Timely Schwann cell division drives peripheral myelination *in vivo* via the laminin/cAMP pathway

Aya Mikdache, Marie-José Boueid, Emilie Lesport, Brigitte Delespierre, Julien Loisel-Duwattez, Cindy Degerny and Marcel Tawk
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Original submission

First decision letter

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MS TITLE: Timely Schwann cell division during migration drives peripheral myelination in vivo via Laminin/cAMP pathway

AUTHORS: Aya Mikdache, Marie-Jose Boueid, Emilie Lesport, Brigitte Delespierre, Julien Loisel-Duwattez, Cindy Degerny, and Marcel Tawk

I have now received all the referees' reports on the above manuscript, and 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.

As you will see, the referees express considerable interest in your work, but have many significant criticisms and extensive suggestions for improving your manuscript. Although challenging to address all the referee concerns, I think their comments and suggestions are constructive and, if addressed, would considerably improve the study. Consequently, if you are able to revise the manuscript along the lines suggested, I will be happy receive a revised version of the manuscript. 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 by Mikdache et al., the authors describe a role for sil, a spindle pole protein, in Schwann cell division and myelination. Using the strengths of the zebrafish model, the authors show

that SCs fail myelinate peripheral axons in sil mutant larvae. However, this defect can be rescued by cAMP administration or overexpression of laminin in both muscle and SCs. This work is interesting in that we still don't fully understand the cellular and molecular mechanisms that mediate peripheral myelination, and this paper describes a role for sil in this process.

Comments for the author

This is an interesting manuscript looking at the role of sil in SC development along the PLLn. While the paper includes many techniques, there are many missing links, poorly described experiments, and confusing data that reduce my enthusiasm. I am also confused how a mutation in sil can lead to a cAMP/Laminin deficiency, and the paper doesn't go into a clear discussion of a mechanism. Also, there are many experiments that need SC markers in the background and there is a complete lack of reporting of n and statistics in the text and main figure legends (although they are in the supplemental legends). For these reasons, the work feels incomplete. Below are my comments:
1. There are several instances where the text needs editing - places where words are left out (like like 79 - "we took advantage of the Tg(foxd3:gfp)." Do you mean to say you took advantage of the line?)
2. There are no n or stat values reported in the text or main figure legends and this is necessary.
3. I have questions about many of the images. For example - in Figure 1A, the scale bars are different for WT and csp mutants. Does this mean the SCs are different sizes? They look the same in the images, but with different size scale bars, that suggests they are different.
4. In Figure 2H, the authors report that csp mutants lose specifically small diameter axons but don't explain why that might be.
5. For this manuscript, the authors focus solely on PLLn SCs. However, motor SCs must also express sil. Are there defects with these SCs as well?
6. A critical piece of data missing is expression of sil in SCs. I appreciate it is ubiquitously expressed. However, given the superficial location of the PLLn SC expression would be easy to see. Alternatively, is sil found in SC RNAseq databases?
7. In the supplied movies, arrows would be very helpful to denote what the reader should be looking at. Additionally, some movies would benefit from other transgenes to label cells - including axons and/or SCs. It's hard to know that the cells being imaged are 100% SCs with no other markers as confirmation.
8. To rescue the phenotype, the authors use a pTol2-sox10:sil-P2A-mcherry-CaaX construct to drive sil in SCs. However, there is no supporting data that this construct actually rescued. Confirmation of sil expression is needed.
9. Along these same lines, higher resolution images of the SCs in Figure 3 are needed.
10. I'm also confused when looking at Figure 3. You show a complete rescue of myelination in csp mutants injected with sox10:sil. This is crazy given the study is transient and mosaic. But then in the images in F-G, you only see single cells rescued along the PLLn. How does that work if sil is required cell autonomously in SCs and you're only getting rescue in a small number of SCs in any given larva?
11. Some images are lacking scale bars, Figure 4, and they also don't describe where or how cells were quantified.
12. When assaying the role of SC division using aphidicolin, the authors state they observed an expected reduction in neuron number. Is this because PLLg neurons are still dividing and are thus affected, or is it because you are halting division of SCs?
13. Interestingly, in these same studies, the authors state that SCs can still myelinate nerves. However, is the myelin complete along the axons?
Discontinuous? Are they internodes longer since there are fewer SCs?
14. I'm confused between the link of sil and cAMP. The authors don't describe a clear mechanism, and this is needed to follow their reasoning.
15. As above, I'm wary of the Rac rescue experiments. How do they know their rescue worked?
16. Figure 9 and 11, and many others, really need a SC markers.
For these reasons, the work feels incomplete and premature. The question is interesting but the manuscript as it stands right now is not convincing.

Reviewer 2

Advance summary and potential significance to field

"Timely SC division during migration drives peripheral myelination in vivo via Laminin/cAMP pathway" by Mikdache, Boueid et al provides new insights into the mechanisms of Schwann cell development and myelination along peripheral nerves in zebrafish. The study focuses on how cell division of Schwann cells during their migration along axons is important for their differentiation and myelination, and the data indicate that activation of cAMP signalling and the expression of Laminin can reduce the effects of impaired proliferation, placing these pathways downstream of Schwann cell proliferation in the transition to myelination. There are interesting data in the manuscript and upon revision I think that this manuscript will make a contribution to the field. This is because it has remained unclear whether it is proliferation, the timing thereof, or simply the number of Schwann cells along a nerve that is the key regulator of the transition to myelination.

Comments for the author

The strongest aspects of this manuscript lie in the analysis of the csp mutant which disrupts the mitotic spindle protein Sil, and in the cell-type specific rescue experiments that point towards roles in Schwann cells for Sil, which helps support the premise that the timing of Schwann cell proliferation is key to the transition to myelination. Although there are a lot of data in this manuscript, the depth of analyses across distinct experiments is not equitable, and at times the flow of the narrative was difficult to deconstruct, but both of these issues should be possible to address with experimental and text revisions.

