Rnd2 differentially regulates oligodendrocyte myelination at different developmental periods

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)
RE: Manuscript #E20-05-0332  
TITLE: Rnd2 uniquely regulates myelination by oligodendrocytes depending on myelinating process

Dear Dr. Yamauchi:

Two reviewers have assessed your manuscript and both are in agreement that it represents a substantial amount of work that addresses the role of RND2 in myelination. They were also in agreement about a number of changes that are needed to make the manuscript suitable for publication in MBOC. These include changes to the writing and organization to provide additional clarity, increased and more precise discussion of statistics and n's, and increased validation of the mouse models and the co-culture model. Please find their detailed comments attached. If you can address these concerns, we would be happy to reconsider this manuscript for publication. If you have any questions about how to proceed (especially in light of potential delays in revising due to the pandemic), please don't hesitate to contact me through MBOC.

Sincerely,

Terry Lechler  
Monitoring Editor  
Molecular Biology of the Cell

Dear Dr. Yamauchi,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers’ comments have been addressed. (The file type for this letter must be “rebuttal letter”; do not include your response to the Monitoring Editor and reviewers in a “cover letter.”) Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

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Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker  
Journal Production Manager  
MBoC Editorial Office  
mbc@ascb.org

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Reviewer #1 (Remarks to the Author):

This manuscript concerns a member of the small guanosine triphosphatase family (GTPase) and its effect on myelination of oligodendrocytes. Other members of this family have been shown to promote or obstruct myelination but the effect of Rnd2 on myelination has not been tested. The authors showed that Rnd2 was expressed in oligodendrocytes throughout the lineage, increasing with differentiation. The authors created an oligodendrocyte-specific Rnd2 knockout. At P14, MBP and CNP expression was down compared to controls, g ratios were elevated indicating thinner myelin whereas Olig2 numbers were the same. Oligodendrocyte-neuronal co-cultures also showed decreases in MBP positive segments when treated with Rnd2 shRNA. These co-culture experiments are problematic if the images shown represent the cultures. See below. However, just 14 days later in the knockout, MBP and CNP levels were increased and myelin thickness was increased. This was mirrored by coculture results. To further confirm their results, the authors made a Rnd2 transgenic mouse on an MBP promoter. The results were the opposite of the knockout, myelin protein levels were increased at 14 days and decreased at 21 days. Does this go through Rho kinase? Phosphorylated Rho kinase levels increased at P14 in the KO mouse and decreased at P28. The authors then conclude that in early development, Rnd2 inhibits Rho and at later development, Rnd2 promotes Rho signaling. In one final experiment, the authors made a tamoxifen inducible Rnd2 KO and deleted the gene at 8-9 weeks. There was less myelin proteins and slightly higher g ratios. Just four weeks later, this had flipped again and there was more myelin protein.

There is a lot of data in this manuscript including three unique genetically altered strains of mice. The observations nicely complement each other in that initially Rnd2 appears to promote myelination somehow through Rho and then not all that much later, it inhibits it. This is a very fast turn around and one wonders what role this mechanism plays in overall oligodendrocyte development and why it is present. Given the switch from promoting to inhibiting in just two weeks, it would have been beneficial to take the models out to longer than 28 days which is still mostly adolescence in a mouse. The comparison to the McLane paper in the discussion is not totally fair because the mTOR studies delete TSC1 from different stages of the lineage which is not happening in this manuscript. Overall, the authors should have speculated on their odd findings more and how they could have evolved to benefit myelination. Could Rnd2 perhaps put the brakes on excessive myelination after axons are appropriately myelinated?

There are a number of major issues with this manuscript as described below. They limit enthusiasm for an otherwise interesting story.

Individual points:

In general, the manuscript needs to be edited by a native speaker of English who is also a scientist. There are numerous grammatical mistakes, punctuation mistakes and awkward phrases.

The title is not clear. What does “depending on myelinating process” mean? A process is both the membrane extension of the cell and the progression that a cell goes through. Which do the authors mean here? Depending on stage of the lineage? Also, “myelination by oligodendrocytes” sounds wrong to the ear. Do they mean myelination of oligodendrocytes?

Statistics:
The methods say that at least three experiments were carried out. Does that mean three mice per age group? Were they from the same or different litters?

In the figures, numbers of slices for independent experiments are given. Does this mean sections stained and counted per animals? More details are necessary.

The cocultures do not look like they have myelinated in the pictures. Just because one seeds oligodendrocytes on top of neurons does not mean myelination proceeds and the appearance of the MBP positive processes do not look like they have wrapped around neurofilaments. Oligodendrocyte processes which myelinated neurons generally appear as straight lines of fluorescence. To see what actual myelinating co-cultures look like, please see Tyler et al, J Neurosci, 29, 2009, Figure 7. Also, why is the merge blue? The merge should be the combination of the two colors used to demonstrate the fluorescence of MBP and NF as shown in Figure 1 of this manuscript. DAPI labeling should be in blue so nuclei can be seen. As shown, none of the coculture data is believable.

There seems to be no logic as to which figures are in the manuscript and which are supplemental. For example, for the Rnd2 transgenic mouse, figure 6 in the manuscript shows that at early time points, there is an increase in MBP and myelin but the next logical figure showing that at later time points, there is a decrease with Rnd2 overexpression is relegated to the supplemental figures (Figure 6). Why is expression at one time point more important than another?

Figure 1D:
The P7 PDGFR images are hard to interpret. The Rnd2 labeling is no so cellular and looks very different than the labeling at 14 and 21 days. It is hard to see what is co-labeled. At 21 days: why is there no yellow in the merged imaged? It does not compare to the one above it.

Many of the MBP blots are overexposed: Figure 4, Figure S2, Figure S3, Figure S4, Figure S5. A lower or shorted exposure might
have shown more subtle changes and more accurate quantification.

