

3. Figure 1B and C, it is unclear that the experiments were done with only one human Group3 medulloblastoma line, D283. I would state which line was used in the text and possibly the figure. Figure 1G, I assume that PAX6 is not expressed. I barely see one dot in cluster 9- is this right? If so, I would circle it since it is barely visible in my copy.

Response: The figure legend (page 40) and results section (page 7) have been edited to reflect the use of D283 tumorspheres specifically for Figure 1b and c. PAX6 expression indicated in Figure 1g is indeed very low and therefore the dots are very small. For Figure 1g, we have increased the scale of the image and added an arrow to denote the small, but visible, circle for cluster 9.

4. Figure 2, many of the writing in the panels A, B, C and D cannot be read which makes the analysis of the Figures difficult to evaluate. This will be worse when the figures are reduced. I suggest you increase the fonts. Figure 2G, protein levels for PAX3 and PAX6 for all three human medulloblastoma cell lines D283, HDMB03 and MB3W1 should be presented.

Response: The font sizes have been increased to enhance readability. Protein levels for PAX3 and PAX6 in OTX2 silenced cells have been assessed by western blot. The new results have been incorporated into Figure 2h of the revised manuscript for both D283 and HDMB03. Of note, despite significant increases in PAX3 and PAX6 transcript levels following OTX2 silencing, only PAX3 sustained this increase at the protein level. These results underscore the importance of following up our epigenetic studies with both protein data and functional assays.

5. Figure 3. Figure 3C provides pictures of tumorsphere formation after PAX3 and PAX6 overexpression in HDMB03 cells: I do not find them convincing whereas the numbers in Figure 3D are. In the text, the authors state that upon secondary passage, there was a significant reduction in tumorsphere number in PAX3 overexpressed cells. Looking at the numbers in Figure 3D, only in PAX6 overexpressed cells do I see a reduction of the number of spheres. I see no difference in the number of spheres in PAX3 overexpressed HDMB03. The authors should comment. Figure 3H and 3K, whereas the p value is less than 0.05, the number of animals tested (n=5 or 7 per cohort) is far too low to reach a conclusion. The authors should increase the number of animals. Similarly, the experiments should be repeated minimally with another line, for example D283 since data are presented in Figures 1 and 3.

Response: In reference to the tumorsphere data, we apologize for the confusion. The tumorsphere numbers at secondary passage were compared to DMSO controls, and not the corresponding

numbers from the previous passage. In support of the stronger "stemness" related phenotype for PAX3 cells, we observe complete abolishment of SOX2 levels both *in vivo* (Figure 3k) and *in vitro* (Figure 4f-g). SOX2 levels are retained, to some extent, in PAX6 cells which may explain a shift towards differentiation in the residual stem cell population in the form of increased DCX and βIII tubulin levels (Figure 4f-g). To strengthen our findings, we also performed βIII tubulin staining on our PAX3 and PAX6 xenografts and observed similar patterns *in vivo*.

Regarding the *in vivo* data, we have increased the number of animals from N=5 to N=8 for Figure 3J, and this has been incorporated into the revised manuscript. While we have made several attempts to generate PAX3 overexpressing D283 and MB3W1 cells via lentiviral transduction, this has been very challenging due to significant inhibitory effects on cell growth (see response to reviewer 1, point #8). While RFP and PAX6 lentiviral transduction were successful, PAX3 overexpression in these lines ultimately led to cell senescence, and they could not be successfully passaged. So, unfortunately, we have not been able to add these data.

6. Figure 4, panel C, I would state that the comparison was done in HDMBO3 overexpressing PAX3 and PAX6.

Response: We have added this to the figure legend for clarity.

7. Figure 5, same criticism as above: I am not able to read numbers and genes in panels A-C and G-I preventing me from evaluating the data. I assume that PAX3 like PAX6 is not expressed in any of the three lines. Is this true? If the speck for PAX3 I see is real, I would circle it.

Response: Thank-you for highlighting this. Indeed, we have we increased the visibility of the data and have strengthened our results with the additional plots described in Response #2 to Reviewer #1. These results suggest that the undifferentiated cell population is inversely correlated with cell cycle but positively correlated with protein synthesis genes. As OTX2 and mTORC1 genes are heterogeneously expressed across all the clusters, these data suggest that the OTX2/PAX/mTOR axis preferentially targets stem/progenitor cells.

