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miR-124-regulated RhoG reduces neuronal process complexity via ELMO/Dock180/Rac1 and Cdc42 signaling

Kristin Franke, Wolfgang Otto, Sascha Johannes , Jan Baumgart, Robert Nitsch and Stefan Schumacher

Corresponding author: Stefan Schumacher, University of Ulm

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1st Editorial Decision 19 October 2011

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see, while there is an interest in the study there are also many concerns raised with it that I am afraid preclude publication at this stage. The referees find that further experiments are needed to demonstrate that RhoG is a direct target of miR-12, to validate the specificity of the RNAi effects, this included rescue experiments and quantifying the efficiency of the knockdown approach and that we need to better understand how RhoG affects dendritogenesis and axonogenesis. So while the study is potential interesting it is also too preliminary to consider publication in the EMBO Journal. Given these opinions from good experts in the field, I am afraid that I can't offer to consider the manuscript further at this stage.

Thank you in any case for the opportunity to consider this manuscript. I am sorry we cannot be more positive on this occasion, but I hope nevertheless that you will find our referees' comments helpful.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

In the present manuscript, Franke et al. investigate the function of the small GTPase RhoG in dendrite and axon development, as well as its regulation by miR-124. The authors show that, in contrast to previous published results, RhoG acts as a negative regulator of dendritic and axonal complexity. Expression of RhoG appears to be inhibited at the post-transcriptional level by miR-124, a microRNA which itself promotes dendritogenesis (but not axon development). Finally, the authors propose that the effects of RhoG on dendrite and axon development are mediated by different downstream signaling modules, the ELMO/DOCK180/Rac1 and Cdc42 signaling pathways, respectively.
This is a well thought out and technically solid study that adds yet another example of a microRNA-dependent mechanism that appears to be crucial for proper neuronal morphogenesis. Although a function of miR-124 in neuronal process development has been reported previously, the identification of a direct link to the cytoskeletal regulator RhoG, and the description of the relevant downstream signaling pathways would be significant. However, some of the key conclusions drawn by the authors are not fully supported by the presented experiments, as outlined in more detail below. Therefore, I feel that this manuscript has to be extensively revised before it can be considered for publication in the EMBO Journal.

Major concerns:
1. The validation of RhoG as a direct target for miR-124 has to be significantly improved. The only data presented are microRNA sensor assays, using GFP reporter constructs that only contain a partial RhoG 3'UTR (e.g. missing the endogenous polyadenylation signal). It would be important to show that inhibition of miR-124 leads to an increase in RhoG protein and/or mRNA expression in primary neurons. Additionally, quantitative reporter gene assays should be repeated with constructs encompassing the full-length RhoG 3'UTR as well as the respective mutants.
2. The observation that knockdown of RhoG leads to increased dendritogenesis/axonogenesis is interesting and challenges previous reports from the literature. To further substantiate these results, the authors have to unequivocally demonstrate the specificity of the RNAi effects, e.g. by performing rescue experiments by expressing an RNAi-resistant RhoG protein in the context of RhoG siRNAs.
3. The observation that different downstream signaling pathways mediate RhoG effects on dendritogenesis and axonogenesis is an important part of the paper that warrants further investigation. To more convincingly demonstrate that Rac1 is not required for dendritogenesis, the authors should knockdown Rac1 in the presence of RhoG. The lack of an effect of the RhoGF37A mutant is clearly not sufficient here.
4. The fact that increasing RhoG levels (e.g. by anti-miR-124) is sufficient to induce dendritogenesis, but not axonogenesis, is puzzling. As stated by the authors, a possible explanation could be that RhoGEFs are limiting specifically during axonogenesis, which would lead to the effect that excessive RhoG protein cannot be activated. However, this hypothesis is not supported by experimental evidence. One way to address this would be to assess the activation status of RhoG (e.g. GTP loading) under the different experimental conditions.

Minor concerns:
1. Dendritic complexity should be analyzed by Sholl analysis, which is a more accepted method that gives additional information about effects on branching and length.
2. The authors mention a previous study (Edbauer et al.) which failed to observe any dendritic phenotype upon miR-124 inhibition. It would be nice if the authors could recapitulate these findings in their own hands.
3. The efficiency and specificity of the RhoG knockdown constructs should also be assessed on the endogenous RhoG protein in primary hippocampal neurons.

Referee #2

The authors describe the effects the microRNA miR-124 on the expression of the GTPase RhoG, and identify RhoG as an inhibitor of neuritic branching both in cultured hippocampal neurons and in vivo. The data show that knockdown of RhoG induces an increase in dendritic and axonal

branching. Overexpression of either the WT or a constitutively active form of RhoG reduces dendritic branching, while only constitutively active RhoG affects axonal branching. The authors have further explored the role of RhoG in neurite development by combination of overexpression of mutants and knockdown by shRNA to indicate the involvement of the ELMO/Dock180/Rac1 and Cdc42 in the regulation of branching in axons and dendrites, respectively.
The finding that RhoG acts as a negative regulator of neuritic branching in hippocampal neurons Is quite interesting, and is in apparent contrast with previous studies showing positive stimulatory effects of RhoG on neurite outgrowth in the rat PC12 cell line and in SCG neurons.
Essential controls for the specificity of the downregulation of RhoG and other endogenous proteins in neurons are missing. Moreover, the rationale to explain some of the results on the function of the ELMO complex is not clear.