Major points.
1. Throughout all of the analyses across multiple experiments, I think it would be very useful/ essential for the authors to show axon number with respect to axon diameter, binned, as is typical for the field in much smaller ranges and not simply as % of axons above and below one cut-off diameter. This would allow us to see much more clearly the effect on axon number, axon diameter etc. and relate those observations to myelination status in various experiments. These data will be to hand already.
2. It would be important to provide consistent depth of analyses across the key experiments, csp mutants forskolin rescues, laminin rescues, and also the laminin rescue of the cell division blocking experiment. This would include analyses of the TEM data, Including axonal analysis as point 1, and various time-lapse analysis of cell division. It would be advisable to carry out analysis of mitosis per se (pH3 staining) for key experiments, where it has not yet been carried out, e.g. to show that it is not affected in the laminin rescues.
3. While the genetic analyses are generally strong and appropriate experimental tools used, the aphidicolin data need to be strengthened. It would be important to know how specific the treatments are on animals at all the stages examined, by assessing the effect on proliferation (pH3 staining), but also to assess for potential toxicity, TUNEL labelling for example. I was quite surprised to see that the later treatments of aphidicolin reduced axon (and neuron?) number so severely. Wouldn't most neurons and axons have been born and extended their axons prior to 45 hpf? Why would proliferation (n the PLLg?) have had such a strong effect in a 9 hour window (45-54 hpf) on axon number? I fear that this later dataset could be particularly confounded.
Indeed, I am generally quite confused by this analysis at the later stage, and am not sure it adds much positive to the overall story. In any case, it would be important to provide much more assurance that the effects seen are specific to a disruption of mitosis. The fact that some axons are myelinated (real number needs to be clear as per point 1), may reflect the fact that some radial sorting and myelination started before these late treatments even went on. Also, myelination continues along the nerve for greatly extended periods of time, and most proliferation actually occurs independently of migration. The conclusion that proliferation of early migrating cells is the key to all myelination and that later proliferation is not seems very unlikely to hold true. My advice would be to cut this analysis. Nonetheless, a rigorous assessment of aphidicolin is required for the earlier stage analyses, although there it is reassuring that myelination can be rescued.
4. The order of the narrative is at times hard to follow. This may be less of an issue if my suggestion regarding later aphidicolin experiments is taken on board, but if the authors do validate those better and keep the data in, I would suggest that this part go at the end. Irrespective of that, a schematic showing SC development proliferation, migration, myelination along the nerve and

where the molecular factors come in to play would be useful. Also, with respect to the narrative, I'm not sure I quite agree with the linking of proliferation and the cAMP signalling and the Laminin effects. The rescue experiments carried out are very nice, but to me they simply show that both of these pathways can function downstream of proliferation and actually, quite strikingly, can over-ride extensive disruption to proliferation. This is a different interpretation to that given which is that proliferation drives these steps. I would argue almost the opposite. proliferation comes before but as the rescue experiments show, it is not strictly necessary. Again, filling out the phenotypic analyses as per point 1 may clarify things, e.g. if those factors do in fact drive proliferation prior to myelinaton. The current data suggest not, (panel M in laminin rescue of csp), but the key (few in number) cells that myelinated maybe did divide? (Data not there, asper point 1, for aphidicolin rescue) In any case, some thought should be given to interpretation
5. A minor point, but the authors should presumably be speaking of cytokinesis rather than mitosis when describing the dynamics of cells dividing in under ten minutes in their controls. Certainly all the phases of mitosis don't happen in <10 minutes.

Reviewer 3

Advance summary and potential significance to field

This manuscript from Mikdache et al. nicely characterizes the loss of Sil in Schwann cells (SCs) and the influence of its function in radial sorting and myelination in the posterior lateral line nerve (pLLn). Understanding the interplay between cell proliferation and the cellular rearrangements that drive SC sorting and wrapping is a critically important and complex question within the field of neurodevelopment. Here, the authors have time-lapse data showing SC dynamics, elegantly combined with transmission electron microscopy (TEM) data to precisely investigate axon number and SC developmental state. These data are generally rigorous and convincing. The discovery that cells with reduced proliferation also have reduced Laminin expression is especially critical for the field, given the bimodal role that Laminin has in different stages of SC development. This paper is a tour de force of experiments on the whole, and I am very enthusiastic about this part of the paper and believe it is well done.

The concept of temporal control of cell division and myelination in SC has potential to be intriguing, given prior findings that oligodendrocytes have a similarly narrow window in CNS myelination. However, that argument is less clear and I am not sure the data are convincing enough to support that idea. I also don't think that part of the story is necessary to understand the Sil and Laminin story, so fleshing out those data to support the arguments might compose a separate study. If left in this paper, I recommend toning down the argument within the text, as it would need more data, and focus the story here on csp-/- and implications with cAMP and Laminin, particularly how this interacts with the Gpr126-mediated cAMP pathway.

Comments for the author

Data and interpretation:
1. One issue I have with this paper's arguments overall is that it often treats SC development too discretely by chronological time. For instance, Fig. 1 suggests that radial sorting is an event occurring between 48-72 hpf. Current data from other sources show sorted cells are apparent at 48 hpf, and myelinated cells are present at 3 dpf. So, the active processes of sorting and myelination must happen before 48 and 72 hpf, respectively, and of course continue beyond those timepoints as well for other axons. Additionally, those data came from a single consistent position in the anterior-posterior axis, rather than the entire nerve at once. This becomes a problem when the authors argue that a certain time period represents migration/division and not a later time period given that they are scoring the entire animal at times. This also is a guiding principle for their drug treatments but I believe this leads to some over-interpretation.
So, to support some of the claims the authors are making about temporal windows for division, some sort of single-cell labeling and tracking should be done. I would like to know if there are migratory cells later in development, the actual window in which single cells migrate and divide, whether these windows occur at different chronological times, etc. This reviewer appreciates how challenging it would be to investigate sorting in this context - it is, of course, extremely difficult to know with any confidence that a labeled cell via fluorescence corresponds to a sorted cell via TEM.

However, a baseline level of fluorescence live-imaging analysis, combined with mbp expression (via double transgene perhaps?), would bolster the claim about temporal windows of development if the authors keep this argument in their paper.

