RNF220 is required for cerebellum development and regulates medulloblastoma progression through epigenetic modulation of Shh signaling

Pengcheng Ma, Tao An, Liang Zhu, Longlong Zhang, Huishan Wang, Biyu Ren, Bin Sun, Xia Zhou, Yan Li and Bingyu Mao
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AUTHORS: Pengcheng Ma, Tao An, Liang Zhu, Longlong Zhang, Huishan Wang, Biyu Ren, Xia Zhou, Bin Sun, Yan Li, and Bingyu Mao

I have now received the reports of two referees on your manuscript and I have reached a decision. The reports are appended below and you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express great interest in your work, but they also have significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. In particular, referee 1 requests that the cerebellar phenotype of RNF220 conditional knockout mice and the medulloblastoma phenotype of RNF220+/-;Ptch1+/- mice are analysed more thoroughly, as detailed in referee1, points 1 and 2. This referee also asks that the ChIP-PCR analysis in Fig. 4 examines additional Gli1 binding sites in the Gli1 and Ptch1 promoters and other Shh target promoters and includes appropriate controls. Referee 2 asks that the RNF220 antibody should be validated and IHC analysis in Figure 1 should be improved. This referee also recommends several other ways to improve significantly this study. A ChIP-Seq analysis for multiple histone marks, suggested in referee 2 point 4, seems however an excessive request for revision of an article. Improving the ChIP-PCR analysis is nevertheless important, as requested by referee 1. The Ethical team of Development has alerted me to a possible mistake in Fig S4 and I am also asking that you repeat the ChIP-PCR experiments that have produced this figure.

If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy to receive a revised version of the manuscript. Your revised paper will be re-reviewed by the original referees, and its acceptance will depend on your addressing

satisfactorily all their major concerns. Please also note that Development will normally permit only one round of major revision.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this manuscript, the authors presented mouse mutant and cell culture data suggesting that RNF220, a ubiquitin E3 ligase, acts positively in Shh signaling to promote cerebellar granule cell proliferation and medulloblastoma (MB) tumorigenesis. These observations are interesting because the same authors have previously demonstrated that RNF220 opposes Shh signaling by K63-linked polyubiquitination and nuclear export of Gli transcription factors during ventral neural patterning. Therefore, RNF220 appears to serve dual roles in Shh signaling depending on the cellular context. The most significant points in this study are the identification of EED, an essential component of the PRC2 complex, as a target of RNF220 in cerebellar granule cells and the Shh MB cell line DAOY, and the demonstration that knockdown of EED can ameliorate the KO/KD effects of RNF220 on Gli1 and Ptch1 expression as well as the epigenetic modifications at their promoters. Shi et al. (Nature Commun. 2014) demonstrated that Shh signaling programs an epigenetic switch from PRC2 repression to JMJD3 activation at Gli target genes including Gli1 and Ptch1. Thus, the current study illustrates another level of RNF20 action in Shh signaling by targeting the PRC2 complex at Gli target promoters in addition to the polyubiquitination and nuclear export Gli transcription factors. The authors proposed that RNF220 could represent a potential new therapeutic target and diagnostic marker for SHH MB.
These results are novel and significant to the Hh and SHH MB fields.

Comments for the author

Overall, the study is solid and has good biochemistry data. Nonetheless, I think the analysis of the cerebellar and MB phenotypes is somewhat superficial, and the ChIP-qPCR study needs additional data. I will recommend the following points for the revision.

1. A more detailed characterization of the cerebellar phenotype is essential. In Fig. 1A, what are the RNF220-expressing cells at E18.5? In Fig. 1C, how the EGL length is measured? Are they normalized to body size? Is there any width/cell number difference in the EGL? Marker gene analysis should be used to establish the defects in proliferation and Hh target gene expression in the mutant cerebellum. Ideally, analysis of the cerebellar phenotype at postnatal stages should be included, since these data are relevant to the interpretation of the MB data in Fig. 3.

2. The MB data are not clearly described. In the abstract, the authors stated “We provided evidence that RNF220+/-;Ptch1+/- mice showed higher spontaneous MB occurrence comparing to Ptch1+/- mice”, however Fig. 3A suggests the opposite. It is important to explain how cumulative MB occurrence is measured. In Fig. S2E, it is difficult to understand why Ptch1 expression is down regulated in Ptch1+/- MB? Since Ptch1 is a well established marker unregulated in SHH MB, this result is very confusing. It will be important to characterize the size of the MB in Ptch1+/- vs. RNF220+/-;Ptch1+/- mice as well as Gli1 and Ptch1 expression in their MB. The authors should explain how many MB samples are used for the analysis in Fig. 3B and C. The correlation of RNF220 vs. GAB1 expression should also be validated using available MB datasets. Since the authors showed that EED is a target of RNF220 in SHH MB, it will be highly relevant to extend the correlation expression analysis to EED, EZH2 and SUZ12 in the human MB samples.

3. The ChIP-qPCR analysis needs careful explanation. Why the single Gli1 and Ptch1 promoter sites are chosen for the analysis? For these ChIP experiments, the authors should include additional Gli

protein binding sites (Gli1/Ptch1 and other Gli target genes) as well as appropriate controls. More detail about the quantification methods will be essential.

4. In Fig. S3, the authors only showed the data of RNF220's effects on the polyubiquitination and nuclear export of Gli1. Are Gli2 and Gli3 also affected similarly to Gli1 in these cerebellar cells?

5. The western blot data in Fig. 5 and Supplementary figures should be quantified.

Reviewer 2

Advance summary and potential significance to field

The mechanisms regulating Shh signaling in distinct circumstances are of great interest in developmental biology. The authors have previously published that RNF220 is a ubiquitin ligase that targets Gli1 and so downregulates Shh signaling in the developing spinal cord.
Here, they present data that RNF220 alters ubiquitination of EED and thereby potentiates Shh signaling in developing cerebellar GCPs and in medulloblastoma. This dichotomy is interesting.