8. Figure 6. Figure 6D and 6E, I would indicate the line used possibly on top of the left panels.

Response: We have marked the panels accordingly. This is important as we have now added more data to the figure including western blots for SOX2, EPHB2, DCX, βIII tubulin, RHEB, p-Raptor, and total Raptor following OTX2 silencing using 2 siRNAs in both HDMB03 (d) and D283 (e) tumorspheres. The text has been updated in the figure legend (page 45) and in the results section (page 17) of the revised manuscript.

9. Figure 7. Whereas I agree that the AZD8055 drug efficiently suppresses pS6 and tumorsphere number in HDMB03 and MB3W1 cells, the required concentrations are high and even greater for PQR620 raising questions as to the validity in using mTORC inhibitor for the treatment of Group 3 medulloblastoma. In addition, the survival curves for mice implanted with HDMB03 treated with PQR620 show no difference in the response using 50 or 100 mg/kg with a relatively small p value. The number of animals per cohort is too small and should be increased. How toxic is the treatment?

Response: In general, we find that higher drug concentrations are typically needed for 3D tumorspheres as opposed to adherent cultures. In light of this however, we chose to reduce the concentration range for AZD8055 to 25-100 nM. These results still showed very strong inhibition of p-

S6 (Supplementary Figure 7), while still enabling us to passage the cells to assess secondary tumorsphere formation (Figure 7i-j). For consistency, we have also extended our western blot data in Figure 7k to assess SOX2, βIII tubulin and cleaved caspase 3 as a measure of viability over a wider range of concentrations for both AZD8055 and PQR620. In accordance with PAX3 overexpressing cells, we observe a decrease in SOX2 without a significant increase in βIII tubulin, particularly at sublethal doses.

PQR620 concentrations were selected based on recent studies (Brandt et al., Neuropharmacology, 2018) in which the drug was utilized up to 100 mg/kg with no toxicity issues in mouse models. We have kept the number of mice to a minimum, as PQR620 is incredibly expensive ($10 000 CA dollars for a 3-week experiment). However, as these studies are important to demonstrate the potential therapeutic benefit of mTOR inhibitor treatment to Group 3 medulloblastoma, we agree with the reviewer and have also repeated our PQR620 drug experiment in Figure 7. Specifically, the lower concentration of 50 mg/kg is now significant (N=10, $p<0.001$***) and the 100 mg/kg is stronger (N=9 at $p<0.01$**) relative to the vehicles (N=17). While we did observe some mild toxicity (ie. 10% weight loss) in the 100 mg/kg group during the 2X daily treatments, the “drug holiday” on weekends alleviated these issues and the animals consistently recovered, which was very encouraging. Of note, in our original submission, only the 100 mg/kg was significant at $p<0.05$ *. Thus, we feel that we have substantially strengthened our findings. The new data have been added to Figure 7d of the revised manuscript. In addition, we performed one additional study in which the animals were euthanized at day 14 to assess Ki67, STEM121, SOX2 and βIII tubulin at a time point when the tumor size difference is quite significant. These new data have also been incorporated into Figure 7c and Supplementary Figure 7 of the revised manuscript. We believe these results significantly strengthen the impact of our study.

10. Figure 8. Because many different cell lines are using for different assays, I would list the cell line used in the panels of the Figures when possible.

Response: The figures have been updated for clarity.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors addressed my suggestios appropriately.

Reviewer #2 (Remarks to the Author):

Thank you very much to the authors for the revisions to the manuscript and addressing the comments.

Regarding the new scRNA-seq analysis of tumorshperes in Fig.5, I am still wondering what the indicated clusters correspond to, e.g. the authors state that it is a mix of "stem cells, progenitors and more differentiated progeny". Are there particular clusters that show markers for the different states? Are there cell-type marklers for the clusters? If there are no markers the authors might consider merging clusters post clustering or using lower resolution for custering. This might also make interpretation of the presented data easier since to me it is not clear from the presented analysis how this was determined: "the notion that mTORC1 signalling is a major component of a self-renewal program". Here, detailed description of expression of mTORC1 genes in individual clusters would be helpful in addition to cluster annotations.
The correlation analysis between undifferentiated metagene program GO Ribosome Biogenesis seems to be circular since according to the cited Hovestadt et al paper this program was primarily characterized by ribosomal genes. How do the authors interpret that thee undifferentiated score is higher for all cells compared to the differentiated score.