2. While the data regarding Laminin are generally well done and convincing, I would restate some of the arguments related to figures 8-9. First, the conclusion on p. 10 lines 233-235 is slightly overstated. These experiments shows simply that csp-/- SC are still capable of activating the transcriptional program for terminal differentiation. It doesn't indicate that the transcription factors aren't entering the nucleus normally in csp-/- without cAMP - this might still be happening. I would leave it at a sufficiency argument. Second, the argument on p. 11 lines 264-265 could be better synthesized with previous data showing that laminin overexpression can also deplete cAMP (likely dependent on Gpr126 signaling state). While Laminin mutants have sorting defects, Laminin overexpression can prevent cAMP accumulation, and potentially terminal gene expression/differentiation without wrapping. So this should be softened in the results and included in the discussion section, because I disagree with the overstated "novel on/off mechanism" claim in the final sentence.

3. Aside from the role of Sil, I am puzzled by the reduction in total SC from 48-72 hpf in wild-type (Fig. 4B). This would be opposed to the theoretical idea that the expansion of axons in the pLLn would need additional SC for myelination
(as all axons will eventually be myelinated) as well as data showing increased Mbp expression as zebrafish larvae age. Is this a consequence of differentiated cells no longer expressing foxd3:gfp, or are there truly fewer SC overall? Could these data be corroborated by counting the SC present in TEM? If there are truly fewer cells, where are they going? This seems antithetical to the argument that SC proliferation in a critical window is important for myelination, as clearly the sibling SCs are able to myelinate a "normal" extent while the overall number of cells is decreasing. It would be good to have these data and to discuss in the context of their model.

4. Some data appear incorrectly represented and should be re-analyzed appropriately, though I am not sure if that will change the interpretations.
Fig. 2H, 5H, and 7E are showing all axons in the nerve binned into either > or <0.4 microns in diameter. This means the data are paired proportions and should be represented in a stacked bar graph to 100%, rather than points in different categories. Additionally, I think a proportional test, potentially Fisher's Exact, might be more appropriate than whatever is used (ideally the test should be reported in the figure captions throughout).

5. It is unfortunate that csp-/- embryos die at 5 dpf as that precludes answering some interesting questions. Is it possible to knockout Sil specifically in SC? These animals would potentially survive to allow later analysis (addressing delay vs. block) and help answer some interesting autonomy questions that are not directly addressed in the rescue experiments. Transplants with mosaic analysis could also potentially work (though technically challenging and less likely to ensure survival).

Organization and presentation:
1. I would prefer to see data on Schwann cell numbers in wild-type and csp-/- at the very beginning of the paper rather than in Fig. 4 (potentially with additional markers, as noted below about my confusion re: foxd3+ decreasing).
The first several figures have an alternative model that could be explained by few to no SC populating the PLLn. Fig. 4 demonstrates that SC numbers are halved at 48 hpf and similar at 72 hpf in csp -/- relative to sibs, so putting that information first will support the written interpretations better. As an example, I was at first unimpressed by Mbp expression by the single labeled csp+
cell in the rescue experiment in Fig. 3 because I didn't know how many other csp-/-; foxd3:gfp cells were around.

2. Fig. 6 seems redundant to Fig. 1. If there are differences between the two they are too subtly presented for the reader to understand. The in-text arguments are also very similar. These should be combined, or the arguments between the two made more clear.

3. While I understand that data values are reported in a supplementary file, the scientific arguments would be better supported by reporting mean +/- standard deviation parenthetically in-text. When the text says “significant increase” the scope of difference is not clear without those data. Additionally, standard deviation is more intuitive and appropriate to report on graphs than standard error of the mean. At minimum report the SD in the text or change the graphs to show SD.
Additional minor points:
p. 6 line 133, “cells” is missing from Schwann cells
Ages of larvae are missing in Fig 1.
Fig. 3 needs a control image to correspond to the graph.

First revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:
In this manuscript by Mikdache et al., the authors describe a role for sil, a spindle pole protein, in Schwann cell division and myelination. Using the strengths of the zebrafish model, the authors show that SCs fail myelinate peripheral axons in sil mutant larvae. However, this defect can be rescued by cAMP administration or overexpression of laminin in both muscle and SCs. This work is interesting in that we still don't fully understand the cellular and molecular mechanisms that mediate peripheral myelination, and this paper describes a role for sil in this process.

Reviewer 1 Comments for the Author:
This is an interesting manuscript looking at the role of sil in SC development along the PLLn. While the paper includes many techniques, there are many missing links, poorly described experiments, and confusing data that reduce my enthusiasm. I am also confused how a mutation in sil can lead to a cAMP/Laminin deficiency, and the paper doesn't go into a clear discussion of a mechanism. Also, there are many experiments that need SC markers in the background and there is a complete lack of reporting of n and statistics in the text and main figure legends (although they are in the supplemental legends). For these reasons, the work feels incomplete. Below are my comments:

1. There are several instances where the text needs editing - places where words are left out (like like 79 - "we took advantage of the Tg(foxd3:gfp)." Do you mean to say you took advantage of the line?)

We have added the word “line” in several instances where it was missing.

2. There are no n or stat values reported in the text or main figure legends and this is necessary.

Given that the previous draft was way too elaborated, there was no room for n and stats in the main text and so we were forced to add them in a supplementary file.
We have now modified the text and figures according to reviewers’ suggestions and we include all n and stats in the main text (text and figure legends).

3. I have questions about many of the images. For example - in Figure 1A, the scale bars are different for WT and csp mutants. Does this mean the SCs are different sizes? They look the same in the images, but with different size scale bars, that suggests they are different.

We apologize for this error, scale bars were supposed to be 25 and 20 um respectively, so we have standardized both to 20 um. This has now been rectified.