Two different shRNAs for RhoG have been used to downregulate endogenous RhoG, but the effects of these shRNAs on neuronal RhoG is not shown. Moreover, rescue experiments to prove the specificity of the RhoG shRNAs in neurons have not been included. Although rescue experiments in primary neurons may be particularly difficult, clear reduction of the endogenous neuronal protein either by biochemistry, or by immunofluorescence must be shown for both shRNAs.
These observations hold true for all the shRNA constructs used in the manuscript.

In addition, an estimate of the efficiency of the two shRNAs in knocking down RhoG should be included, by quantification from distinct experiments as the one shown in Fig. 2B.

Figure 4B: how do the authors explain the strong positive effect on branching observed in neurons overexpressing the RhoG-F37A and RhoG-G12V/F37A mutants that are unable to bind the ELMO complex? Wouldn't endogenous RhoG interacting with the ELMO complex be sufficient to keep branching normal?
In the same palel, it is shown that Rac1 KD increases axonal branching compared to control KD; but then Rac1 KD does not seem to significantly affect branching compared to the KD control + RFP: this part of the presentation of the data needs revision.

Along the same line, also some of the data presented in Figure 6B are confusing: in the first group of bars on the left, anti-miR-124 + RhoG KD is expected to decrease endogenous RhoG and therefore to increase branching, but it does not (only a small increase between first and fourth bars is shown, but is not indicated as significant). This indicates that the reproducibility of the effects on branching following alteration of RhoG levels is quite weak.

In Figure S3, what is axonal length? Does it represent the length of the longest axonal branch, or the total length of the axonal tree? This should be clearly specified in the text and legend.

Referee #3
This manuscript by Franke et al. is a study of the role of the GTPase RhoG in axonal and dendritic structure using in vivo and in vitro techniques. RhoG has previously been implicated in controlling apoptosis and engulfment of cell corpses. Here, the authors show that RhoG activity inhibits axonal and dendritic branching which, at least in vertebrates, has not been previously described. Indeed, in PC12 cells, RhoG was found to stimulate, not inhibit, axonal outgrowth. The authors also show that RhoG is a target of downregulation by a micro RNA, miR-124, and that miR-124 inhibits RhoG activity in repression of axonal and dendritic branching. The regulation of axon and dendrite branching is an important topic with broad interest, and the role of micro RNAs in neuronal development adds to interest and significance.

The authors use a nice combination of cell culture, in vivo electroporation, shRNA knockdown, and overexpression of mutant proteins to mechanistically dissect the roles of these molecules in axon and dendrite branching. A strength of the paper is the extensive use of epistasis analysis to demonstrate that the effects of one treatment requires the functions of downstream molecules. For example, the authors showed that activated RhoG G12V did not inhibit axon branching if combined with Rac1 knockdown; and that activated RhoG G12V expression reduced the amount of ectopic branching caused by miR-124 overproduction. Overall, the experiments are strong and the conclusions reached are consistent with the results.
The main findings of the paper are:

1) RhoG inhibits axonal branching via a DOCK180/ELMO complex and Rac1.
2) RhoG inhibits dendritic branching via Cdc42.
3) RhoG is a target of downregulation by miR-124 mediated by binding sites in the 3' UTR.
4) miR-124 downregulation of RhoG causes dendritic and axonal branching.

The last finding is particularly important, as the roles of micro RNAs in development, especially neuronal development, are in the beginning stages of being fleshed out.

While generally a strong manuscript, there are some issues that need to be addressed.
Comments:
1) I would recommend a restructuring of the manuscript. As it is, the authors first discuss downregulation of RhoG by miR-124, and then launch into a dissection of RhoG activity, and then come back to miR-124 regulation of RhoG in branching. I would recommend that all of the RhoG studies come first, and then the issue of what regulates RhoG (i.e. miR-124) be the last topic. In other words, Figure 1 could be placed between Figures 5 and 6. Thus, the manuscript would first discuss RhoG signaling in branching, and then discuss a regulator of RhoG. I think this flows better than splitting up the miR-124 data to the beginning and the end.

2) Is there an independent means to show that miR-124 is being "captured" by the anti-sense technique? A missing control here is treatment with a non-miR-124-specific anti-sense miRNA to show that the effect of antisense treatment was specific to miR-124 and not just general antisense RNA treatment.