Comments for the author

There are major issues with the data presented and the conclusions that are drawn.

Specific issues:

1. Figure 1. The IHC presented is not very convincing. The Ki67 stain- looks like meninges which is often background staining, and the RNF220 antibody validation with knock out would be helpful for interpreting this, and other images.

2. BrdU incorporation in dissociated GCPs cultured for 3 days in 10% serum is largely independent of Shh signaling. This makes the conclusions in Figure 1 difficult. Perhaps this could be done either in vivo, or in vitro under more physiologic conditions.

3. While the genetic knockout of RNF220 is clear, in many cases the SiRNA for RFN220 is not very effective- making the conclusions difficult to assess. (Figure 5)

4. ChIP-PCR has several limitations particularly for the conclusions on binding of specifically modified histones. Seeing effects across genome using ChIP-Seq and highlighting those on genes of interest would be much more convincing (Figures 4,6,7)

5. IHC correlation of clinical cases is not so convincing. To look at large datasets it might be best to examine RNF220 correlates with the distinction of Shh+ versus Shh- MB groups by RNA analysis.
I know that the authors state that “Although RNF220 mRNA levels were decreased in Ptch1+/- 159 MB tissues (Fig. S2G), the RNF220 160 protein was markedly upregulated (Fig. 5C), which suggests that RNF220 is stabilized at a post-translational level in MBs.”-However this is completely dependent on appropriate validation of antibody.

6. A major point of the paper is that RNF220, in addition to the previously published effect on GLi1 ubiquitination, also has an indirect effect on Shh signaling through changes in histone marks. However, in Figure 5i and j- not clear that sh to RNF220 changes ubiquitination of EED.
It is clear that shRNF220 changes gli-ubiqutin as previously published. Certainly quantification of this effect would be helpful.

7. The authors show that double heterozygotes RNF220+/-Ptc+/-, have a lower rate of medullo than the tTc+/- mice. However the effects is fairly small, and the incidence of medullo in PTc+/- mice is fairly variable. Given that the authors have a floxed RNF220 allele and atoh-Cre-ER is a great driver in MBs, that would be a more convincing way to do experiment

Minor issues:
1. The authors state that “IP indicated that RNF220 directly interacts with EED both in vitro and in vivo in CGNPs, Daoy, HEK293 cells and E18.5 210 mouse cerebellum tissues (Fig. 5D-G and S5A-B). “ These data do not indicate direct interaction- as many other components are present in the lysate and could be responsible for forming a larger complex.

2. The authors state “Our work provides a new potential drug target for Shh-group MB treatment. “Which is the target- clearly not RNF220, since that is bivalent. Are they suggesting EED?
3. The authors reference (Lisa V Goodrich 1999) this should be a citation of the paper (; L V Goodrich L Milenković, K M Higgins, M P Scott, 1997, Altered Neural Cell Fates and Medulloblastoma in Mouse Patched Mutants, Science, 1997, 277 (5329), 1109-13)

First revision

Author response to reviewers' comments

Response Letter

Reviewer 1
Advance Summary and Potential Significance to Field:
In this manuscript, the authors presented mouse mutant and cell culture data suggesting that RNF220, aubiquitin E3 ligase, acts positively in Shh signaling to promote cerebellar granule cell proliferation and medulloblastoma (MB) tumorigenesis. These observations are interesting because the same authors have previously demonstrated that RNF220 opposes Shh signaling by K63-linked polyubiquitination and nuclear export of Gli transcription factors during ventral neural patterning. Therefore, RNF220 appears to serve dual roles in Shh signaling depending on the cellular context. The most significant points in this study are the identification of EED, an essential component of the PRC2 complex, as a target of RNF220 in cerebellar granule cells and the Shh MB cell line DAOY, and the demonstration that knockdown of EED can ameliorate the KO/KD effects of RNF220 on Gli1 and Ptch1 expression as well as the epigenetic modifications at their promoters. Shi et al. (Nature Commun. 2014) demonstrated that Shh signaling programs an epigenetic switch from PRC2 repression to JMJD3 activation at Gli target genes including Gli1 and Ptch1. Thus, the current study illustrates another level of RNF20 action in Shh signaling by targeting the PRC2 complex at Gli target promoters in addition to the polyubiquitination and nuclear export Gli transcription factors. The authors proposed that RNF220 could represent a potential new therapeutic target and diagnostic marker for SHH MB. These results are novel and significant to the Hh and SHH MB fields.
Thank you very much for your comments and supports.

Comments for the Author:
Overall, the study is solid and has good biochemistry data. Nonetheless, I think the analysis of the cerebellar and MB phenotypes is somewhat superficial, and the ChIP-qPCR study needs additional data. I will recommend the following points for the revision.

1. A more detailed characterization of the cerebellar phenotype is essential.
In Fig. 1A, what are the RNF220-expressing cells at E18.5?
We performed the co-staining of RNF220/BrdU and RNF220/NeuN antibodies in the developing cerebellum at P0, P5 and P10 and found that RNF220 signals overlapped well with that of BrdU which labels the proliferating CGNPs, but not with that of NeuN which is a marker of mature granular neuron at IGL (Figure 1A and B). Therefore, we conclude that RNF220 is highly expressed in the CGNP cells of the developing cerebellum.

In Fig. 1C, how the EGL length is measured? Are they normalized to body size?
Thank you for your comments. We collected all the transverse sections of a cerebellum and measured the cerebellar perimeter on the midsagittal area section containing the largest cerebellum (Quaranta et al., 2017, Cell Death Dis) using CellSens software equipped with Olympus

IX73 microscope. And the cerebellum length is normalized with their body weight respectively. More details on this assays is included in the revised manuscript.

Is there any width/cell number difference in the EGL?
The RNF220 knockout mice died shortly after their birth. As the EGL have not been fully formed at E18.5 or P0 stage, we have not conducted the width or cell number comparison. However, we counted the BrdU positive cell at P0 cerebellum and found that the incorporation of BrdU was reduced in RNF220 knockout cerebellums compared to controls (Figure 1E-G). These results were included in the revised Figure 1 and also the description on this assay was included in the revised manuscript.