Discussion:
The section on plexins is not well-integrated into the overall discussion. Plexins were not tested in this manuscript so why waste three-quarters of a page on them. This is also true of some of the discussion of transcription factors. Since the authors did not examine the expression of the major oligodendrocyte transcription factors in this manuscript, it is not necessary to go into such detail about them. Mentioning that they exist and could be looked at is sufficient.

Reviewer #2 (Remarks to the Author):

Miyamoto et al. ask an important question in this manuscript - how does the small GTPase RND2 affect myelination? RND2 is highly expressed in OPCs and oligodendrocytes by many transcriptomics databases, but its function in these cells is largely unclear. To address this question, the authors generated two novel mouse models, a RND2 knockout and a RND2 overexpressor, both with conditional potential. The authors cross the KO with the MBP-Cre line and the inducible PLP-CreERT line; both crosses demonstrate that ~P14, RND2 KO results in thinner myelin, but that ~P28, RND2 KO results in thicker myelin. In contrast, overexpression of RND2 by crossing with the MBP-Cre line results in thicker myelin ~P14. These results are consistent with each other and indicate that RND2 has opposing role at different developmental stages. Holistically, these results likely indicate that RND2 plays a role in the timing of myelination and may regulate the cessation of wrapping.

This manuscript certainly contains an impressive amount of data and goes above and beyond by adding the PLP-CreERT crosses. However, I have some technical concerns and requests for control data to validate the mouse models. In addition, it would benefit from some streamlining in terms of text edits and figure layouts. Nevertheless, largely based on the heroic amount of robust data, I recommend publication with major revisions.

Major points:
1. Technical concerns regarding the embryonic oligodendrocyte cultures. As the authors cite their own papers in the methods sections, it is important to validate this approach (i.e. the isolated cells express OPC/oligo markers). In contrast to most oligodendrocyte primary cultures, which are derived from postnatal brains, this technique uses embryonic brain (cortex). As the authors show in Fig. 1A, RND2 protein increases postnatally with no expression at P7 and low expression at P14. In addition, the authors grow their cells initially in serum; postnatal OPCs grown in serum will differentiate into Type 2 astrocytes (many papers from Martin Raff lab around the 1990s-2000s). One way to validate is via immunostaining (e.g. the isolated cells express OPC/oligo markers, no astrocyte markers); this experiment can also be used to address Point #3. Maybe this has already been done; if so, please state in methods. Another way is using the transcriptome data (Table 1).
2. Co-culture interpretation. These are nice experiments so they do not need to be over-interpreted. For example, the authors repeatedly assert that Day 28 cultures are representative of P28 in vivo. This type of language needs to be removed due to caveats to co-culture experiments. First, this is a Frankenstein experiments with CNS oligos (from the cortex) with PNS axons (DRGs) and therefore is non-physiological. Second, as tissues came from embryonic tissue, very little myelination is happening before birth and this point is connected to Point #1. Third, the timing of in vitro and in vivo experiments are not linked; Day 28 in vitro can be interpreted as looking at more mature oligos, but does not necessarily correlate with a particular developmental day in vivo.
3. In Western blots, CNP levels correlate with RND2 status and g-ratio changes (e.g. thicker myelin and more CNP). Recent work from Mika Simon's lab shows that CNP is crucial for maintaining cytoplasmic channels in the myelin sheath and in fact facilitates actin "struts" that hold up these tunnels. Microtubules are also found inside these cytoplasmic channels so an increase in CNP would follow up on this with brain histology and cell culture. It would be unreasonable to perform these experiments on all 3 mouse lines (and multiple time points); thus, it would be sufficient to show staining for either one line/time or with shRNA. Please also show quantification of the CNP Westerns.
4. Validation of KO mice with histology. Does RND2 staining (as shown in Fig. 1) go away in KO mice?
5. Please revise the title ("RND2 uniquely regulates myelination by oligodendrocytes depending on myelinating process"). Currently, the title is repetitive and “uniquely” should be removed as Rnd2 is not the only protein that regulates myelination this way. A suggestion is "RND2 differentially regulates myelination at different developmental periods".

Minor points (optional to address unless otherwise noted):
1. (Need to address) Please check the n's for the EMs. I think you mean that 300+ axons were quantified, not nerves (a bundle of axons; e.g. sciatic nerve, optic nerve, etc.).
2. The authors would benefit from a professional editing service (also see Point #5). The manuscript is difficult to follow at times, perhaps owing to the authors not being native English speakers. An example: Intro, 2nd paragraph: "Myelin formation begins with the first contact of oligodendrocyte processes along axons and proceeds to further processes." This phrasing and repetition of "processes" is a bit confusing. I think you mean "further steps" (not additional cellular processes/arms).
3. Table 1 could be introduced with more detail. This what the authors did, how they sorted the hits, and what they expect to find (i.e. mRNAs whose expression increases in Day 3 vs. Day 0 cells). This is also not the only transcriptome showing very high
levels of RND2 in OPCs/oligos so it would strengthen the paper to mention those studies too.

4. The manuscript would be a lot clearer with a table summarizing the results (with g-ratios, MBP expression, CNP expression, etc.). This would help to breakdown the already confusing concepts and more convincingly show that the data are internally consistent.

5. Cre validation. Fig. 2C genotyping validation using tail genomic DNA only shows that the Flp step is efficient. To show Cre is active in MBP-expressing tissues or cells, the authors can design a primer set flanking the Cre-excised region.

6. Fig. 2E quantification. MBP typically does not label cell bodies well. So I think what you are quantifying is relative intensity and not necessarily number of cells.