3) The results that RhoG inhibits branching go against previous work on PC12 cells, which suggest a positive role. The authors discuss only work with vertebrate models, but there is a body of literature using flies and worms with the functional equivalent of RhoG called Mtl (mig-2-like) and Rac GTPases. In C. elegans and Drosophila, Mtl/mig-2 also inhibits axon branching, as mutants have extra axon branches (e.g. Ng et al., 2002; Lundquist et al., 2001; Struckhoff et al., 2003). These data would support the authors' argument that RhoG inhibits branching. They should be discussed.

4) The section on p. 8 dealing with the RhoG-F37A mutant protein was unclear. Are the authors saying that overexpression of F37A acts as a "dominant positive" independently of Rac1/ELMO/DOCK180? This should be explained more clearly. Also, in this section and on the Figure, it is unclear if the F37A mutant is used in all experiments (in the legend for Figure 5B, the last two treatments just say RhoG, but I think these are supposed to be RhoG-F37A).

5) I could not find a definition of pCLEG. I infer it is the vector used to overexpress molecules. It would be helpful to describe these experiments more succinctly in the figures, legends, and text.

6) There are statistical analyses that should be added in the figures. Figure 3B should compare kdcontrol with RhoG-kd1 and EGFP with EGFP+RhoG; and Figure 6D should compare miR-124 EGFP with miR-124 + RhoG G12V.

7) In the abstract, the last statement is a "discernible developmental phenotype". It might be more clear to say "to control axon and dendritic branching."

8) On p. 5, the mutations abolishing the 3' UTR binding sites should be referenced in the methods here, or described here.

9) On p. 7, "To finally proof the importance..." should be changed to something like "To further demonstrate the importance of..."

Resubmission 27 February 2012

Referee #1

Major concerns:
1. The validation of RhoG as a direct target for miR-124 has to be significantly improved.
The only data presented are microRNA sensor assays, using GFP reporter constructs that

only contain a partial RhoG 3'UTR (e.g. missing the endogenous polyadenylation signal). It would be important to show that inhibition of miR-124 leads to an increase in RhoG protein and/or mRNA expression in primary neurons. Additionally, quantitative reporter gene assays should be repeated with constructs encompassing the full-length RhoG 3'UTR as well as the respective mutants.
We now showed that the inhibition of miR-124 leads to an increase in RhoG protein in primary hippocampal neurons (new Figure 5G and new Supplementary Figure S8B). We repeated the quantitative reporter gene assays with a new sensor construct [EGFP-3´UTRfull(RhoG)] encompassing the full-length RhoG 3´UTR and including the endogenous polyadenylation signal (new Figure 5E and F). In addition, we cloned and analyzed the respective mutant EGFP-3´UTRfull(RhoG)-Mut4.1 in our sensor assay (new Figure 5E and F). The cloning details of both new constructs are described in the *Materials and methods*. The data generated with the old sensor constructs (old Figure 1D and E) is now transferred to the Supplement (now Supplementary Figure S8A). When comparing the quantifications of the sensor assay using the old sensor constructs EGFP-3´UTR(RhoG) and EGFP-3´UTR(RhoG)-Mut4.1 (new Supplementary Figure S8A) with the reporter assay employing the new sensor constructs EGFP-3´UTRfull(RhoG) and EGFP-3´UTRfull(RhoG)-Mut4.1 (new Figure 5E and F), almost identical results were obtained.

2. The observation that knockdown of RhoG leads to increased dendritogenesis/axonogenesis is interesting and challenges previous reports from the literature. To further substantiate these results, the authors have to unequivocally demonstrate the specificity of the RNAi effects, e.g. by performing rescue experiments by expressing an RNAi-resistant RhoG protein in the context of RhoG siRNAs.
We now better validated the specificity and efficiency of the RhoG-specific knockdown constructs (new Supplementary Figure S2). (1) We demonstrated that the RhoG-specific knockdown constructs RhoG-kd1 and RhoG-kd4 reduce the expression of endogenous RhoG in primary hippocampal neurons (new Supplementary Figure S2A and B). (2) We showed that RhoG-kd1 and RhoG-kd4 reduce the expression of overexpressed HA-tagged RhoG in hippocampal neurons (new Supplementary Figure S2C, D, E, and F). (3) We established the RhoG-specific rescue construct RhoGresist(kd4) (new Supplementary Figure S2C and D). (4) We quantified the Western blot data for knockdown documentation (new Figure 1B and C versus old Figure 2B). With these validated constructs we performed functional rescue experiments (new Figure 1A, C, and D). We found that, although the rescue construct HA-RhoGresist(kd4) did not express as well as HA-RhoG (new Supplementary Figure S2D), this construct nevertheless led to a rescue of RhoG function in the presence of the stronger knockdown construct RhoG-kd4 (new Figure 1A, C, and D). Since the weaker knockdown construct RhoG-kd1 only led to a reduction of approximately 40% of overexpressed HA-RhoG (Supplementary Figure S2F), a functional rescue in the presence of RhoG-kd1 could be achieved simply by overexpression of RhoG (new Figure 1A, C, and D).