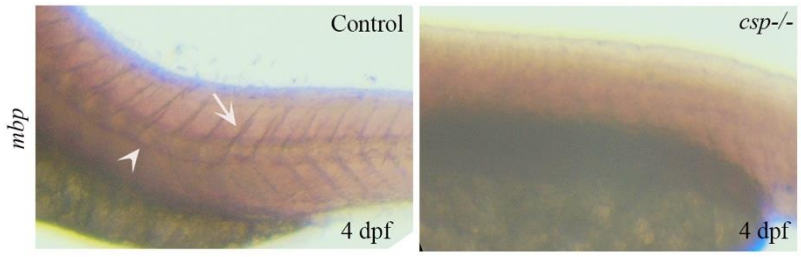
4. In Figure 2H, the authors report that csp mutants lose specifically small diameter axons but don't explain why that might be.

We have now added a new data showing the distribution of axons per diameter in the different experimental conditions. It is possible that some small caliber axons do extend more later than big caliber ones and/or small caliber axons are more fragile in csp mutants. It is also possible that

reduced neuronal numbers in the PLLg reflects a reduction in respective small caliber axons. We do not know exactly why this happens.
Importantly, the axons of bigger caliber that are supposed to be myelinated (≥0.4 μm) are present in the right proportions in these mutants so the lack of myelin do not result from lack of high caliber axons. This is the main message presented here.

5. For this manuscript, the authors focus solely on PLLn SCs. However, motor SCs must also express sil. Are there defects with these SCs as well?

Yes, we can also see a significant reduction in mbp expression along spinal cord motor axons (figure below). Arrowhead represents mbp expression along PLLn and arrow represents mbp expression along motor axons in control that is significantly reduced in csp mutants.

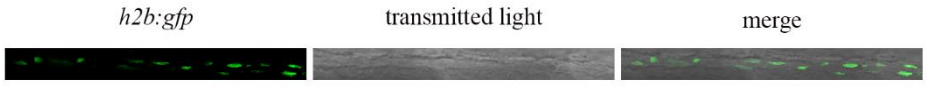


6. A critical piece of data missing is expression of sil in SCs. I appreciate it is ubiquitously expressed. However, given the superficial location of the PLLn, SC expression would be easy to see. Alternatively, is sil found in SC RNAseq databases?

We could not really pinpoint its expression in SC since it is ubiquitously expressed and enriched in the nervous system.
As suggested, we can refer to https://snat.ethz.ch/search.html?q=sil SC database of mice development.

7. In the supplied movies, arrows would be very helpful to denote what the reader should be looking at. Additionally, some movies would benefit from other transgenes to label cells - including axons and/or SCs. It's hard to know that the cells being imaged are 100% SCs with no other markers as confirmation.

Even though all data from movies are highlighted and denoted in figures, we have now added arrows in all movies.
As for the ones that do not show SC' markers we refer to transmitted light images to ensure that these cells sit in the PLLn (figure below). This information has now been added to the main text (materials and methods section).



8. To rescue the phenotype, the authors use a pTol2-sox10:sil-P2A-mcherry-CaaX construct to drive sil in SCs. However, there is no supporting data that this construct actually rescued. Confirmation of sil expression is needed.

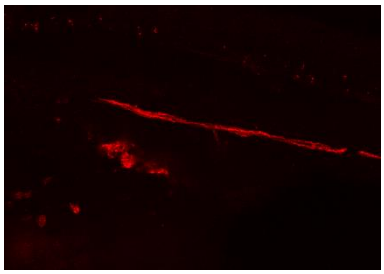
We refer to the expression of mcherry since the construct has a P2A crosslink; mcherry expression MUST follow sil one. In other words, there is NO mcherry expression without sil one.
Unfortunately, we do not have an antibody against sil to check its expression. The carac1 (rac1V12) construct is a well-established one (Boueid et al., 2020) and we used it previously to rescue radial sorting/myelin defect in elmo1 mutant (Mikdache et al., 2019).

9. Along these same lines, higher resolution images of the SCs in Figure 3 are needed.

Figure 3 includes high resolution images for myelinated axons as for TEM. The fluorescent ones are highlighted by co-localized Mbp expression.

10. I'm also confused when looking at Figure 3. You show a complete rescue of myelination in csp mutants injected with sox10:sil. This is crazy given the study is transient and mosaic. But then in the images in F-G, you only see single cells rescued along the PLLn. How does that work if sil is required cell autonomously in SCs and you're only getting rescue in a small number of SCs in any given larva?

When injected, we normally obtain a mosaic expression of mcherry in few SCs along the PLLn. In our first experiments, we analyzed over several hundred embryos to obtain only three showing continuous mcherry expression along the PLLn in the area analyzed so we chose these embryos for further TEM analysis (figure below). This information has now been added to the main text in the results section.



11. Some images are lacking scale bars, Figure 4, and they also don't describe where or how cells were quantified.

We have now added a scale bar for figure 2 (previous figure 4) in the merge still (third from top, left). We describe in the text (materials and methods section) where and how Schwann cells have been counted and quantified.

12. When assaying the role of SC division using aphidicolin, the authors state they observed an expected reduction in neuron number. Is this because PLLg neurons are still dividing and are thus affected, or is it because you are halting division of SCs?

We have now scrapped all Aphidicolin analysis according to reviewers 2 and 3 suggestion. However, to answer this question, it is important to note that neurons do not divide but progenitors do; in our analysis there are more neurons added to the PLLg between 2 and 3 dpf (Mikdache et al., 2019), they only reach their final number at 3 dpf, at least we can firmly say that there are no more neurons added to the PLLg between 3 and 5 days.
45-72 hpf aphidicolin treatment would definitely affect the number of axons as more neurons are added within this timeframe.

13. Interestingly, in these same studies, the authors state that SCs can still myelinate nerves. However, is the myelin complete along the axons?
Discontinuous? Are they internodes longer since there are fewer SCs?

We have not looked at myelin in longitudinal sections, as this is beyond the scope of this analysis. Other studies have looked at the importance of SC' numbers in radial myelin extension.

14. I'm confused between the link of sil and cAMP. The authors don't describe a clear mechanism, and this is needed to follow their reasoning.