7. Co-culture quantification. This would be strengthened by additional analysis of length of myelin segments. Also related to Point #3 on CNP. This would be consistent with recent work from the Barres Lab showing that microtubules along myelin sheaths (the ones inside the cytoplasmic channels) facilitate myelin sheath elongation. Fig. S3 is a very nice experiment and the authors might consider moving it to a main figure; to make room, perhaps move the GFAP/NeuN images to the supplement. Also, it’s unclear how many days the authors expect the shRNA to be effective (this could be clarified simply by stating what/when the Western blot samples are from, for example in Fig. S2A).

8. Figure reorganization. It would be helpful for the reader if Figures were organized by each line/time.

9. For future directions (not for this paper), the authors might think about crossing their mice with mutants that create thicker myelin. For example, is this a separate pathway from PTEN (cKOs also have thicker myelin)?
Responses to the reviewers’ comments

Reviewer #1
First, we sincerely thank the reviewer for the many helpful suggestions. After carefully considering the reviewer’s comments, we have reorganized our coculture immunofluorescence data and added magnified images to the new figures. To further characterize Rnd2 signaling, we have added immunoblots and statistical data for Tg mice to the reorganized figures. We believe that these data emphasize the role of Rho kinase in Rnd2 signaling at the cell and tissue levels. We would like to respond to specific queries as follows.

To further confirm their results, the authors made a Rnd2 transgenic mouse on an MBP promoter. The results were the opposite of the knockout, myelin protein levels were increased at 14 days and decreased at 21 days. Does this go through Rho kinase?

We thank the reviewer for pointing this out. We performed immunoblotting for an active phosphorylated Rho kinase and a phosphorylated form of Mbs in Rnd2 Tg mice. The phosphorylation levels of Rho kinase and Mbs were decreased at postnatal day 14 in the Tg mice and increased at postnatal day 28. It is thus suggested that the myelin protein levels were changed through Rho kinase. We have added these data in the present Figure 8, G-L.

This is a very fast turn around and one wonders what role this mechanism plays in overall oligodendrocyte development and why it is present. Given the switch from promoting to inhibiting in just two weeks, it would have been beneficial to take the models out to longer
than 28 days which is still mostly adolescence in a mouse.

The issue of whether the myelination thickness remains increased in adolescent mice is an important one. We assessed myelin ultrastructure in two-month-old Rnd2 KO mice. At two months, KO mice exhibited decreased g-ratios similar to those seen at 28 days, demonstrating that the increase in myelin thickness persists in adolescence. We have added these data in the present Figure5, J-M.

The comparison to the McLane paper in the discussion is not totally fair because the mTOR studies delete TSC1 from different stages of the lineage which is not happening in this manuscript. Overall, the authors should have speculated on their odd findings mouse and how they could have evolved to benefit myelination. Could Rnd2 perhaps put the brakes on excessive myelination after axons are appropriately myelinated?

We agree with the reviewer’s suggestion. We deleted the description of the McLane paper and TSC1 signaling form the Discussion section. In addition, based on the downstream signaling of Rho and Ras GTPases, we speculated on the possible reasons for Rnd2’s dual role in myelination. As suggested by the reviewer, we added a discussion of this matter to the first and last paragraphs of the Discussion section.

Individual points

In general, the manuscript needs to be edited by a native speaker of English who is also a scientist. There are numerous grammatical mistakes, punctuation mistakes and awkward phrases.

We appreciate this helpful comment. We have sent the manuscript to a professional scientific editing service and have rewritten many sentences throughout the manuscript.
The title is not clear. What does “depending on myelinating process” mean? A process is both the membrane extension of the cell and the progression that a cell goes through. Which do the authors mean here? Depending on stage of the lineage? Also, “myelination by oligodendrocytes” sounds wring to the ear. Do they mean myelination of oligodendrocytes?

We are sincerely appreciative of the reviewer’s comment on the title. We have revised the title to “Rnd2 differentially regulates oligodendrocyte myelination at different developmental periods”, as proposed by reviewer #2.

Statistics: The methods say that at least three experiments were carried out. Does that mean three mice per age group? Were they from the same or different litters? In the figures, numbers of slices for independent experiments are given. Does this mean sections stained and counted per animals? More details are necessary.

Thank you for these questions, which helped us improve our statistical reporting. In the Methods section, “three experiments” means “three different sets of mice, each containing mice of the same age (wild-type and knockout or transgenic mice) from the same litters”. In the figures, “numbers of slices for independent experiments” means “the total numbers of sections stained and counted from three different sets of mice, each set containing mice of the same age (wild-type and knockout or transgenic mice)”. We have carefully checked the Methods section and the figure legends throughout the manuscript and have updated the phrases we use to refer to these topics to avoid any confusion.

The cocultures do not look like they have myelinated in the pictures. Just because one
seeds oligodendrocytes on top of neurons does not mean myelination proceeds and the appearance of the MBP positive processes do not look like they have wrapped around neurofilaments, Oligodendrocyte processes which myelinated neurons generally appear as straight lines of fluorescence. To see what actual myelinating co-cultures look like, please see Tyler et al, J Neurosci, 29, 2009, Figure 7). Also, why is the merge blue? The merge should be in the combination of the two colors used to demonstrate the fluorescence of MBP and NF as shown in Figure 1 of this manuscript. DAPI labeling should be in blue so nuclei can be seen. As shown, none of the coculture data is believable.

We agree with these comments on the coculture data. As in the pictures of myelinating coculture in Tyler’s paper, our cocultures appeared as straight lines of MBP fluorescence (new Figure 4, B and C, with magnified pictures and new Figure 6, A and B, with magnified pictures). We have carefully checked the quality of all pictures and have added additional magnified photographs as new panels. We have also checked and added some merged images as well as the respective MBP, NF, and DAPI images.