3. The observation that different downstream signaling pathways mediate RhoG effects on dendritogenesis and axonogenesis is an important part of the paper that warrants further investigation. To more convincingly demonstrate that Rac1 is not required for dendritogenesis, the authors should knockdown Rac1 in the presence of RhoG. The lack of an effect of the RhoGF37A mutant is clearly not sufficient here.
Now, we added data about Rac1 knockdown, Rac1 knockdown combined with RhoG overexpression, and Cdc42 knockdown combined with RhoG-F37A overexpression (new Figure 4A, B, and C). We found that Rac1 knockdown alone decreased the number of dendritic end tips (TNDET) and that Rac1 knockdown in the presence of overexpressed RhoG synergistically affects TNDET (new Figure 4B). One explanation for the synergism of Rac1 knockdown and RhoG overexpression could simply be that Rac1 can be driven independently of RhoG to increase dendritic tree complexity. Interestingly however, this synergism applied mainly to the distal part of the dendritic tree as shown by Sholl analysis (new Figure 4C). Another interpretation for the synergism of Rac1 knockdown and RhoG overexpression could be that RhoG overall decreases dendritic branching in the proximal to middle part of the dendritic tree (this can be seen in the Sholl curves for RhoG overexpression and RhoG knockdown; new Figure 1D), but that RhoG may also stimulate Rac1 to some extent for increasing complexity in the distal part of the dendritic tree. This

interpretation could also explain the subtle difference that we observed when comparing the effects of RhoG and RhoG-F37A on dendritic tree analysis. The F37A mutant, not able to activate Rac1 via ELMO/Dock180 signaling (even functioning dominant-negative for Rac1 signaling), decreased complexity also in the distal part of the dendritic tree as shown by Sholl analysis (new Figure 4C). Finally, although Cdc42 knockdown rescued complexity under both RhoG and RhoG-F37A overexpression (new Figure 4C, lower panel), in the distal part a difference in complexity remained between the experimental conditions Cdc42-kd + RhoG versus Cdc42-kd + RhoG-F37A. This difference might stem from the contrasting effects of RhoG and RhoG-F37A in activating Rac1.

4. The fact that increasing RhoG levels (e.g. by anti-miR-124) is sufficient to induce dendritogenesis, but not axonogenesis, is puzzling. As stated by the authors, a possible explanation could be that RhoGEFs are limiting specifically during axonogenesis, which would lead to the effect that excessive RhoG protein cannot be activated. However, this hypothesis is not supported by experimental evidence. One way to address this would be to assess the activation status of RhoG (e.g. GTP loading) under the different experimental conditions.
In several published studies on RhoG function only activated RhoG (RhoG-G12V or RhoGQ61L) but not wild-type RhoG was used to elicit biological effects in different experimental settings (e.g. Katoh and Nesgishi, 2003; Meller *et al*, 2008; van Buul *et al*, 2007; Yamaki *et al*, 2007). A recent study showed that dominant-positive RhoG-G12V but not wild-type RhoG promotes the proliferation of neural progenitor cells whereas reduction of endogenous RhoG expression via RNA interference decreased this proliferation (Fujimoto *et al*, 2009). From there, it seems not to be unusual that only activated RhoG (RhoG-G12V) but not wild-type RhoG was able to initiate a biological response. We addressed the possible explanation that RhoG-specific GEFs are limiting factors specifically during axonogenesis by two means: (1) According to reviewer's suggestion, we tried to assess the activation status of RhoG by pulling down GTP-loaded RhoG from hippocampal cell cultures under the different experimental conditions (meaning here: early cultures around DIV2 for axonogenesis versus older cultures around DIV8 for dendritogenesis). To our best knowledge, no pulldown of RhoG-GTP from primary hippocampal cell culture is published so far. Nevertheless, we attempted to pulldown RhoG-GTP with a recombinant GSTELMO1 construct (Meller et al., 2008) from hippocampal cell culture. Yet we failed to get reproducible signals in the immunoblot strong enough for quantification because the total amount of RhoG-GTP in the cultures was too low for this type of assay. As a positive control we performed pulldowns of RhoG-GTP from hippocampal tissue – with success. However, hippocampal tissue consists of a mixture of different neuron populations that are born at different times and do not develop axons and dendritic trees in a synchronized fashion. Therefore, it is not possible with the biochemical RhoG-GTP pull down assay starting from hippocampal tissue to discriminate axonogenesis and dendritogenesis in a sufficient way. (2) Assuming that RhoG-specific GEFs are limiting factors during axonogenesis, we expressed a RhoG-activating GEF in hippocampal neurons at DIV2. We decided to use a construct of SGEF, because SGEF seems to have a high preference for RhoG activation when compared to other Rho GTPases (Ellerbroek et al., 2004). We employed the SGEFΔN construct because removal of amino-terminal sequences upstream from the DH domain seems to be an activating event for many GEFs (Ellerbroek et al., 2004). SGEFΔN was found to reduce axonal branching and co-expression of SGEFΔN together with RhoG was detected to significantly reduce axonal branching when compared to RhoG expression alone (new Supplementary Figure S4). Finally, we performed epistasis analysis by overexpressing SGEFΔN under RhoG knockdown (RhoG-kd4). We found that RhoG knockdown rescued axonal branching in the presence of SGEFΔN (Supplementary Figure S4). Together, these experiments strengthen the hypothesis that RhoG-specific GEFs are limiting factors for the activation of overexpressed RhoG during axonogenesis.