Reviewer #3 (Remarks to the Author):

Thank you for appropriately addressing my criticisms and those of the other reviewers that I believe has improved the manuscript
Martine F Roussel

We thank the reviewers for all the positive feedback. As Reviewer #1 and Reviewer #3 are satisfied with the revisions, we have specifically responded to the remaining questions from Reviewer #2 regarding our single cell RNA sequencing (scRNA-seq) data. We have addressed the issue of cluster identity and how the mTORC1 pathway is associated with the clusters to the best of our abilities. All new data have been highlighted in blue throughout the revised manuscript.

Reviewer #1 (Remarks to the Author):

The authors addressed my suggestions appropriately.

Response: Thank-you very much.

Reviewer #2 (Remarks to the Author):

Thank you very much to the authors for the revisions to the manuscript and addressing the comments.

Regarding the new scRNA-seq analysis of tumorspheres in Fig.5, I am still wondering what the indicated clusters correspond to, e.g. the authors state that it is a mix of "stem cells, progenitors and more differentiated progeny". Are there particular clusters that show markers for the different states? Are there cell-type markers for the clusters? If there are no markers the authors might consider merging clusters post clustering or using lower resolution for clustering. This might also make interpretation of the presented data easier since to me it is not clear from the presented analysis how this was determined: "the notion that mTORC1 signalling is a major component of a self-renewal program". Here, detailed description of expression of mTORC1 genes in individual clusters would be helpful in addition to cluster annotations.
The correlation analysis between undifferentiated metagene program GO Ribosome Biogenesis seems to be circular since according to the cited Hovestadt et al paper this program was primarily characterized by ribosomal genes. How do the authors interpret that thee undifferentiated score is higher for all cells compared to the differentiated score.

Response: Thank-you very much for the positive feedback. Indeed, we appreciate the suggestion to merge the clusters as this enabled us to better assess the identity of the clusters and how an mTORC1 gene signature is associated with them. Accordingly, we have integrated the scRNA-seq data from all 3 cell lines and now present the results as a new Figure 5. The old Figure 5 has been moved, in its entirety, to the Supplemental data (new Supplementary Figure 6). Please let us know if a different figure arrangement is preferred. We have summarized the additional integrated scRNA-seq data as follows. We now provide:

1. **UMAP representations of integrated tumorsphere data from D283, HDMB03 and MB3W1, cell cycle phases from integrated data and transcriptionally distinct cell populations from the integrated data (Figure 5a-c, and the results section on pages 16-17 of the revised manuscript).**
2. **Correlation plots from the integrated data displaying the similar relationships between the undifferentiated program and cell cycle as well as the undifferentiated program and translation initiation. Importantly, we observe the same positive correlation between the undifferentiated program and our mTORC1 gene signature and the same negative correlation between cell cycle and our mTORC1 gene signature (Figure 5d-g and the results section on pages 16-17 of the revised manuscript).**