There seems to be no logic as to which figures are in the manuscript and which are supplemental. For example, for the Rnd2 transgenic mouse, figure 6 in the manuscript shows that at early time points, there is an increase in MBP and myelin but the next logical figure showing that at later time points, there is a decrease with Rnd2 overexpression is relegated to the supplemental figures (Figure 6). Why is expression at one time point more important than another?

We agree with the reviewer’s comment and have carefully reorganized the figures so that each time period is adequately represented.
6) **Figure 1D:** The P7 PDGFRalpha images are hard to interpret. The Rnd2 labeling is no so cellular and looks very different than the labeling at 14 and 21 days. It is hard to see what is co-labeled. At 21 days: why is there no yellow in the merged imaged? It does not compare to the one above it.

We agree with the reviewer’s comment regarding the immunohistochemical images. We carefully cut the tissues and performed immunostaining for Rnd2 and PDGFalpha. We have included a new image in Figure 1D to present the new data, along with another image taken at P21 showing the clear yellow color that represents equal quantities of green and red staining.

*Many of the MBP blots are overexposed: Figure 4, Figure S2, Figure S3, Figure S4, Figure S5. A lower or shorted exposure might have shown more subtle changes and more accurate quantification.*

We thank the reviewer for this very helpful comment. We have included short-exposure images of MBP blots in the present Figures, 4H, 6G, and 7K and the new ones in Figure 5D as well as supplemental Figure S6D.

**Discussion:** The section on plexins is not well-integrated into the overall discussion. Plexins were not tested in this manuscript so why waste three-quarters of a page on them. This is so true of some of the discussion of transcription factors. Since the authors did not examine the expression of the major oligodendrocyte transcription factors in this manuscript, it is not necessary to go into such detail about them. Mentioning that they exist and could be looked at is sufficient.

We thank the reviewer this valuable comments. We have omitted the discussion on
Plexins and transcription factors in the Discussion section. In its place, we now explore the potential roles of Ras and Rho GTPases as they affect Rnd2 in oligodendrocyte differentiation and myelination as well as the possible roles of Rnd2 in these processes.

Other modifications:
1. We have added new panels to the present Figures 2E, 2F, 2H, 4F, 4I, 5E, 5 J-M, 6E, 6H, 7 C-F, and 7 M-O. We have also added new panels to the present supplemental Figures S1C, S1D, S3 A-D, S6E, and S7E.
2. In keeping with all these additions to the figures, we have updated the numbering of the figures and the lettering of their panels.
3. For every new figure or addition to a preexisting figure, we have updated our explanation of each figure’s contents in the Results section and Figure legends.
4. We have created a new Table 2.

We sincerely appreciate the reviewer’s input, which has enabled us to strengthen our argument for the proposed role of Rnd2 in oligodendrocyte myelination.

Reviewer #2
First, we would like to thank the reviewer for commenting that “This manuscript certainly contains an impressive amount of data, and goes above and beyond by adding the PLP-CreERT crosses.” We would like to respond to specific queries as follows.

Major points:
1) Technical concerns regarding the embryonic oligodendrocyte cultures. As the authors
cite their own papers in the methods sections, it is important to validate this approach (i.e. the isolated cells express OPC/oligo markers). In contrast to most oligodendrocyte primary cultures, which are derived from postnatal brains, this technique uses embryonic brain (cortex). As the authors show in Fig. 1A, RND2 protein increases postnatally with no expression at P7 and low expression at P14. In addition, the authors grow their cells initially in serum; postnatal OPCs grown in serum, will differentiated into Type 2 astrocytes (many papers from Martin Raff lab around the 1990s-2000s). One way to validate is via immunostaining (e.g. the isolated cells express OPC/oligo markers, no astrocyte markers); this experiment can also be used to address Point#3. Maybe this has already been done; if so, please state in methods. Another way is using the transcriptome data (Table 1).

The reviewer pointed out several important issues concerning the characterization of isolated cells expressing OPC/oligodendrocyte markers. Transcriptome data suggested the presence of the oligodendrocyte lineage cell markers Olig1 and Olig2 (see Table 1, blue colors) and the absence of the astrocyte markers Nfia, Sox9, Gfap, and Aqp4 (see Table 1, green colors). Differentiating oligodendrocyte markers such as Mbp, Mag, and Plp1 (Table 1) are also strongly upregulated following the induction of differentiation.

2) Co-culture interpretation. These are nice experiments so they do not need to be over-interpreted. For example, the authors repeatedly assert that Day28 cultures are representative of P28 in vivo. This type of language needs to be removed due to caveats to co-culture experiments.

We thank the reviewer for this comment. We agree and have removed the unsupported interpretations from the manuscript.
First, this is a Frankenstein experiments with CNS oligos (from the cortex) with PNS axons (DRGs) and therefore is non-physiological.

We agree with the reviewer’s comment. While sensory neuronal axons in the spinal cord are wrapped with central nervous system oligodendroglial cells, those in the peripheral tissues are wrapped with peripheral nervous system glial Schwann cells. It is thus thought that cocultures *in vitro* can partially, but not completely, mimic the formation of myelin in the central nervous system. This is explained in the “Oligodendrocyte-neuronal cocultures” subsection of the Materials and Methods section.

Second, as tissues came from embryonic tissue, very little myelination is happening before birth and this point is connected to Point #1. Third, the timing of *in vitro* and *in vivo* experiments are not linked; Day28 *in vitro* can be interpreted as looking at more mature oligos, but does not necessarily correlate with a particular developmental day in vivo.

We promptly thank the reviewer for raising this important issue. We have removed the interpretation concerning the timing of *in vitro* and *in vivo* experiments. As we only intended to suggest the different effects of Rnd2 on myelination in earlier and later periods of myelination, following the reviewer’s suggestion allowed us to emphasize our conclusions more effectively.