Minor concerns:
1. Dendritic complexity should be analyzed by Sholl analysis, which is a more accepted method that gives additional information about effects on branching and length.
We conducted Sholl analysis for all constructs used in this manuscript with respect to dendritogenesis (29 new Sholl curves!; new Figures 1D, 4C, 6C, and Supplementary Figure 9B). Although this was actually a great deal of work, we thank this referee for this

recommendation, because the impact of RhoG and RhoG-driven signal transduction on dendritic tree complexity is now specified more precisely (see above).

2. The authors mention a previous study (Edbauer et al.), which failed to observe any dendritic phenotype upon miR-124 inhibition. It would be nice if the authors could recapitulate these findings in their own hands.
We have recapitulated the experiment performed by Edbauer et al., 2010 (Supplementary Figure S2 from Edbauer et al., 2010). We have performed Sholl analysis and have determined TNDET (new Supplementary Figure S9) Basically, we got the same result as Edbauer et al.: miR-124 expression did not significantly increase dendritic branching in DIV14+3 hippocampal neurons. However, miR-124 still led to a small (although not significant) shift of the Sholl curve to more dendritic tree complexity at DIV14+3. This result is concordant to our conclusion that miR-124 has an impact on the establishment of dendritic tree complexity, but no significant impact on dendritic arborization during the time when dendritogenesis is largely completed but spinogenesis proceeds.

3. The efficiency and specificity of the RhoG knockdown constructs should also be assessed on the endogenous RhoG protein in primary hippocampal neurons.
We performed these experiments (see answer to Major concern 2).

All full citations of the literature are given in the References.

Referee #2

Essential controls for the specificity of the downregulation of RhoG and other endogenous proteins in neurons are missing.
Two different shRNAs for RhoG have been used to downregulate endogenous RhoG, but the effects of these shRNAs on neuronal RhoG is not shown. Moreover, rescue experiments to prove the specificity of the RhoG shRNAs in neurons have not been included. Although rescue experiments in primary neurons may be particularly difficult, clear reduction of the endogenous neuronal protein either by biochemistry, or by immunofluorescence must be shown for both shRNAs.
These observations hold true for all the shRNA constructs used in the manuscript.
In addition, an estimate of the efficiency of the two shRNAs in knocking down RhoG should be included, by quantification from distinct experiments as the one shown in Fig. 2B.

We now better validated the specificity and efficiency of the RhoG-specific knockdown constructs (new Supplementary Figure S2). (1) We demonstrated that the RhoG-specific knockdown constructs RhoG-kd1 and RhoG-kd4 reduce the expression of endogenous RhoG in primary hippocampal neurons (new Supplementary Figure S2A and B). (2) We showed that RhoG-kd1 and RhoG-kd4 reduce the expression of overexpressed HA-tagged RhoG in hippocampal neurons (new Supplementary Figure S2C, D, E, and F). (3) We established the RhoG-specific rescue construct RhoGresist(kd4) (new Supplementary Figure S2C and D). (4) We quantified the Western blot data for knockdown documentation (new Figure 1B and C versus old Figure 2B). With these validated constructs we performed functional rescue experiments (new Figure 1A, C, and D). We found that, although the rescue construct HA-RhoGresist(kd4) did not express as well as HA-RhoG (new Supplementary Figure S2D), this construct nevertheless led to a rescue of RhoG function in the presence of the stronger knockdown construct RhoG-kd4 (new Figure 1A, C, and D). Because the weaker knockdown construct RhoG-kd1 only led to a reduction of approximately 40% of overexpressed HA-RhoG (Supplementary Figure S2F), we could achieve a functional rescue simply by overexpressing RhoG in the presence of RhoG-kd1 (new Figure 1A, C, and D).
In addition, we also validated the other knockdown constructs used in this study to and found that Dock180-kd, Rac1-kd, and Cdc42-kd reduce the expression of endogenous Dock180, Rac1, and Cdc42, respectively, in primary hippocampal neurons (new Supplementary Figure S5). For the validation of the Cdc42-kd construct, we could not determine the fluorescence intensity after staining for endogenous Cdc42 because of a lack of a specific anti Cdc42 antibody functioning in immunocytochemistry (we tried the monoclonal anti Cdc42 antibody, clone B-8 from Santa Cruz Biotechnology, but without success). Therefore we expressed a

T7-tagged Cdc42 construct and determined the relative fluorescence intensity after staining with a monoclonal antibody to the T7 tag (#69522-3 from Novagen). This antibody unspecifically stained nuclei but produced virtually no background staining in neuronal processes (Supplementary Figure S5C). Thus, here we measured the fluorescence signals in the primary dendrites.