Sil controls timely cell division and by doing so it controls the timely expression of at least one key radial sorting/myelin driver such as Laminin. In $csp^{-/-}$ mutant, Laminin expression is significantly reduced at a critical time when its polymerization is needed to set up the process of SC differentiation. Hence, the link here is between sil and Laminin expression and not sil and cAMP per se. The latter is dependent on Laminin /Gpr126 activation. When Laminin or Gpr126 is missing,

then cAMP activity is downregulated and peripheral myelination is altered. This has been discussed in the paper (results and discussion).

15. As above, I'm wary of the Rac rescue experiments. How do they know their rescue worked?

This construct as outlined above has been tested in previous work and it does rescue a radial sorting defect (Mikdache et al. 2019).

16. Figure 9 and 11, and many others, really need a SC markers.

What these images show is Laminin expression within the muscles which is the source of Laminin in this case (green labeling) and the Laminin secreted towards the PLLn (red labeling) that sits between these muscles. This has been explicitly explained in the text and has also been shown and demonstrated by Monk's lab (Petersen et al. 2015). SC in figure 11 (now figure 7) are highlighted by Mbp co-expression.

Mikdache et al., 2019. Elmo1 function, linked to Rac1 activity, regulates peripheral neuronal numbers and myelination in zebrafish. Cellular and Molecular life sciences. 77, 161-177 (2020)

Boueid MJ et al, 2020. Rho GTPases Signaling in Zebrafish Development and Disease. Cells. 2020 Dec 8;9(12):2634. Doi: 10.3390/cells9122634.

Petersen et al. 2015. The adhesion GPCR GPR126 has distinct, domain-dependent functions in Schwann cell development mediated by interaction with laminin-211. Neuron 2015 Feb 18;85(4):755-69.

For these reasons, the work feels incomplete and premature. The question is interesting but the manuscript as it stands right now is not convincing.

Reviewer 2 Advance Summary and Potential Significance to Field:
"Timely SC division during migration drives peripheral myelination in vivo via Laminin/cAMP pathway" by Mikdache, Boueid et al provides new insights into the mechanisms of Schwann cell development and myelination along peripheral nerves in zebrafish. The study focuses on how cell division of Schwann cells during their migration along axons is important for their differentiation and myelination, and the data indicate that activation of cAMP signalling and the expression of Laminin can reduce the effects of impaired proliferation, placing these pathways downstream of Schwann cell proliferation in the transition to myelination. There are interesting data in the manuscript and upon revision I think that this manuscript will make a contribution to the field. This is because it has remained unclear whether it is proliferation, the timing thereof, or simply the number of Schwann cells along a nerve that is the key regulator of the transition to myelination.

Reviewer 2 Comments for the Author:
The strongest aspects of this manuscript lie in the analysis of the csp mutant which disrupts the mitotic spindle protein Sil, and in the cell-type specific rescue experiments that point towards roles in Schwann cells for Sil, which helps support the premise that the timing of Schwann cell proliferation is key to the transition to myelination. Although there are a lot of data in this manuscript, the depth of analyses across distinct experiments is not equitable, and at times the flow of the narrative was difficult to deconstruct, but both of these issues should be possible to address with experimental and text revisions.

Major points.
1. Throughout all of the analyses across multiple experiments, I think it would be very useful/ essential for the authors to show axon number with respect to axon diameter, binned, as is typical for the field in much smaller ranges and not simply as % of axons above and below one cut-off diameter. This would allow us to see much more clearly the effect on axon number, axon diameter etc. and relate those observations to myelination status in various experiments. These data will be to hand already.

First, we would like to thank reviewer 2 for his constructive reviewing that helped us improve the paper. We have now added this data showing the distribution of axons according to their diameter (0.1 um range).
The reason why we present the percentage of axons above and below the 0.4 cut off diameter is the fact that the smallest axon to be myelinated at 3 dpf has a diameter of 0.4.
We now keep both data.

2. It would be important to provide consistent depth of analyses across the key experiments, csp mutants, forskolin rescues, laminin rescues, and also the laminin rescue of the cell division blocking experiment. This would include analyses of the TEM data, Including axonal analysis as point 1, and various time-lapse analysis of cell division. It would be advisable to carry out analysis of mitosis per se (pH3 staining) for key experiments, where it has not yet been carried out, e.g. to show that it is not affected in the laminin rescues.

We have now added new data including total number of Schwann cells, number of PH3+, ratio of PH3+/SCs, time to divide and axonal analysis in all experimental conditions.

3. While the genetic analyses are generally strong and appropriate experimental tools used, the aphidicolin data need to be strengthened. It would be important to know how specific the treatments are on animals at all the stages examined, by assessing the effect on proliferation (pH3 staining), but also to assess for potential toxicity, TUNEL labelling for example. I was quite surprised to see that the later treatments of aphidicolin reduced axon (and neuron?) number so severely. Wouldn't most neurons and axons have been born and extended their axons prior to 45 hpf? Why would proliferation (n the PLLg?) have had such a strong effect in a 9 hour window (45-54 hpf) on axon number? I fear that this later dataset could be particularly confounded.
Indeed, I am generally quite confused by this analysis at the later stage, and am not sure it adds much positive to the overall story. In any case, it would be important to provide much more assurance that the effects seen are specific to a disruption of mitosis. The fact that some axons are myelinated (real number needs to be clear as per point 1), may reflect the fact that some radial sorting and myelination started before these late treatments even went on. Also, myelination continues along the nerve for greatly extended periods of time, and most proliferation actually occurs independently of migration. The conclusion that proliferation of early migrating cells is the key to all myelination and that later proliferation is not seems very unlikely to hold true. My advice would be to cut this analysis. Nonetheless, a rigorous assessment of aphidicolin is required for the earlier stage analyses, although there it is reassuring that myelination can be rescued.

It is important to note that progenitors still divide in the PLLg and more neurons are added to the PLLg between 48 and 72 hpf (Mikdache et al., 2019). No more neurons are added between 3 and 5 dpf, the latest point analyzed in the lab. So, when blocking cell division from 45hpf onwards, we are altering the number of neurons and therefore extended axons along the PLLn. The area where we analyze myelination and part of Schwann cell numbers/PH3+ has no radial sorting at 48 hpf. However, we have cut out the pharmacological analysis, as this would be part of another study and we focus on the genetic analysis in this paper as suggested by reviewer 3 too.