3) In Western blots, CNP levels correlate with RND2 status and g-ratio changes (e.g. thicker myelin and more CNP). Recent work from Mikael Simon’s lab shows that CNP is crucial for maintaining cytoplasmic channels in the myelin sheath and in fact facilitates actin “struts” that hold up these tunnels. Microtubules are also found inside these
cytoplasmic channels so an increase in CNP could indicated that more cytoplasmic channels are being created and therefore more wraps and thicker myelin. The authors should follow up on this with brain histology and cell culture. It would be unreasonable to perform these experiments on all 3 mouse lines (and multiple time points); thus, it would be sufficient to show staining for either one line/time or with shRNA. Please also shown quantification of the CNP westerns.

We agree with the reviewer’s comments concerning the CNP levels. We performed immunostaining for CNP in 14-day-old mice and found that its expression levels were lower in knockout mice. Conversely, Rnd2 Tg mice exhibited increased CNP expression, consistent with their MBP expression levels, at day 14. We have added these data to the present Figure 2, E and F, and Figure 7, D-F, respectively.

We performed immunofluorescence of CNP in cocultures. Knockdown of Rnd2 in oligodendrocytes decreased the number of CNP-positive cells and the number of Olig2-positive cells expressing CNP at 21 days. The formation of CNP-positive myelin segments and the total lengths of myelin sheaths were also decreased. We have added these data in the new supplemental Figure S3, A-C. Notably, the opposite results were observed in cultures at 28 days, as shown in the present Figure 6.

Further, we have added quantification of our CNP immunoblot data to the present Figures 2H, 4I, 5E, 6H, and 7C as well as to the new supplemental Figures S6E and S7E.

4) Validation of KO mice with histology. Does RND2 staining (as shown in Fig. 1) go away in KO mice?
We performed coimmunostaining for Rnd2 with CC1 in Rnd2 knockout mice and controls. Rnd2 staining yielded weaker positive signals in conditional knockout mice. These data have been added to the new supplemental Figure 1D.

5) Please revise the title ("Rnd2 uniquely regulates myelination by oligodendrocytes depending on myelinating process"). Currently, the title is repetitive and “uniquely” should be removed as Rnd2 is not the only protein that regulates myelination this way. A suggestion is “RND2 differentially regulates myelination at different developmental periods”.

We sincerely thank the reviewer for this valuable comment. We revised the title to “Rnd2 differentially regulates oligodendrocyte myelination at different developmental periods”.

Minor points (optional to address unless otherwise noted):

1) (Need to address) Please check the n’s for the EMs. I think you mean that 300+ axons were quantified, not nerves (a bundle of axons; e.g. sciatic nerve, optic nerve, etc.)

We thank the reviewer for this comment. We have changed “nerves” to “axons” throughout the figure legends.

2) The authors would benefit from a professional editing service (also see Point #5). The manuscript is difficult to follow at times, perhaps owing to the authors not being native English speakers, An example: Intro, 2nd paragraph: “Myelin formation begins with the first contact of oligodendrocyte processes along axons and proceeds to further processes.” This phrasing and repetition of “process” is a bit confusing. I think you mean “further steps” (not additional cellular processes/arms).
We appreciate this helpful comment. We changed the phrase “further processes” to “further steps” in the Introduction section (2nd paragraph) as recommended. We also sent our manuscript to a professional scientific English editing service and edited a large number of sentences throughout the manuscript.

3) **Table 1 could be introduced with more detail.** This what the authors did, how they sorted the hits, and what they expect to find. (i.e. mRNAs whose expression increases in Day 3 vs. Day 0 cells). This is also not the only transcriptome showing very high levels of RND2 in OPCs/oligos so it would strengthen the paper to mention those studies too.

We thank the reviewer for this comment, with which we agree. We performed transcriptome analysis using primary oligodendrocyte culture mRNAs before and after the induction of differentiation (day 0 vs. day 3). We sorted them by fold increase values, focusing on signaling molecules that have not yet been studied. We have mentioned this in the figure legend of Table 1.

Dugas et al. have analyzed gene profiles in differentiating oligodendrocytes using microarrays (2006). We explored the publicly available data in their paper but could not find evidence for any Rnd2 expression. We have cited Dugas’s paper and mentioned them in the Reference section. The reason for the discrepancy between their findings and ours might be due to the use of a different apparatus for mRNA analysis or to a difference in experimental conditions.

4) **The manuscript would be a lot clearer with a table summarizing the results (with g-ratios, MBP expression, CNP expression, etc.).** This would help to breakdown the already
confusing concepts and more convincingly show that the data are internally consistent. We agree with this comment and have made a new table summarizing these results, which appears as the new Table 2.

5) Cre validation. Fig. 2C genotyping validation using tail genomic DNA only shows that the Flp step is efficient. To show Cre is active in MBP-expressing tissues or cells, the authors can design a primer set flanking the Cre-excised region. As indicated by the reviewer, this issue of whether Cre is active in the MBP-expressing corpus callosum is an important one. We designed a new genomic PCR primer pair for the Cre-excised region and performed PCR amplification using the mouse corpus callosum genome to confirm actual conditional deletion. We identified the deleted alleles in the corpus callosum and shown them in panel C of the new supplemental Figure S1.

6) Fig. 2E quantification. MBP typically does not label cell bodies well. So I think what you are quantifying is relative intensity and no necessarily number of cells. We thank the reviewer for this comment. We quantified the relative intensity of MBP in the present Figure 2C, the new Figure 5B, and the new supplemental Figures S6B and S7B. We have left the cell number data in place to enable comparisons between control and KO or Tg mice.