Marker gene analysis should be used to establish the defects in proliferation and Hh target gene expression in the mutant cerebellum.
Thank you for your suggestion. Indeed, we tried IF and ISH assays using Gli1/Ptch1 antibody or probes and unfortunately we found that both their antibodies and probes didn't work well in our hands. However, we examined the expression level of Shh target genes Gli1, Ptch1 and Hhip1 using realtime PCR assays and found that all the three genes are down-regulated in RNF220$^{-/-}$ cerebellum compared to controls (Figure 1H-J). We include these results in the revised Figure 1.

Ideally, analysis of the cerebellar phenotype at postnatal stages should be included, since these data are relevant to the interpretation of the MB data in Fig. 3.
Thank you for your comment. We agree that it would be ideal to follow the postnatal cerebellar phenotype using the Atoh-CreER line for example, which we will try in the future when possible. We have provided evidence that RNF220 is highly expressed at EGL in the developing cerebellum and is over-expressed in medulloblastoma tissues (Figure 1 and 3). Also, our results showed that RNF220 is required for the full activation of Shh signaling in CGNP/Daoy cells, developing cerebellum and medulloblastoma tissues (Figure 1-3 and Supplementary Figure 1-2). Collectively, we think that the above data sufficiently support the conclusion and we hope you could understand and support us.

2. The MB data are not clearly described.
In the abstract, the authors stated "We provided evidence that RNF220+/-;Ptch1+/- mice showed higher spontaneous MB occurrence comparing to Ptch1+/- mice", however Fig. 3A suggests the opposite. It is important to explain how cumulative MB occurrence is measured.
Thank you for your careful reading and comment. The statement in the abstract is incorrect, the medulloblastoma occurrence rate in RNF220$^{+/-}$; Ptch1$^{+/-}$ mice is lower than that inPtch1$^{+/-}$ mice. The statement has been corrected in the MS.
We took a one-year long observation of the siblings with different genotypes since their birth. The mice were killed only when observed paralyzed and examined for the occurrence of medulloblastoma. At the end of our observation, none of the 37 RNF220$^{+/-}$ mice suffered medulloblastoma; 18/56 of the Ptch1$^{+/-}$ mice and 6/28 of the RNF220$^{+/-}$; Ptch1$^{+/-}$ mice were found to bear medulloblastoma. More details on this assay are included in the revised manuscript.

In Fig. S2E, it is difficult to understand why Ptch1 expression is down regulated in Ptch1+/- MB? Since Ptch1 is a well-established marker unregulated in SHH MB, this result is very confusing. It will be important to characterize the size of the MB in Ptch1+/- vs. RNF220+/-;Ptch1+/- mice as well as Gli1 and Ptch1 expression in their MB.
Thank you for your comment and suggestion.
There is only one allele contributing Ptch1 expression in Ptch1$^{+/-}$ mice and it is reasonable that Ptch1$^{+/-}$ medulloblastoma expressed less Ptch1 mRNA though Shh signaling is up-regulated. Since the medulloblastoma tissue were dissected whenever the mice paralyzed during our observation, which varied a lot in size irrespective of their genotype, it makes little sense to compare their size directly. We compared the expression of Shh signaling targets Gli1, Ptch1 and Hhip1 in medulloblastoma with different genotypes using realtime PCR, and they all showed reduced expression in tissues from RNF220$^{+/-}$; Ptch1$^{+/-}$mice compared to that from Ptch1$^{+/-}$ mice (Figure 3B-D). The related results were included in the revised Figure 3.

The authors should explain how many MB samples are used for the analysis in Fig. 3B and C.
Thank you for your suggestion. The tissue ChIP slide (Cat No. BC17012b, Alenabio) includes 32 independent human medulloblastoma cases and every case has two samples on the slide. The dots

in the original Figure 3B present the average evaluation of the two samples for each case. The number in the original Figure 3C present the order of the samples on the slide. We revised the number in the original Figure 3C with the indicated sample number. More details on this assay are included in the method and material part of the revised manuscript.

The correlation of RNF220 vs. GAB1 expression should also be validated using available MB datasets.
Thank you for your suggestion. We analyzed the correlation of RNF220 and GAB1 in five different medulloblastoma datasets and one available Shh-subgroup medulloblastoma dataset. The result shows that only in the Shh-subgroup medulloblastoma (GSE49234) the expression of RNF220 positively correlated with that of GAB1 (R=0.2624, P=0.0249) while in other datasets no such correlation was observed (Figure R1). We argue that the correlation of RNF220 and GAB1 expression should be examined at the protein level, since the expression of RNF220 is regulated also post-transcriptionally. For example, the RNF220 protein level increased in Ptch1$^{+/-}$ medulloblastoma while its mRNA level reduced (Figure 5C and Supplementary Figure S2H), likely due to reduced Smurfs which serve as RNF220 E3-ubiquitin ligases (Figure R2, unpublished observations).