Mikdache et al., 2019. Elmo1 function, linked to Rac1 activity, regulates peripheral neuronal numbers and myelination in zebrafish. Cellular and Molecular life sciences. 77, 161-177 (2020)

4. The order of the narrative is at times hard to follow. This may be less of an issue if my suggestion regarding later aphidicolin experiments is taken on board, but if the authors do validate those better and keep the data in, I would suggest that this part go at the end. Irrespective of that, a schematic showing SC development, proliferation, migration, myelination along the nerve and where the molecular factors come in to play would be useful. Also, with respect to the narrative, I'm not sure I quite agree with the linking of proliferation and the cAMP signalling and the Laminin effects. The rescue experiments carried out are very nice, but to me they simply show that both of these pathways can function downstream of proliferation and actually, quite strikingly, can over-ride extensive disruption to proliferation. This is a different interpretation to that given, which is that proliferation drives these steps. I would argue almost the opposite. proliferation comes before, but as the rescue experiments show, it is not strictly necessary. Again, filling out the phenotypic analyses as per point 1 may clarify things, e.g. if those factors do in fact drive proliferation prior to

myelinaton. The current data suggest not, (panel M in laminin rescue of csp), but the key (few in number) cells that myelinated maybe did divide? (Data not there, asper point 1, for aphidicolin rescue) In any case, some thought should be given to interpretation

We have now cut out the pharmacological analysis.
We have also changed the order of the results (as suggested by reviewer 3 too) and added new results to fill out the phenotypic analyses in all experimental conditions.
We have modified the discussion accordingly.

5. A minor point, but the authors should presumably be speaking of cytokinesis rather than mitosis when describing the dynamics of cells dividing in under ten minutes in their controls. Certainly all the phases of mitosis don't happen in <10 minutes.

We have now modified the text accordingly.

Reviewer 3 Advance Summary and Potential Significance to Field:
This manuscript from Mikdache et al. nicely characterizes the loss of Sil in Schwann cells (SCs) and the influence of its function in radial sorting and myelination in the posterior lateral line nerve (pLLn). Understanding the interplay between cell proliferation and the cellular rearrangements that drive SC sorting and wrapping is a critically important and complex question within the field of neurodevelopment. Here, the authors have time-lapse data showing SC dynamics, elegantly combined with transmission electron microscopy (TEM) data to precisely investigate axon number and SC developmental state. These data are generally rigorous and convincing. The discovery that cells with reduced proliferation also have reduced Laminin expression is especially critical for the field, given the bimodal role that Laminin has in different stages of SC development. This paper is a tour de force of experiments on the whole, and I am very enthusiastic about this part of the paper and believe it is well done.

The concept of temporal control of cell division and myelination in SC has potential to be intriguing, given prior findings that oligodendrocytes have a similarly narrow window in CNS myelination. However, that argument is less clear and I am not sure the data are convincing enough to support that idea. I also don't think that part of the story is necessary to understand the Sil and Laminin story, so fleshing out those data to support the arguments might compose a separate study. If left in this paper, I recommend toning down the argument within the text, as it would need more data, and focus the story here on csp-/- and implications with cAMP and Laminin, particularly how this interacts with the Gpr126-mediated cAMP pathway.

Reviewer 3 Comments for the Author:
Data and interpretation:

1. One issue I have with this paper’s arguments overall is that it often treats SC development too discretely by chronological time. For instance, Fig. 1 suggests that radial sorting is an event occurring between 48-72 hpf. Current data from other sources show sorted cells are apparent at 48 hpf, and myelinated cells are present at 3 dpf. So, the active processes of sorting and myelination must happen before 48 and 72 hpf, respectively, and of course continue beyond those timepoints as well for other axons. Additionally, those data came from a single consistent position in the anterior-posterior axis, rather than the entire nerve at once. This becomes a problem when the authors argue that a certain time period represents migration/division and not a later time period, given that they are scoring the entire animal at times. This also is a guiding principle for their drug treatments but I believe this leads to some over- interpretation.

So, to support some of the claims the authors are making about temporal windows for division, some sort of single-cell labeling and tracking should be done. I would like to know if there are migratory cells later in development, the actual window in which single cells migrate and divide, whether these windows occur at different chronological times, etc. This reviewer appreciates how challenging it would be to investigate sorting in this context - it is, of course, extremely difficult to know with any confidence that a labeled cell via fluorescence corresponds to a sorted cell via TEM. However, a baseline level of fluorescence live-imaging analysis, combined with mbp expression (via double transgene perhaps?), would bolster the claim about temporal windows of development if the authors keep this argument in their paper.

We would like to thank reviewer 3 for his valuable comments and constructive reviewing that helped us improve this paper.

As suggested by reviewer 2 too, we have scrapped all pharmacological analysis in this study as this would be part of another study. We focus on the genetic analysis (sil mutant) and the link between timely division, Laminin expression and activity for myelination.
As for chronological SC division and development, it is certain from our studies and others (Lyons et al. 2005, Raphael et al.,2011) that SC divide during migration and during radial sorting. What is the role of these two particular divisions is left for another study. It is true that radial sorting and myelination do not occur at the same time along the AP axis and one has to be careful in interpreting results. One way of studying cell division, radial sorting and myelination is to choose a specific location along the AP axis and analyze the data accordingly. For example, the area where we chose to study myelination by TEM shows no radial sorting at 48 hpf. We leave these data out now as part of another study.

Lyons et al., 2015. Erbb3 and erbb2 are essential for Schwann cell migration and myelination in zebrafish. Curr Biol. 2005 Mar 29;15(6):513-24.
Raphael et al., 2011. ErbB signaling has a role in radial sorting independent of Schwann cell number. Glia. 2011 Jul;59(7):1047-55.