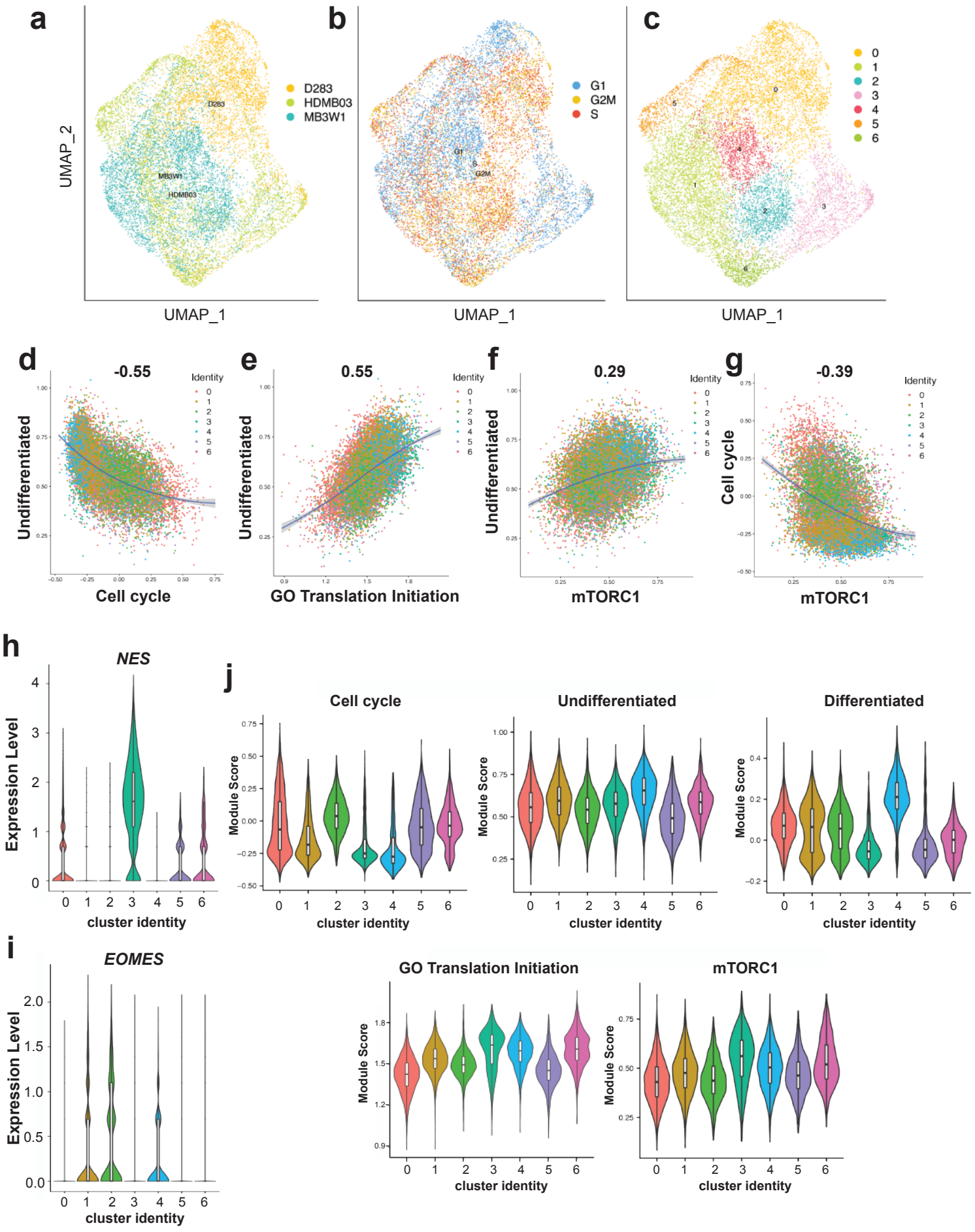
3. **Annotation of stem/progenitor cell and more differentiated cell clusters within the integrated tumorsphere data. Expression of the neural stem cell marker Nestin (*NES*) and the differentiated unipolar brush cell marker Eomesodermin (*EOMES*) was used to identify specific clusters. Importantly, the translation initiation signature as well as our more specific mTORC1 gene signature, while expressed across all clusters, are highest in the *NES*+ lower/non-cycling cell compartment. (Figure 5h-j and the results section on pages 16-17 of the revised manuscript).**

Tumorspheres, by their very nature, are enriched for stem/progenitor cells and are less heterogeneous than a typical patient sample. This is attributed to the defined serum-free media in which tumorspheres are grown. Nevertheless, we are pleased that despite the *in vitro* nature of our tumorsphere model, we were still able identify different cell populations in our integrated data that mirror the recent findings in Group 3 medulloblastoma primary samples from Hovestadt et al. We chose day 5 tumorspheres as there is an appropriate balance between undifferentiated stem cells and progenitors. Leaving the tumorspheres a few more days would increase the number of differentiated cells; however, this is also accompanied by a significant increase in cell death which is not ideal for scRNA-seq. The smaller proportion of terminally differentiated cells is also evident throughout the manuscript in our Western blots. For example, under "wild type conditions," DCX, a more primitive neuronal precursor marker is often expressed, while βIII tubulin, which marks even more differentiated cells, is not. Thus, our control tumorspheres consist mostly of stem and progenitor cells as opposed to much more terminally differentiated progeny.

Reviewer #3 (Remarks to the Author):

Thank you for appropriately addressing my criticisms and those of the other reviewers that I believe has improved the manuscript
Martine F Roussel

Response: Thank-you very much.


a
b
c
UMAP_2
UMAP_1
UMAP_1
UMAP_1
D283
HDMB03
MB3W1
G1
G2M
S
0
1
2
3
4
5
6
d
-0.55
e
0.55
f
0.29
g
-0.39
Identity
Undifferentiated
Cell cycle
GO Translation Initiation
mTORC1
h
NES
Expression Level
cluster identity
j
Cell cycle
Undifferentiated
Differentiated
Module Score
cluster identity
i
EOMES
GO Translation Initiation
mTORC1

REVIEWERS' COMMENTS:

Reviewer #2 (Remarks to the Author):

Thank you very much to the authors for the revisions on the manuscript and for addressing the comments appropriately.