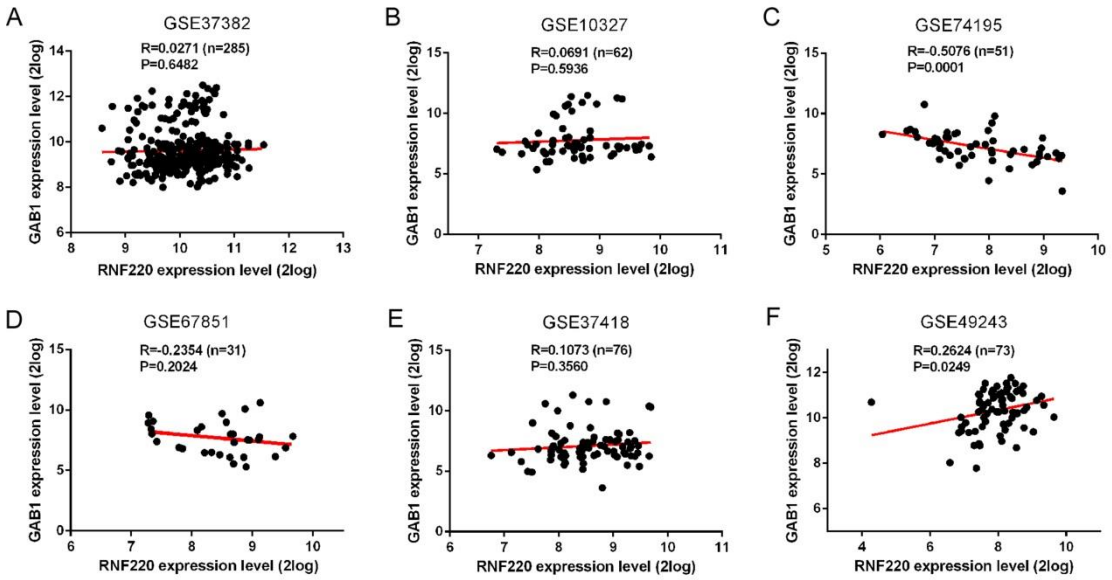

Figure R1. Correlations between RNF220 and GAB1 expression level in different MB datasets from R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl). Among them, MB samples from GSE49243 are all Shh-subtype, and MB samples from other five datasets are mixed. Dataset accession number, correlation coefficient (R), sample numbers (n) and P value are shown.

NOTE: We have removed unpublished data that had been provided for the referees in confidence.

Since the authors showed that EED is a target of RNF220 in SHH MB, it will be highly relevant to extend the correlation expression analysis to EED, EZH2 and SUZ12 in the human MB samples.
Thank you for your suggestion. We tried to order more tissue Chip slides for these analyses; however the slides were no longer available currently. We were told by the company that it may take a long time to collect enough samples for new slide preparation since medulloblastoma is relatively rare. We examined the correlation between EED/SUZ12/EZH2 with RNF220/GAB1 in Shh-subgroup medulloblastoma dataset in which RNF220 positively correlates with GAB1. The results showed that only EED-GAB1 (R=-0.2688, P=0.0215) and SUZ12-RNF220 (R=-0.2598, P=0.0264) showed weak negative correlations (Figure R3). Again we do not consider these data conclusive since only transcriptional data available. Although it is important to provide the co-relation analysis between RNF220 and EED in human clinic samples, our available data from both cancer cells and Ptch1$^{+/-}$ medulloblastoma tissues have already provided enough supports for our conclusions. We hope you could understand this situation and support us.

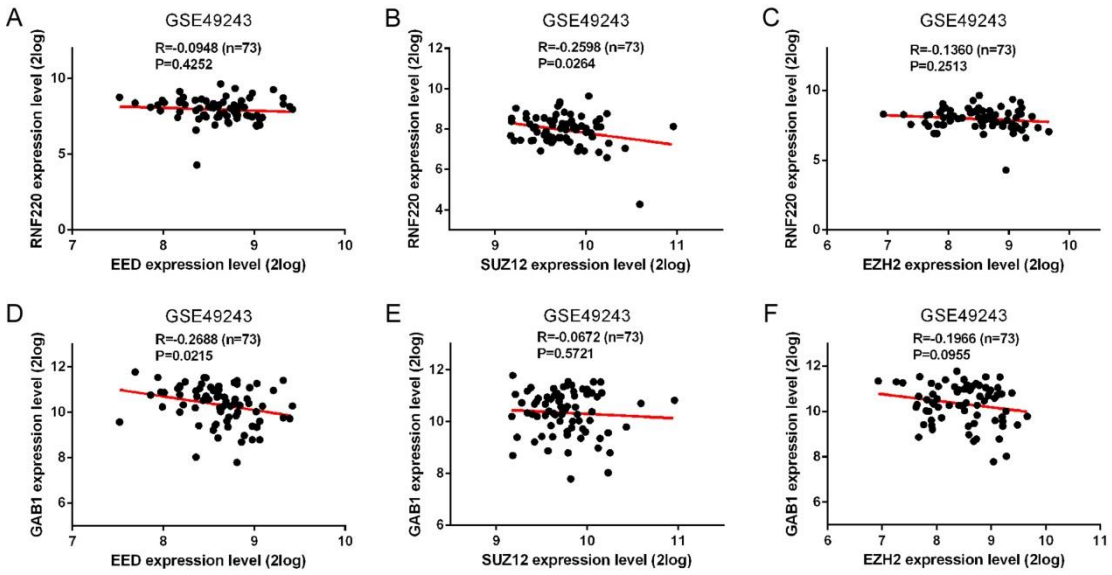

Figure R3. Correlation analysis between EED/EZH2/SUZ12 and GAB1/RNF220 expression level in Shh-driven MB dataset GSE49243 from R2. Dataset accession number, correlation coefficient (R), sample numbers (n) and P value are shown.

3. The ChIP-qPCR analysis needs careful explanation.
Why the single Gli1 and Ptch1 promoter sites are chosen for the analysis? For these ChIP experiments, the authors should include additional Gli protein binding sites (Gli1/Ptch1 and other Gli target genes) as well as appropriate controls. More detail about the quantification methods will be essential.

Thank you for your comment and suggestion. Indeed, we first examined the epigenetic modification status of two sites for both Gli1 and Ptch1 based on semi-quantification ChIP-PCR. When our manuscript was reviewed in another journal, we were asked to provide the realtime ChIP-PCR results. To make the figure concise, we only chose one promoter sites of Gli1 and Ptch1 for validation by realtime ChIP-PCR. In the revised MS, we re-substituted the realtime ChIP-PCR results with our original semi-quantification ChIP-PCR results which includes two different promoter sites for both Gli1 and Ptch1 in the revised Figures 4, 6, 7 and supplementary Figures 4, 6, 7 and 9. We also included two promoter sites of an additional Shh signaling target, Hhip1, in the related assays (Figures 4, 6, 7 and Supplementary Figures 4, 6, 7, 9).