2. While the data regarding Laminin are generally well done and convincing, I would restate some of the arguments related to figures 8-9. First, the conclusion on p. 10 lines 233-235 is slightly overstated. These experiments shows simply that csp-/- SC are still capable of activating the transcriptional program for terminal differentiation. It doesn't indicate that the transcription factors aren't entering the nucleus normally in csp-/- without cAMP - this might still be happening. I would leave it at a sufficiency argument. Second, the argument on p. 11 lines 264-265 could be better synthesized with previous data showing that laminin overexpression can also deplete cAMP (likely dependent on Gpr126 signaling state). While Laminin mutants have sorting defects, Laminin overexpression can prevent cAMP accumulation, and potentially terminal gene expression/differentiation without wrapping. So this should be softened in the results and included in the discussion section, because I disagree with the overstated "novel on/off mechanism" claim in the final sentence.

We might have been misunderstood, the FSK treatment is to test whether the transcriptional activity is completely lost in sil mutant following a long delay in M2 phase or whether this could still be activated following cAMP activation that is upstream of transcriptional activity.
We have amended this part.
We have now added new data showing that Laminin overexpression as well as FSK treatment would reduce proliferation, not only in sil mutants but also in controls. We have now scrapped the 'novel ON/OFF mechanism' and amended the text.

3. Aside from the role of Sil, I am puzzled by the reduction in total SC from 48-72 hpf in wild-type (Fig. 4B). This would be opposed to the theoretical idea that the expansion of axons in the pLLn would need additional SC for myelination (as all axons will eventually be myelinated) as well as data showing increased Mbp expression as zebrafish larvae age. Is this a consequence of differentiated cells no longer expressing foxd3:gfp, or are there truly fewer SC overall? Could these data be corroborated by counting the SC present in TEM? If there are truly fewer cells, where are they going? This seems antithetical to the argument that SC proliferation in a critical window is important for myelination, as clearly the sibling SCs are able to myelinate a "normal" extent while the overall number of cells is decreasing. It would be good to have these data and to discuss in the context of their model.

We apologize for this error, we have now analyzed more embryos at different stages, and it is certain that the number of SCs do not significantly change in controls between 48 and 72 hpf. This has been amended, and data now show that these numbers are still reduced in sil mutants at 72 hpf reflecting the fewer number of axons too. However, the number of PH3 positive as well as the ratio of PH3+/SCs is comparable to controls suggesting that SC do exit mitosis and do not show signs of apoptosis following the delay in M2 phase contrary to neuronal cells.

4. Some data appear incorrectly represented and should be re-analyzed appropriately, though I am not sure if that will change the interpretations.
Fig. 2H, 5H, and 7E are showing all axons in the nerve binned into either > or <0.4 microns in diameter. This means the data are paired proportions and should be represented in a stacked bar graph to 100%, rather than points in different categories. Additionally, I think a proportional test, potentially Fisher's Exact, might be more appropriate than whatever is used (ideally the test should be reported in the figure captions throughout).

We have now added this data as suggested by reviewer 2 too. We also keep the previous data, the reason why we present the percentage of axons above and below the 0.4 cut off diameter is the fact that the smallest axon to be myelinated at 3 dpf has a diameter of 0.4.

5. It is unfortunate that csp-/- embryos die at 5 dpf as that precludes answering some interesting questions. Is it possible to knockout Sil specifically in SC? These animals would potentially survive to allow later analysis (addressing delay vs. block) and help answer some interesting autonomy questions that are not directly addressed in the rescue experiments. Transplants with mosai c analysis could also potentially work (though technically challenging and less likely to ensure survival).

Unfortunately, this kind of approach is not well developed in zebrafish. Future mice studies might answer these questions.

Organization and presentation:

1. I would prefer to see data on Schwann cell numbers in wild-type and csp-/- at the very beginning of the paper rather than in Fig. 4 (potentially with additional markers, as noted below about my confusion re: foxd3+ decreasing).
The first several figures have an alternative model that could be explained by few to no SC populating the PLLn. Fig. 4 demonstrates that SC numbers are halved at 48 hpf and similar at 72 hpf in csp -/- relative to sibs, so putting that information first will support the written interpretations better. As an example, I was at first unimpressed by Mbp expression by the single labeled csp+ cell in the rescue experiment in Fig. 3 because I didn't know how many other csp-/-; foxd3:gfp cells were around.

We have now changed the order of the results, we start off by showing the pattern of division and numbers.

2. Fig. 6 seems redundant to Fig. 1. If there are differences between the two, they are too subtly presented for the reader to understand. The in-text arguments are also very similar. These should be combined, or the arguments between the two made more clear.

They represent the pattern of division during radial sorting and during migration respectively. We have now merged these two into Figure 1.

3. While I understand that data values are reported in a supplementary file, the scientific arguments would be better supported by reporting mean +/- standard deviation parenthetically in-text. When the text says "significant increase" the scope of difference is not clear without those data. Additionally, standard deviation is more intuitive and appropriate to report on graphs than standard error of the mean. At minimum report the SD in the text or change the graphs to show SD.

The previous version was too elaborate with so many different results, there was no room for n and stats in the main text, now that we have removed the pharmacological analysis and re-organized the paper according to reviewers' comments we include these data values in the main text.

Additional minor points:

p. 6 line 133, "cells" is missing from Schwann cells
This has been amended accordingly.

Ages of larvae are missing in Fig 1.
The timing and length of timelapse recording is highlighted in movies' legends.

Fig. 3 needs a control image to correspond to the graph.
This has been added to Figure 4 now.

Second decision letter

MS ID#: DEVELOP/2022/200640

MS TITLE: Timely SC division drives peripheral myelination in vivo via Laminin/cAMP pathway

AUTHORS: Aya Mikdache, Marie-Jose Boueid, Emilie Lesport, Brigitte Delespierre, Julien Loisel-Duwattez, Cindy Degerny, and Marcel Tawk

I have now received all the referees reports on the above manuscript, and 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 are largely happy with your revisions and and we would like to publish a revised manuscript in Development, provided that the remaining referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response.