7) Co-culture quantification. This would be strengthened by additional analysis of length of myelin segments. Also related to Point#3 on CNP. This would be consistent with recent work from the Barres Lab showing that microtubules along myelin sheaths (the ones inside the cytoplasmic channels) facilitate myelin sheath elongation.
We agree with the reviewer’s suggestion. We quantified the total lengths of myelin segments in cocultures. Compared with those in controls, the total sheath lengths in Rnd2 knocked down cocultures were decreased at 21 days and increased them at 28 days, being compared with controls. These data have been added to the new Figures 4F and 6E.

Fig. S3 is a very nice experiment and the authors might consider moving it to a main figure.; to make room, perhaps move the GFAP/NeuN images to the supplement.
We thank the reviewer for this suggestion. We have moved the former supplemental Figures S2 and S3 to become main figures (the present Figures 4 and 6, respectively).

Also, it’s unclear how many days the authors expect the shRNA to be effective (this could be clarified simply by stating what/when the Western blot samples are from, for example in Fig. S2A).
We agree with the reviewer’s comments. We performed immunoblotting using the coculture samples. The expression levels of Rnd2 were decreased in Rnd2 knocked down cocultures at 28 days, suggesting that the shRNAs for Rnd2 are effective within 28 days. We have added these data to the new Figure 6G.

8) Figure reorganization. It would be helpful for the reader if Figures were organized by each line/time.
We thank the reviewer for this comment. We have revised the panel and figure placement and accordingly, their number and letter designations, throughout this manuscript. In accordance with these changes and our addition of new data, we have updated the explanations of each figure in the Results section and in the Figure legends.
9) For future directions (not for this paper), the authors might think about crossing their mice with mutants that create thicker myelin. For example, is this separate pathway from PTEN (cKOs also have thicker myelin)?

We thank the reviewer for this idea. As the reviewer pointed out, conditional knockout of PTEN in mice typically results in enhanced myelin thickness. Rho GTPase Rnd2 signaling is a negative regulator in the later stages of myelination in development and maintenance. The presence of regulatory networks underlying Rho and Ras GTPases as well as the downstream signals on-and-off mechanisms in myelination are more complicated than initially expected. It is possible that small GTPase networks and PTEN/PI3K signaling are crosstalking. We have added this information, mainly in the second paragraph of the Discussion section.

Other modifications:
1. We have added new panels to the present Figures 4B, 4C, 6A, 6B and 8 G-L. We have also added new images to the present Figures 1D and 5D.
2. Accordingly, we have changed the numbering of the figures and panels.
3. In accordance with these changes to the figures, we have updated our explanations of their contents in the Results section and in the Figure legends.

We sincerely appreciate the reviewer’s input, which has enabled us to strengthen our argument for the proposed role of Rnd2 in oligodendrocyte myelination.
Dear Dr. Yamauchi:

The reviewers have found the manuscript much improved. They still have a number of suggestions to improve the manuscript - however, these all appear to be textual changes that do not require additional experiments. I expect to be able to make an editorial decision on the revised manuscript without going back to reviewers. My apologies in the delay of the review over the holidays.

Sincerely,

Terry Lechler
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Yamauchi,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

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In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL): Link Not Available

Authors of Articles and Brief Communications whose manuscripts have returned for minor revision ("revise only") are encouraged to create a short video abstract to accompany their article when it is published. These video abstracts, known as Science Sketches, are up to 2 minutes long and will be published on YouTube and then embedded in the article abstract. Science Sketch Editors on the MBoC Editorial Board will provide guidance as you prepare your video. Information about how to prepare and submit a video abstract is available at www.molbiolcell.org/science-sketches. Please contact mboc@ascb.org if you are interested in creating a Science Sketch.

Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

------------------------------------------------------------------------
Reviewer #1 (Remarks to the Author):

This manuscript is a resubmission of a previous version. The reviewers of the last version had many specific criticisms and the authors have taken these seriously and the manuscript is definitely improved, the data more solid and the figures more interpretable. Also, the English is much improved and the manuscript is much easier to read. The authors have now shown using knockout mice, transgenic mice, inducible mice, and myelinating co-cultures at various time points that Rnd2, an atypical branch of the Rho GTPase family, both promotes and inhibits oligodendrocyte maturation, depending on the stage of development or time post deletion. This is a complicated story yet the data is convincing.

Some lingering issues remain.

1) The role of Rnd2 as identified by the authors is very complex and varies with the age of the mice and the length of time Rnd2 is expressed. A figure or graphic depiction of the role of Rnd2 would be very helpful to readers.

2) P6 towards the bottom: Rnd2 co stained with O4 and CC1. “in contrast, approximately 60% of PDGFalpha OPCs expressed Rnd2 at P7.” Does this mean the all the O4 and CC1+ cells expressed Rnd2? The number that co-stained with O4 or CC1 is not stated, so “in contrast” does not make sense. Please count the O4+/Rnd2+ cells and the CC1+/Rnd2+ cells.

3) Figure S1D could use a merged image and cell counts.

4) In many of the figures, immunoblots were shown for MBP and CNP but only CNP was quantified. Why? For example, in Figure 2G and H, the text says there is a decrease in both MBP and CNP but only CNP is quantified and the blot shown does not look like there is much of a decrease in MBP.

5) What is the actual difference between shRNA Rnd2#1 and Rnd2#2? It appears in most of the figures that shRNA Rnd2#2 is much more effective but there does not appear to be a comment about this. For example, in Figure 4B, the myelinating sheaths look completely different with Rnd2#1 than Rnd2#2. Rnd2#1 looks like the controls whereas Rnd2#2 looks like cells that have not wrapped, yet the cell counts are the same for the 2 shRNAs. Either the cell counts are not accurate or a more representative image is needed. Likewise, it looks like there is a large decrease in MBP with Rnd2#2 and hardly a decreased in MBP at all with Rnd2#1 in Figure 4H. Again, this a representative blot? Please also see Figure 6, G and H.