To better control our experiments, we examined an unrelated promoter site of the house-keeping gene Actin and found that the epigenetic modification of the site is not affected by either RNF220 or EED knockdown in both Daoy and CGNP cells. Here, we provide some of the results for your reference (Figure R4). In addition, an IgG antibody was used in our ChIP assay as a negative control and we found that no specific PCR product was detected at 35 cycles in the related assays (Figures 4, 6, 7 and Supplementary Figures 4, 6, 7, 9).

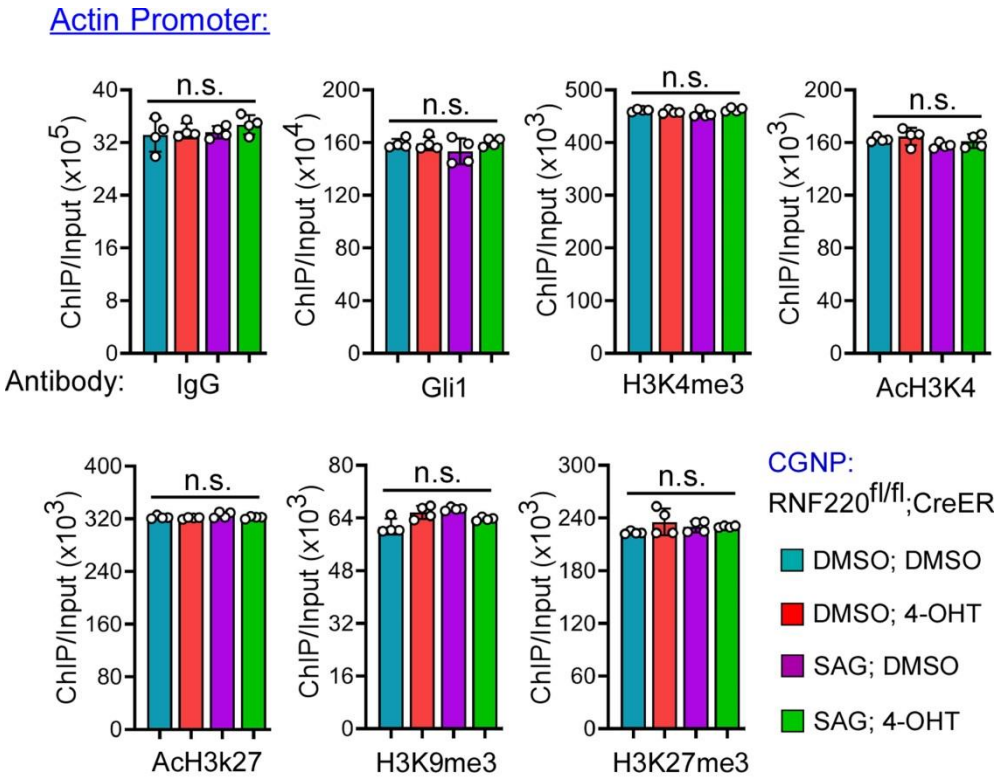

Figure R4. ChIP-qPCR analysis of the indicated histone modification marks in Actin promoter in CGNPs. CGNPs were treated with SAG or DMSO for 24 h before cells were harvested. 4-OHT was used to induce RNF220 knockout in CGNPs. Cells were harvested and followed by nuclear purification, chromosome fragments and immuniprecipitation with the indicated antibodies. n.s., not significant difference.

4. In Fig. S3, the authors only showed the data of RNF220's effects on the polyubiquitination and nuclear export of Gli1. Are Gli2 and Gli3 also affected similarly to Gli1 in these cerebellar cells?
Thank you for your comment. We examined the effects of RNF220 on the protein level, polyubiquitination status and nuclear export of all the Glis and got similar results. The data of all Glis are now included in the revised Figure S3.

5. The western blot data in Fig. 5 and Supplementary figures should be quantified.
Thank you for your suggestion. The quantification data were included in the revised Figure 5 and Supplementary Figure 5.

Reference

Ma P, Song NN, Cheng X, Zhu L, Zhang Q, Zhang L, Yang X, Wang H, Kong Q, Shi D et al. (2019). ZC4H2 stabilizes RNF220 to pattern ventral spinal cord through modulating Shh/Gli signaling. J Mol Cell Biol mjz087

Ma P, Song NN, Li Y, Zhang Q, Zhang L, Kong Q, Ma L, Yang X, Ren B, Li C et al. (2019b). Fine-Tuning of Shh/Gli Signaling Gradient by Non-proteolytic Ubiquitination during Neural Patterning. Cell Rep 28:541-553 e544.

Quaranta R, Pellulo M, Zema S, Nardozza F, Checquolo S, Lauer DM, Bufalieri F, Palermo R, Felli MP, Vacca A et al. (2017) Maml1 acts cooperatively with Gli proteins to regulate sonic hedgehog signaling pathway. Cell Death Dis 8, e2942.

Reviewer 2

Advance Summary and Potential Significance to Field:

The mechanisms regulating Shh signaling in distinct circumstances are of great interest in developmental biology. The authors have previously published that RNF220 is a ubiquitin ligase that targets Gli1 and so downregulates Shh signaling in the developing spinal cord. Here, they present

data that RNF220 alters ubiquitination of EED and thereby potentiates Shh signaling in developing cerebellar GCPs and in medulloblastoma. This dichotomy is interesting.
Thank you very much for your comments and supports.