Moreover, the rationale to explain some of the results on the function of the ELMO complex is not clear.
Figure 4B: how do the authors explain the strong positive effect on branching observed in neurons overexpressing the RhoG-F37A and RhoG-G12V/F37A mutants that are unable to bind the ELMO complex? Wouldn't endogenous RhoG interacting with the ELMO complex be sufficient to keep branching normal?

The ELMO1/Dock180 complex is described in the literature as an unconventional guanine nucleotide exchange factor (GEF) for the small GTPase Rac1, which can be activated by RhoG after forming a ternary complex which is composed of RhoG, ELMO1, and Dock180 (Katoh and Negishi, 2003, and literature cited therein). We analyzed in this study the differentiation of axons and dendritic trees. Up to now, RhoG was published to stimulate neurite growth in PC12 cells via ELMO1/Dock180/Rac1 signaling. This was the motivation for us to explore whether the ELMO1/Dock180/Rac1 pathway has any relevance for the differentiation of axons and dendrites in primary neurons, especially because we figured out that the effect of RhoG on axonal and dendritic branching is inhibitory. For this reason, we interfered with the RhoG/ELMO1/Dock180/Rac1 signaling pathway at different points: (1) We interfered with Rac1 function as the final effector either by expressing the dominant-negative construct Rac1-T17N or by reducing endogenous Rac1 via the Rac1-kd knockdown construct. (2) We reduced the expression of endogenous Dock180 to prevent activation of Rac1. (3) We inhibited the activation of Dock180 by expressing a truncated ELMO1 construct (ELMO1-D625), which can bind RhoG but not Dock180. (4) We used the RhoG-F37A and RhoG-G12VF37A constructs to inhibit signal transduction from RhoG to ELMO1/Dock180/Rac1 because both constructs cannot bind to ELMO1. Both constructs carry a point mutation in the effector region of RhoG preventing ELMO1 binding. However, binding of RhoG-activating GEFs can still occur. This way, F37A mutants of RhoG titrated away RhoG-activating GEFs precluding access of these GEFs to endogenous RhoG. Consequently, F37A mutants of RhoG function in a dominant-negative manner in the RhoG/ELMO1/Dock180/Rac1 pathway. Endogenous RhoG interacting with the ELMO complex is not sufficient to keep branching normal because of a lack of activated RhoG. What we cannot exclude is that the F37A mutants of RhoG, in addition to function dominantnegative in the RhoG/ELMO1/Dock180/Rac1 signaling pathway, affects other (and so far unknown) signal transduction events. Based on studies of a F37A mutant derived from Rac1, which is structurally similar to RhoG, it is likely that RhoGF37A can still activate several signaling molecules (independent from ELMO1; Lamarche *et al*, 1996; Schwartz *et al*, 1998). We have extended and improved this issue in the *Discussion* to display it in a more precise manner.

In the same panel, it is shown that Rac1 KD increases axonal branching compared to control KD; but then Rac1 KD does not seem to significantly affect branching compared to the KD control + RFP: this part of the presentation of the data needs revision.

We had compared significance within experimental groups (separated by an “empty bar). Rac1-kd also affects axonal branching significantly when compared to kdcontrol + RFP (the statistical analysis is now included in Figure 3B).

Along the same line, also some of the data presented in Figure 6B are confusing: in the first group of bars on the left, anti-miR-124 + RhoG KD is expected to decrease endogenous RhoG and therefore to increase branching, but it does not (only a small increase between first and fourth bars is shown, but is not indicated as significant). This indicates that the reproducibility of the effects on branching following alteration of RhoG levels is quite weak.