6) Figure 5D: the text says the increase in MBP is “slight” but the increase shown on the blot is very large. Which is correct?

7) Figure 5L: the variability is so large that it is surprising that the P value is 0.01.

8) P12 at the top “thus it has been inferred that knockout of Rnd2 is mice upregulates Rho kinase signaling and inhibits myelination at PD14.” Inferred is an odd word to use here. Does this mean hypothesized? Or seems possible or is likely that?

9) The last experiment in which Rnd2 was knockout by tamoxifen injection in the adult is a bit unclear. It appears that 2 weeks after Tx administration, KO of Rnd2 decreased MBP whereas just two weeks later, the MBP levels were decreased. If this is true, this turnaround is quite remarkable. Given how quickly this happens, it might be difficult to imagine using Rnd2 as a target for intervention.

Reviewer #2 (Remarks to the Author):

Overall, the authors have successfully integrated the major changes suggested by both reviewers: to re-organize the figures by developmental time points and to revise the text. In particular, I found the revised coculture figures (Fig. 4 and 6) to be much improved, with additional figures that are very compelling. As a result, the paper is much clearer and presents the paradoxical results at different developmental time points in a coherent and straightforward way. Thus I recommend publication without review. The authors should be really proud of the revised manuscript and their impressive and thorough characterization of Rnd2 in oligodendrocytes.

Minor Points (that the authors may want to address but are not required):

1. I was particularly intrigued by the addition of CNP staining. This could indicate that Rnds are affecting myelination through cytoplasmic channels. I suggest the authors include in the discussion and/or consider for follow-up experiments any connection to Golgi outposts inside the myelin sheath. These organelles were recently described in a Cell paper from the Barres lab and reports similar defects that were described here (shorter sheaths). In addition, in neurons, Rho GTPases likely affect the number of Golgi outposts found in dendrites (Quassollo et al 2015).

2. Please add scale bar to Fig. 1F and clarify whether this is a “cross section” as the legend states. NF here looks filamentous and therefore could be a transverse or longitudinal section. To distinguish between these two types and whether the ring structures are actually cell bodies (i.e., not myelin sheaths), look for DAPI inside the ring (i.e. nucleus inside a cell body).

3. I think the coculture figures would be even clearer if labeled with DIV14 and DIV28 at the top.

4. It is really too bad that the Cre-ERT experiments are in the supplement. If Fig. 2 and 3 are combined (and decrease the size of Fig. 2A or move parts to the supplement), then the Cre-ERT figure could be shifted to a main figure.

5. Consider including in Table 2: the Cre-ERT experiments and the 2-month Cre KO experiments (Fig. 5)

Typos:
1. Abstract: “an atypical branch of the Rho GTPase“ - add “family”
2. CNP is 2',3'-cyclic-nucleotide ... No space after the comma; “cyclic” and “nucleotide” are separate words (sometimes with a space, sometimes with a hyphen in between).
3. Typo: “ratswere"
Responses to the reviewers’ comments

Response to the reviewer #1’s comments

We would like to thank the reviewer for commenting that “This manuscript is a resubmission of a previous version. The reviewers of the last version had many specific criticisms and the authors have taken these seriously and the manuscript is definitely improved, the data more solid and the figures more interpretable. Also, the English is much improved and the manuscript is much easier to read. The authors have now shown using knockout mice, transgenic mice, inducible mice, and myelinating co-cultures at various time points that Rnd2, an atypical branch of the Rho GTPase family, both promotes and inhibits oligodendrocyte maturation, depending on the stage of development or time post deletion. This is a complicated story yet the data is convincing.”

1) The role of Rnd2 as identified by the authors is very complex and varies with the age of the mice and the length of time Rnd2 is expressed. A figure or graphic depiction of the role of Rnd2 would be very helpful to readers.

We sincerely thank the reviewer for this comment and agree with the reviewer’s one. We have added a graphic depiction of the role of Rnd2 to the new Figure 11.

2) P6 towards the bottom: Rnd2 co stained with O4 and CC1. "in contrast, approximately 60% of PDGFralpha OPCs expressed Rnd2 at P7." Does this mean all the O4 and CC1+ cells expressed Rnd2? The number that co-stained with O4 or CC1 is
not stated, so "in contrast" does not make sense. Please count the O4+/Rnd2+ cells and the CC1+/Rnd2+ cells.

We agree with the reviewer’s comments. We have counted the PDGFRα+/Rnd2+ cells, the O4+/Rnd2+ cells and the CC1+/Rnd2+ cells in the present Figure 1E.

3) Figure S1D could use a merged image and cell counts.

We agree with the reviewer’s comment. We have placed a merged image and semi-quantified the intensity of Rnd2 staining in the Figure S1, D and E.

4) In many of the figures, immunoblots were shown for MBP and CNP but only CNP was quantified. Why? For example, in Figure 2G and H, the text says there is a decrease in both MBP and CNP but only CNP is quantified and the blot shown does not look like there is much of a decrease in MBP.

We thank the reviewer for these comments. As pointed out by the reviewer, decrease in CNPase is clearly significant in immunoblotting whereas decrease in MBP is comparatively mild or small. We have changed a blot of Fig. 2G into clearer one.

In 1st round review, another reviewer pointed out them and recommended CNPase quantification. We thus quantified blots for CNPase and placed them in the figures.

5) What is the actual difference between shRNA Rnd2#1 and Rnd2#2? It appears in most of the figures that shRNA Rnd2#2 is much more effective but there does not appear to be a comment about this. For example, in figure 4B, the myelinating sheaths look completely different with Rnd2#1 than Rnd2#2. Rnd2#1 looks like the controls whereas
Rnd2#2 looks like cells that have not wrapped, yet the cell counts are the same for the 2 shRNAs. Either the cell counts are not accurate or a more representative image is needed. Likewise, it looks like there is a large decrease in MBP with Rnd2#2 and hardly a decreased in MBP at all with Rnd2#1 in Figure 4H. Again, is this a representative blot? Please also see Figure 6, G and H.