Comments for the Author:
There are major issues with the data presented and the conclusions that are drawn.
Specific issues:
1. Figure 1. The IHC presented is not very convincing. The Ki67 stain- looks like meninges which is often background staining, and the RNF220 antibody validation with knock out would be helpful for interpreting this, and other images.
Thank you for your comment. We have validated the RNF220 antibody in both Western Blot and IF assays on spinal cord tissues (Ma et. al., 2019, Cell Rep; Ma et. al., 2019, J Mol Cell Biol). And here we also validated its specificity on P0 cerebellum slide (Figure R5A). In addition, we repeated the Ki-67 staining with BrdU on P10 mouse cerebellum slide and found that BrdU staining overlapped well with the Ki-67 staining in the EGL of cerebellum (Figure R5B). Collectively, we found that RNF220 is highly expressed in the EGL of the developing cerebellum which is labeled by Ki-67 and BrdU signals (revised Figure 1).

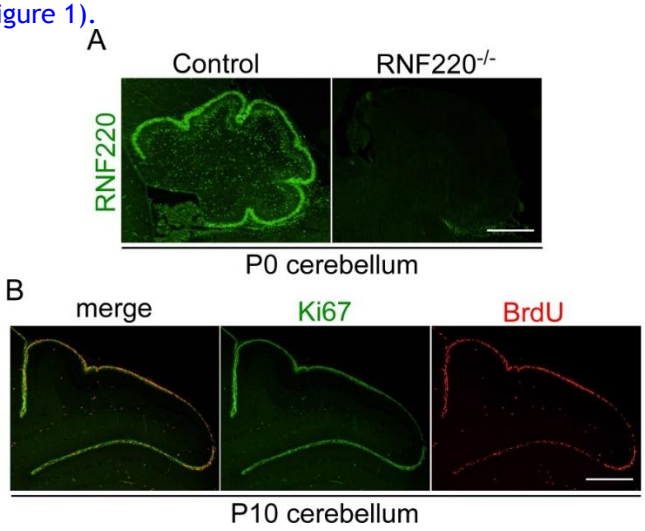

Figure R5. Immunofluorescence assays of the indicated antibody at cerebellum. (A) RNF220 antibody staining on control or RNF220 knockout P0 cerebellum. Scale bar, 170 μm. (B) Ki67 and BrdU co-staining on control P10 cerebellum. Scale bar 350 μm.

2. BrdU incorporation in dissociated GCPs cultured for 3 days in 10% serum is largely independent of Shh signaling. This makes the conclusions in Figure 1 difficult. Perhaps this could be done either in vivo, or in vitro under more physiologic conditions.
Thank you for your comment. We carried out BrdU assays in P0 cerebellum and found that both the total BrdU positive cell number and ratio decreased in RNF220$^{-/-}$ knockout cerebellum compared to controls (Figure 1E-G). Shh signaling is the most important mitogen for CGNP cell proliferation driving during cerebellum development and RNF220 knockout severely disturbed Shh signaling in cultured CGNPs and developing cerebellum (Figure 1 and Supplementary Figure 1). Therefore, we contributed the decrease in proliferation rate in RNF220 knockout CGNP cells and cerebellum development defect in RNF220 knockout mice to the down-regulated Shh signaling. More details on this assay were included in the revised manuscript.

3. While the genetic knockout of RNF220 is clear, in many cases the SiRNA for RFN220 is not very effective- making the conclusions difficult to assess. (Figure 5)
Thank you for your comment. Although the siRNAs work less efficient, we consider their effects clear, reproductive and consistent with that from the KOs. All the experiments have been conducted at least three times. The quantification data have been added in some of the panels in Figure 5 and Supplementary Figure S5. We hope you could understand and support us.

4. ChIP-PCR has several limitations particularly for the conclusions on binding of specifically modified histones. Seeing effects across genome using ChIP-Seq and highlighting those on genes of interest would be much more convincing (Figures 4,6,7)

Thank you for your comment and suggestion. To further confirm our results, we carried out semi-quantitative ChIP-PCR analysis for all 3 Gli target genes (Gli1, Ptch1 and Hhip1), two independent sites each, and IgG pull-down was included as a negative control (revised Figures 4, 6, 7 and Supplementary Figures S4, S6, S7, S9). Our new data are fully consistent with our previous conclusion. Also, the epigenetic modification of the control Actin promoter is not affected by either RNF220 or EED knockdown in both Daoy and CGNP cells (see above Figure R4). Together, we think that the current data above are sufficient to support the conclusion in this manuscript. We hope you could understand and support us.

5. IHC correlation of clinical cases is not so convincing. To look at large datasets it might be best to examine RNF220 correlates with the distinction of Shh+ versus Shh- MB groups by RNA analysis.
Thank you for your suggestion. We analyzed the correlation of RNF220 and GAB1 in five different medulloblastoma datasets and one available Shh-subgroup medulloblastoma dataset. The results showed that only in the Shh-subgroup medulloblastoma the expression of RNF220 positively correlated with that of GAB1 (please refer to the above Figure R1). However, we suggest these data less conclusive, since at least RNF220 is likely regulated post-transcriptionally. We failed to get more clinical samples currently, which might be further examined in the future.

I know that the authors state that "Although RNF220 mRNA levels were decreased in Ptch1+/- MB tissues (Fig. S2G), the RNF220 protein was markedly upregulated (Fig. 5C), which suggests that RNF220 is stabilized at a post-translational level in MBs."-However this is completely dependent on appropriate validation of antibody.
Thank you for your comment. The RNF220 antibody we used is validated by our previous work by both Western Blot and IF staining assays in control, $RNF220^{+/-}$ and $RNF220^{-/-}$ mice (Ma et. al., 2019, Cell Rep; Ma et. al., 2019, J Mol Cell Biol). In Western Blot assays, a single band was observed around the predicted molecular weight (70kD) and this band was totally diminished in $RNF220^{-/-}$ mice (Ma et. al., 2019, J Mol Cell Biol).
We do have data that RNF220 is stabilized at a post-translational level in Daoy, CGNP cells and $Ptch1^{+/-}$ medulloblastoma which is likely mediated by the E3 ligases Smurf1/2 (please refer to the above Figure R2). We think that this is beyond the scope of this manuscript and is not further discussed here.