We assume that lack of presentation of statistical analysis has led to the conclusion drawn by this referee. However, we want to point to the fact that in all presented experiments, the

knockdown of endogenous RhoG by the shRNA construct RhoG-kd4 (and also RhoG-kd1) reproducibly and significantly increases dendritic branching in hippocampal cell culture [RhoG-kd4 versus kdcontrol in new Figure 1C and D (old Figure 2C); anti miR-control + RhoG-kd4 versus anti miR-control + kdcontrol in new Figure 6B and C (old Figure 6B)] and *in vivo* in the intact brain [RhoG-kd4 versus kdcontrol in new Figure 1H (old Figure 2G)]. In all presented experiments, the overexpression of RhoG led to reduced dendritic branching [EGFP + RhoG versus EGFP, and kdcontrol + RhoGresist(kd4) versus kdcontrol in new Figure 1C and D (old Figure 2C); miR-control + pCLEG-RhoG versus miR-control + pCLEGGFP in new Figure 6B and C (old Figure 6B)] in hippocampal cell culture and *in vivo* in the intact brain [pCLEG-RhoG versus pCLEG-GFP in new Figure 1H (old Figure 2G)]. The experimental condition anti miR-124 + RhoG-kd4 was chosen to prove whether knockdown of RhoG did rescue dendritic branching which was reduced due to up-regulated endogenous RhoG expression by anti miR-124 [anti miR-124 + RhoG-kd4 versus anti miR-124 + kdcontrol in new Figure 6B and C (old Figure 6B)]. In fact, RhoG knockdown significantly rescued dendritic branching, proving that anti miR-124 reduced TNDET, at least in part, via up-regulation of endogenous RhoG expression. TNDET under the condition of anti miR-124 + RhoG-kd4 co-expression was not as high as under the condition of anti miR-control + RhoG-kd4 co-expression and, consequently, is not significantly higher than under the condition of anti miR-control + kdcontrol expression. This may be explained by the possibility that RhoG is not the only target of miR-124. It is also feasible that the different kinetics of the anti miR-124 effect (faster because anti miR-124 captures away endogenous miR-124 and allows quickly RhoG expression) and the RhoG-kd4-mediated knockdown effect (slower because RhoG-kd4 reduces production of new RhoG protein, but did not prevent existing RhoG protein from inhibiting dendritic branching; the full knockdown effect comes up when the endogenous RhoG protein is degraded according to the half-life of this protein) contribute to the degree of RhoG-kd4-mediated rescue.
We apologize for contributing to this confusion by not presenting statistical analysis for the experimental conditions anti miR-124 + RhoG-kd4 versus anti miR-124 + kdcontrol and anti miR-control + RhoG-kd4 versus anti miR-control + kdcontrol in the old Figure 6B. We corrected this in the new Figure 6B.

In Figure S3, what is axonal length? Does it represent the length of the longest axonal branch, or the total length of the axonal tree? This should be clearly specified in the text and legend.

The term “axonal length” describes the length of the longest axonal branch emanating from the neuronal cell body (now explained in the legend of Supplementary Figure S3).

All full citations of the literature are given in the References.

Referee #3
1) I would recommend a restructuring of the manuscript. As it is, the authors first discuss downregulation of RhoG by miR-124, and then launch into a dissection of RhoG activity, and then come back to miR-124 regulation of RhoG in branching. I would recommend that all of the RhoG studies come first, and then the issue of what regulates RhoG (i.e. miR-124) be the last topic. In other words, Figure 1 could be placed between Figures 5 and 6. Thus, the manuscript would first discuss RhoG signaling in branching, and then discuss a regulator of RhoG. I think this flows better than splitting up the miR-124 data to the beginning and the end.

We agree – and have re-structured this manuscript according to the recommendation. Since in this revised manuscript the data load is increased in several figures, we have split the old Figure 6 into the new Figures 6 and 7 (separating the analysis of the impact of miR-124 on dendritic versus axonal branching). We placed the new and improved Figure 5 (old Figure 1) between new Figures 4 and 6.

2) Is there an independent means to show that miR-124 is being "captured" by the antisense technique? A missing control here is treatment with a non-miR-124-specific antisense miRNA to show that the effect of antisense treatment was specific to miR-124 and

not just general antisense RNA treatment.

We now included the missing controls (EGFP + anti miR-control and EGFP-3´UTR(RhoG) + anti miR-control) into new Figure 5H and I (old Figure 1F and G).

3) The results that RhoG inhibits branching go against previous work on PC12 cells, which suggest a positive role. The authors discuss only work with vertebrate models, but there is a body of literature using flies and worms with the functional equivalent of RhoG called Mtl (mig-2-like) and Rac GTPases. In C. elegans and Drosophila, Mtl/mig-2 also inhibits axon branching, as mutants have extra axon branches (e.g. Ng et al., 2002; Lundquist et al., 2001; Struckhoff et al., 2003). These data would support the authors' argument that RhoG inhibits branching. They should be discussed.

We are glad that this referee points to the Mtl/mig-2 literature. We were aware of the addressed publications but felt not expert enough how to assess the (strong) phenotypes of the double or triple mutants (Rac1/Rac2/Mtl) in *Drosophila* or *C. elegans* (ced-10/rac2/3/mig-2) compared to the (relatively weak) phenotypes of single Mtl/mig-2 mutants [axonal branching phenotype in the Mtl single mutants in Drosophila (Figure 2C in Ng et al., 2002), but no increase in the percentage of ectopic axons in the mig-2 single mutant mu28 compared to an increase in the percentage of ectopic axons in the ced10/mig-2 double mutant (Table 1 in Struckhoff and Lundquist, 2003). We now included these three publications (Ng et al., 2002; Lundquist et al., 2001; Struckhoff et al., 2003) in the *Discussion*.