Oligodendrocyte lineage cell marker Olig2-positive cells were comparable in control and knocked down cells.

As pointed out by the reviewer, shRNA Rnd2#2 was more effective compared to shRNA Rnd2#1 either morphologically or in expression levels of Rnd2 and marker proteins. Also, in an immunoblotting with an anti-MBP antibody, we would like to obtain four bands of MBP proteins. Thus, it might be difficult to observe a slight difference among data.

Either Rnd2#1 or Rnd2#2 decreased the number of both MBP-positive cells and myelin segments whereas knockdown by shRNA#2, rather than by Rnd2#1, decreased the length of myelin sheaths. And, it is likely that knockdown by Rnd2#2 greatly affects their length and MBP expression. We have emphasized these matters in the Figures 4 and 6.

6) Figure 5D: the text says the increase in MBP is "slight" but the increase shown on the blot is very large. Which is correct?

We thank the reviewer for this comment. We made a mistake. The increase was very large. We have corrected it.
7) Figure 5L: the variability is so large that it is surprising that the P value is 0.01.
We used Student’s t-test to determine the p value. In addition, around three hundred myelinated axons were counted. Despite the small difference of two bars, the p value was indeed less than 0.01.

8) P12 at the top "thus it has been inferred that knockout of Rnd2 is mice upregulates Rho kinase signaling and inhibits myelination at PD14." Inferred is an odd word to use here. Does this mean hypothesized? Or seems possible or is likely that?
We agree with the reviewer’s comments. We have corrected it.

9) The last experiment in which Rnd2 was knockout by tamoxifen injection in the adult is a bit unclear. It appears that 2 weeks after Tx administration, KO of Rnd2 decreased MBP whereas just two weeks later, the MBP levels were decreased. If this is true, this turnaround is quite remarkable. Given how quickly this happens, it might be difficult to imagine using Rnd2 as a target for intervention.
The reviewer pointed out the important issue. We agree with the reviewer’s comments. Rnd2 is not suitable for a target for intervention. We have modified the last paragraph of the Discussion section.

We sincerely appreciate the reviewer’s input, which has enabled us to strengthen our argument for the proposed role of Rnd2 in oligodendrocyte myelination.
Response to the reviewer #2’s comments

First, we would like to thank the reviewer for commenting that “Overall, the authors have successfully integrated the major changes suggested by both reviewers: to re-organize the figures by developmental time points and to revise the text. In particular, I found the revised coculture figures (Fig. 4 and 6) to be much improved, with additional figures that are very compelling. As a result, the paper is much clearer and presents the paradoxical results at different developmental time points in a coherent and straightforward way. Thus I recommend publication without re-review. The authors should be really proud of the revised manuscript and their impressive and thorough characterization of Rnd2 in oligodendrocytes.”

Minor Points (that the authors may want to address but are not required):

1. I was particularly intrigued by the addition of CNP staining. This could indicate that Rnds are affecting myelination through cytoplasmic channels. I suggest the authors include in the discussion and/or consider for follow-up experiments any connection to Golgi outposts inside the myelin sheath. These organelles were recently described in a Cell paper from the Barres lab and reports similar defects that were described here (shorter sheaths). In addition, in neurons, Rho GTPases likely affect the number of Golgi outposts found in dendrites (Quassollo et al 2015).

We thank the reviewer for these comments. These papers give us the great interest concerning Golgi body and Rho family GTPases; it is unlikely that these findings are directly associated with atypical Rho family GTPase Rnd2. In the future, when their relationships become clearer in more detail, I would like to study them in our generated Rnd2 genetically-modified mice.
2. Please add scale bar to Fig. 1F and clarify whether this is a "cross section" as the legend states. NF here looks filamentous and therefore could be a transverse or longitudinal section. To distinguish between these two types and whether the ring structures are actually cell bodies (i.e., not myelin sheaths), look for DAPI inside the ring (i.e. nucleus inside a cell body).

We have added a scale bar to the Figure 1F and have added how to cut nerves to the corresponding figure legend.

3. I think the coculture figures would be even clearer if labeled with DIV14 and DIV28 at the top.

We agree with the reviewer’s comments. We have labeled DIV14 and DIV28 at the top of the panels.

4. It is really too bad that the Cre-ERT experiments are in the supplement. If Fig. 2 and 3 are combined (and decrease the size of Fig. 2A or move parts to the supplement), then the Cre-ERT figure could be shifted to a main figure.

We agree with the reviewer’s comments. We have moved the Cre-ERT experiments to the present Figures 9 and 10.

5. Consider including in Table 2: the Cre-ERT experiments and the 2-month Cre KO experiments (Fig. 5).

We thank the reviewer for this comment. We have included the results for the Cre-ERT experiments in Table 2.
Typos:

1. Abstract: "an atypical branch of the Rho GTPase" - add "family"

2. CNP is 2',3'-cyclic-nucleotide .... No space after the comma; "cyclic" and "nucleotide" are separate words (sometimes with a space, sometimes with a hyphen in between).

3. Typo: "ratwere"

We sincerely thank the reviewer for pointing out these terms. We have corrected them.

We sincerely appreciate the reviewer’s input, which has enabled us to strengthen our argument for the proposed role of Rnd2 in oligodendrocyte myelination.
Dear Dr. Yamauchi:

I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Thank you for resubmitting this work to Molecular Biology of the Cell, and your careful attention to the remaining concerns of the reviewers. Congratulations on the acceptance of this work for publication!

Sincerely,
Terry Lechler
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Yamauchi:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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