6. A major point of the paper is that RNF220, in addition to the previously published effect on GLi1 ubiquitination, also has an indirect effect on Shh signaling through changes in histone marks. However, in Figure 5i and j- not clear that sh to RNF220 changes ubiquitination of EED. It is clear that shRNF220 changes gli-ubiqutin as previously published. Certainly quantification of this effect would be helpful.
Thank you for your comment and suggestion. The quantification data have been added in the revised Figure 5 and Supplementary Figure S5. The effects of RNF220 on EED polyubiquitination hold true in both Daoy and CGNP cells.

7. The authors show that double heterozygotes RNF220+/-; Ptc+/-, have a lower rate of medullo than the Ptc+/- mice. However the effects is fairly small, and the incidence of medullo in Ptc+/- mice is fairly variable. Given that the authors have a floxed RNF220 allele, and atoh-Cre-ERis a great driver in MBs, that would be a more convincing way to do experiment.
Thank you for your comment and suggestion. The incidence of medulloblastoma of $Ptch1^{+/-}$ mice in our hand is about 30% based on our long term (about 3 years) and large amount (about 180 mice) observation. In the $RNF220^{+/-}$; $Ptch1^{+/-}$ mice, we observed 6 out of 28 (21.4%) $RNF220^{+/-}$; $Ptch1^{+/-}$ mice that developed medulloblastom, which is lower than in the $Ptch1^{+/-}$ mice. It would be desirable to carry out the experiment using the Atoh-CreER line, which we may try in the future. We think that our conclusions sufficiently supported and we hope you would understand and support us.

Minor issues:
1. The authors state that "IP indicated that RNF220 directly interacts with EED both in vitro and in vivo in CGNPs, Daoy, HEK293 cells and E18.5 mouse cerebellum tissues (Fig. 5D-G and S5A-B)". These data do not indicate direct interaction- as many other components are present in the lysate and could be responsible for forming a larger complex.

Thank you for careful reading and your comment. We have revised the description in the manuscript accordingly.

2. The authors state “Our work provides a new potential drug target for Shh-group MB treatment. “Which is the target- clearly not RNF220, since that is bivalent. Are they suggesting EED?
Thank you for your comment. We suggest RNF220 to be the target. Although RNF220 shows bivalent effects on Shh signaling in different contexts, it acts as a positive regulator for Shh signalingin Shh-group medulloblastoma and contribute positively to its progression. As in the case in the Ptch1$^{+/-}$; RNF220$^{+/-}$ mice, we think it is possible that targeting RNF220 might inhibit or delay the Shh-group medulloblastoma progression.

3. The authors reference (Lisa V Goodrich 1999) this should be a citation of the paper (; L V Goodrich L Milenković, K M Higgins, M P Scott, 1997, Altered Neural Cell Fates and Medulloblastoma in Mouse Patched Mutants, Science, 1997, 277 (5329), 1109-13)
Thank you for your careful reading. We revised the reference accordingly.

Reference

Ma P, Song NN, Cheng X, Zhu L, Zhang Q, Zhang L, Yang X, Wang H, Kong Q, Shi D et al. (2019). ZC4H2 stabilizes RNF220 to pattern ventral spinal cord through modulating Shh/Gli signaling. J Mol Cell Biol mjz087
Ma P, Song NN, Li Y, Zhang Q, Zhang L, Kong Q, Ma L, Yang X, Ren B, Li C et al. (2019b). Fine-Tuning of Shh/Gli Signaling Gradient by Non-proteolytic Ubiquitination during Neural Patterning. Cell Rep 28:541-553 e544.

Second decision letter

MS ID#: DEVELOP/2020/188078

MS TITLE: RNF220 is required for cerebellum development and regulates medulloblastoma progression through epigenetic modulation of Shh signaling

AUTHORS: Pengcheng Ma, Tao An, Liang Zhu, Longlong Zhang, Huishan Wang, Biyu Ren, Xia Zhou, Bin Sun, Yan Li, and Bingyu Mao

I have now received the reports of two of the referees who reviewed the earlier version of your manuscript and I have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The reviewers’ evaluation is positive and we would like to publish a revised manuscript in Development, provided that you satisfactorily address the remaining suggestions and comments of referee 2, including the need for further discussion on the different roles of RNF220 in regulation of Shh signaling in medulloblastoma and the ventral spinal cord. Please attend to all these comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

This study suggests that RNF220, an ubiquitin E3 ligase, acts positively in Shh signaling to promote cerebellar granule cell proliferation and medulloblastoma (MB) tumorigenesis. These observations are interesting because RNF220 has been previously shown by the same group to oppose Shh signaling through K63-linked polyubiquitination and nuclear export of Gli transcription factors during ventral neural patterning.

Thus, depending on the cellular context, RNF220 can act either positively or negatively on Shh signaling. In this study, the authors identified that EED, an essential component of the PRC2 complex, is a target of RNF220 in cerebellar granule cells and the Shh MB cell line DAOY. These results illustrate that, in addition to the polyubiquitination and nuclear export Gli transcription factors, RNF20 can modulate Shh signaling by targeting the PRC2 complex at Gli target promoters. I think these results are novel to the field of Shh signaling and MB tumorigenesis.

Comments for the author

Overall, I think the authors have addressed most of my concerns in the previous review. However, due to the low resolution of the supplementary figures, especially Fig. S3, I am not able to validate some of the points.

Reviewer 2

Advance summary and potential significance to field

The study Ma et al. explores the roles of RNF220 in the Shh pathway and medulloblastoma progression. The authors determine RNF220 promotes Shh activation through EED ubiquitination and altering Gli activating epigenetic effects. Therefore, the authors propose RNF220 as not only a potential biological marker as well as a novel therapeutic target for Shh-subtype medulloblastoma. Overall, the conclusions and links made in the study are well supported by the data.