Reviewer 1

Advance summary and potential significance to field

In this manuscript by Mikdache et al., the authors describe a role for sil, a spindle pole protein, in Schwann cell division and myelination. Using the strengths of the zebrafish model, the authors show that SCs fail myelinate peripheral axons in sil mutant larvae. However, this defect can be rescued by cAMP administration or overexpression of laminin in both muscle and SCs. This work is interesting in that we still don't fully understand the cellular and molecular mechanisms that mediate peripheral myelination, and this paper describes a role for sil in this process.

Comments for the author

The authors have adequately addressed the previous round of reviewer concerns and it is a much improved paper.

Reviewer 2

Advance summary and potential significance to field

See original review

Comments for the author

The authors have significantly tidied up the manuscript by removing the pharmacological experiments blocking cell proliferation adn by extending the analysis of the core mutant under investigation. Results are presented in better detail and with more rigor. To me the manuscript is acceptable for publication now.

Reviewer 3

Advance summary and potential significance to field

This revised manuscript from Mikdache et al. is improved with respect to both clarify and rigor of scientific conclusions. In this paper, the authors link the cell division function of Sil via the csp-/- mutant to the canonical SC myelination program. This is important as it links cell division to development of the basal lamina, which is essential for SC differentiation. The authors' re-organization of the manuscript and inclusion of new data makes the story clearer, and the novelty of the scientific findings can have greater impact as a result. I only have minor comments remaining with respect to the text and communication.

The one finding that gave me pause was that forskolin decreases SC numbers, but could be attributable to how heightened cAMP via FSK can have detrimental effects. Given that the authors are demonstrating rescue of csp-/-, rather than decrease in SC number, I think this finding is robust even if the controls seem counterintuitive at first. The finding is also important for future studies using FSK.

Comments for the author

Minor points:
Fig. 2D y-axis confuses me, as I think it is a % and not a ratio. (Using my guesses at average values from the graph, for controls ~3 PH3+ / ~75 total = ~4% shown in the graph).
Fig. 3A'' label (with the double-prime '') would suggest that it is a variant of the image in Fig. 3A. However it seems to be a completely different micrograph as I cannot find that SC in both images. The image is good but should be labeled indicating it's a different image.
The heading "Sil is required to initiate radial sorting, myelin gene expression and axonal wrapping by SC via cAMP pathway" is still somewhat overstated. This experiment shows that FSK can rescue but it doesn't show that Sil mediates this pathway, just that this pathway is sufficient to rescue no matter the defects in csp-/-. If csp-/- mutants were demonstrated to have reduced cAMP (analogous to the demonstrated reduction in Laminin), then I would be more convinced, but that's not shown. So, rewording that heading in terms of rescue/sufficiency would be more prudent.

Second revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:
In this manuscript by Mikdache et al., the authors describe a role for sil, a spindle pole protein, in Schwann cell division and myelination. Using the strengths of the zebrafish model, the authors show that SCs fail myelinate peripheral axons in sil mutant larvae. However, this defect can be rescued by cAMP administration or overexpression of laminin in both muscle and SCs. This work is interesting in that we still don't fully understand the cellular and molecular mechanisms that mediate peripheral myelination, and this paper describes a role for sil in this process.

Reviewer 1 Comments for the Author:
The authors have adequately addressed the previous round of reviewer concerns and it is a much improved paper.

We would like to thank reviewer 1 for his helpful comments that helped us improve this paper.

Reviewer 2 Advance Summary and Potential Significance to Field:
See original review

Reviewer 2 Comments for the Author:
The authors have significantly tidied up the manuscript by removing the pharmacological experiments blocking cell proliferation adn by extending the analysis of the core mutant under

investigation. Results are presented in better detail and with more rigor. To me the manuscript is acceptable for publication now.

We appreciate the constructive reviewing of reviewer 2 that considerably improved the paper.

Reviewer 3 Advance Summary and Potential Significance to Field:
This revised manuscript from Mikdache et al. is improved with respect to both clarify and rigor of scientific conclusions. In this paper, the authors link the cell division function of Sil via the csp-/- mutant to the canonical SC myelination program. This is important as it links cell division to development of the basal lamina, which is essential for SC differentiation. The authors' re-organization of the manuscript and inclusion of new data makes the story clearer, and the novelty of the scientific findings can have greater impact as a result. I only have minor comments remaining with respect to the text and communication.

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Reviewer 3 Comments for the Author:
Minor points:
Fig. 2D y-axis confuses me, as I think it is a % and not a ratio. (Using my guesses at average values from the graph, for controls ~3 PH3+ / ~75 total = ~4% shown in the graph).

It is the percentage indeed since ratio values were reported to 100.
We have changed the title of the y axis in Figure 2, 5 and 7 and the corresponding text in the main manuscript (text and Figure legends).

Fig. 3A'' label (with the double-prime '') would suggest that it is a variant of the image in Fig. 3A. However it seems to be a completely different micrograph as I cannot find that SC in both images. The image is good but should be labeled indicating it's a different image.

This has been labeled differently now A''(2) and highlighted in Figure 3 legend as from a different control embryo.

The heading "Sil is required to initiate radial sorting, myelin gene expression and axonal wrapping by SC via cAMP pathway" is still somewhat overstated. This experiment shows that FSK can rescue but it doesn't show that Sil mediates this pathway, just that this pathway is sufficient to rescue no matter the defects in csp-/-. If csp-/- mutants were demonstrated to have reduced cAMP (analogous to the demonstrated reduction in Laminin), then I would be more convinced, but that's not shown. So, rewording that heading in terms of rescue/sufficiency would be more prudent.

This has now been changed to:
'Forcing cAMP activity, via Forskolin treatment, rescues radial sorting, myelin gene expression and axonal wrapping defects in csp$^{-/-}$ '

Third decision letter

MS ID#: DEVELOP/2022/200640

MS TITLE: Timely SC division drives peripheral myelination in vivo via Laminin/cAMP pathway

AUTHORS: Aya Mikdache, Marie-Jose Boueid, Emilie Lesport, Brigitte Delespierre, Julien Loisel-Duwattez, Cindy Degerny, and Marcel Tawk
ARTICLE TYPE: Research Article

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