Comments for the author

There are some issues with data presentation that need to be remedied before publication. In addition, it is critical that the authors provide some further discussion of the duality of RNF220- and why it is a positive contributor to Shh-signaling in medulloblastoma, while they have demonstrated that it is a negative contributor in the ventral motor cord. Issues:
1) Lines 309-313, Materials and Methods and Figure 3A. It is unclear if all the mice were observed and sacrificed at 12 mos, and the brains examined for any evidence of a tumor, or they just used behavior to assess tumor incidence. This distinction is important if they wish to state that this affects tumor occurrence, rather than tumor progression. In addition, the word "suicided" should not be used, rather the word "sacrificed" with mice. These points need to be clear and remedied. The data would also be better supported with the addition of a Kaplan Meir graph. It will also answer the question if RNF220+/- will rescue the chance of survival of Ptch+/- mice.
2) Figure 4B-D, 5A-C, 6F-H, and 7E-G: The fold changes included with the ChIP-PCR and Western blot images should also be represented in graph format with the n denoted as well. These should also include statistical analysis. It is difficult to interpret the data without such graphs.
Minor issues:
1) Figure 2E: Spelling error of "colony"
2) Line 153. Reference needs to be fixed to match the rest of the paper.
3) Line 461-462: Grammatical and spelling errors

Second revision

Author response to reviewers' comments

Response Letter

Reviewer 1
Advance Summary and Potential Significance to Field:
This study suggests that RNF220, an ubiquitin E3 ligase, acts positively in Shh signaling to promote cerebellar granule cell proliferation and medulloblastoma (MB) tumorigenesis. These observations

are interesting because RNF220 has been previously shown by the same group to oppose Shh signaling through K63-linked polyubiquitination and nuclear export of Gli transcription factors during ventral neural patterning. Thus, depending on the cellular context, RNF220 can act either positively or negatively on Shh signaling. In this study, the authors identified that EED, an essential component of the PRC2 complex, is a target of RNF220 in cerebellar granule cells and the Shh MB cell line DAOY. These results illustrate that, in addition to the polyubiquitination and nuclear export Gli transcription factors, RNF220 can modulate Shh signaling by targeting the PRC2 complex at Gli target promoters. I think these results are novel to the field of Shh signaling and MB tumorigenesis.
Thank you very much for your supports.

Comments for the Author:
Overall, I think the authors have addressed most of my concerns in the previous review. However, due to the low resolution of the supplementary figures, especially Fig. S3, I am not able to validate some of the points.
According to the requirements of the editor team, we have combined all supplementary figures and their legends into a single PDF file for your review during the previous revision. Here, we upload all the figures individually and we hope this figure is clear enough for your reference this time.

Reviewer 2
Advance Summary and Potential Significance to Field:
The study Ma et al. explores the roles of RNF220 in the Shh pathway and medulloblastoma progression. The authors determine RNF220 promotes Shh activation through EED ubiquitination and altering Gli activating epigenetic effects. Therefore, the authors propose RNF220 as not only a potential biological marker as well as a novel therapeutic target for Shh-subtype medulloblastoma. Overall, the conclusions and links made in the study are well supported by the data.
Thank you for your supports.

Comments for the Author:
There are some issues with data presentation that need to be remedied before publication. In addition, it is critical that the authors provide some further discussion of the duality of RNF220 and why it is a positive contributor to Shh-signaling in medulloblastoma, while they have demonstrated that it is a negative contributor in the ventral motor cord.
Thank you for your suggestion. More discussion on the duality of RNF220 in Shh signaling regulation is included in the revised manuscript.

Issues:
1) Lines 309-313, Materials and Methods and Figure 3A. It is unclear if all the mice were observed and sacrificed at 12 mos, and the brains examined for any evidence of a tumor, or they just used behavior to assess tumor incidence. This distinction is important if they wish to state that this affects tumor occurrence, rather than tumor progression. In addition, the word “suicided” should not be used, rather the word “sacrificed” with mice. These points need to be clear and remedied. The data would also be better supported with the addition of a Kaplan Meir graph. It will also answer the question if RNF220+/- will rescue the chance of survival of Ptch+/- mice.
Thank you for your suggestion. We observed the mice for a period of 12 months. During the observation the mice were sacrificed only when they showed some paralyzed behavior and at the end of the observation all the remaining mice were sacrificed to assess tumor incidence. We revised the related description.
Rigorously, based on the description above and the results showed in Figure 3A, we think that we could only reach the conclusion that RNF220$^{+/-}$ could rescue the MB occurrence of Ptch1$^{+/-}$ mice but not the conclusion that RNF220$^{+/-}$ could rescue the chance of survival of Ptch1$^{+/-}$ mice. To avoid such mis-understanding, we have not represented the data with a Kaplan Meir graph in the manuscript. We hope you could understand and support us.

2) Figure 4B-D, 5A-C, 6F-H, and 7E-G: The fold changes included with the ChIP-PCR and Western blot images should also be represented in graph format with the n denoted as well. These should also include statistical analysis. It is difficult to interpret the data without such graphs.

Thank you for your suggestions. To make the figure concise, the statistics have not been represented in graph format. We think the results we showed in the manuscript are clear enough to support our conclusions. Each of the ChIP-PCR and Western blot experiments were conducted at least three times with the same conclusion as the results we showed in the manuscript and we add the information in the revise method and material parts. We hope you could understand and support us.

Minor issues:
1) Figure 2E: Spelling error of "colony"
2) Line 153. Reference needs to be fixed to match the rest of the paper.
3) Line 461-462: Grammatical and spelling errors
Thank you for your careful reading. We corrected all the errors and carefully revised the whole manuscript to avoid such errors.

Third decision letter

MS ID#: DEVELOP/2020/188078

MS TITLE: RNF220 is required for cerebellum development and regulates medulloblastoma progression through epigenetic modulation of Shh signaling

AUTHORS: Pengcheng Ma, Tao An, Liang Zhu, Longlong Zhang, Huishan Wang, Biyu Ren, Xia Zhou, Bin Sun, Yan Li, and Bingyu Mao
ARTICLE TYPE: Research Article

I am delighted to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.