4) The section on p. 8 dealing with the RhoG-F37A mutant protein was unclear. Are the authors saying that overexpression of F37A acts as a "dominant positive" independently of Rac1/ELMO/DOCK180? This should be explained more clearly. Also, in this section and on the Figure, it is unclear if the F37A mutant is used in all experiments (in the legend for Figure 5B, the last two treatments just say RhoG, but I think these are supposed to be RhoG-F37A).

We realize that the figuring in our previous version led to some unnecessary misunderstanding. However, the lettering was correct in the old Figure 5. Now, we added data about Rac1 knockdown, Rac1 knockdown combined with RhoG overexpression, and Cdc42 knockdown combined with RhoG-F37A overexpression (new Figure 4A, B, and C). We used the RhoG-F37A mutant to inhibit signal transduction from RhoG to ELMO1/Dock180/Rac1 because RhoG-F37A cannot bind to ELMO1. The F37A mutant carries a point mutation in the effector region of RhoG preventing ELMO1 binding. However, binding of RhoG-activating GEFs can still occur. This way, F37A mutants of RhoG titrated away RhoG-activating GEFs precluding access of these GEFs to endogenous RhoG. Consequently, the F37A mutant of RhoG functions in a dominant-negative manner in the RhoG/ELMO1/Dock180/Rac1 pathway. In addition, our data showed that RhoG-F37A acted independently of ELMO1/Dock180/Rac1 signaling to decrease dendritic branching. The results indicated that RhoG-F37A functioned here via Cdc42 signaling. However, this is not a dominant-positive effect of RhoG-F37A, but a simple overexpression *gain-of-function* effect (like RhoG overexpression). Based on studies of a F37A mutant derived from Rac1, which is structurally similar to RhoG, it is likely that RhoGF37A can still activate several signaling molecules (independent from ELMO1; Lamarche *et al*, 1996; Schwartz *et al*, 1998). The Sholl analysis revealed a subtle difference when comparing the effects of RhoG and RhoG F37A on dendritic tree analysis. Overall, RhoG decreased complexity in the proximal to middle part of the dendritic tree (new Figure 1D). The F37A mutant, not able to activate Rac1 via ELMO/Dock180 signaling (even functioning dominant-negative for Rac1 signaling), decreased complexity also in the distal part of the dendritic tree (new Figure 4C).
We found that Rac1 knockdown alone decreased the number of dendritic end tips (TNDET) and that Rac1 knockdown in the presence of overexpressed RhoG synergistically affects TNDET (new Figure 4B). One explanation for the synergism of Rac1 knockdown and RhoG overexpression could simply be that Rac1 can be driven independently of RhoG to increase dendritic tree complexity. Interestingly however, this synergism applied mainly to the distal part of the dendritic tree as shown by Sholl analysis (new Figure 4C). Another interpretation for the synergism of Rac1 knockdown and RhoG overexpression could be that RhoG overall

decreases dendritic branching in the proximal to middle part of the dendritic tree (this can be seen in the Sholl curves for RhoG overexpression and RhoG knockdown; new Figure 1D), but that RhoG may also stimulate Rac1 to increase complexity in the distal part of the dendritic tree to some extent. Finally, although Cdc42 knockdown rescued complexity under both RhoG and RhoG-F37A overexpression (new Figure 4C, lower panel), in the distal part a difference remained between the experimental conditions Cdc42-kd + RhoG versus Cdc42-kd + RhoG-F37A. This difference might stem from the contrasting effects of RhoG and RhoG-F37A in activating Rac1 which we demonstrated to be stimulating for dendritic branching (a synergistic effect of Rac1 knockdown combined with RhoG overexpression could be observed in the distal part of the dendritic tree (new Figure 4C, lower panel).
We have extended and improved this issue in the *Discussion* to display it in a more precise manner.

5) I could not find a definition of pCLEG. I infer it is the vector used to overexpress molecules. It would be helpful to describe these experiments more succinctly in the figures, legends, and text.
pCLEG is derived from the pCLE vector and used for gain-of-function experiments. It contains a CMV/MLV promoter, an EF1α enhancer, and an internal ribosomal entry site (IRES) for the translation of GFP as a reporter (Chen *et al*, 2005).
We added this information in the *Materials and methods* and in the legend of new Figure 1.

6) There are statistical analyses that should be added in the figures. Figure 3B should compare kdcontrol with RhoG-kd1 and EGFP with EGFP+RhoG; and Figure 6D should compare miR-124 EGFP with miR-124 + RhoG G12V.
We added these statistical analyses. The new Figure 2B (old Figure 3B) now additionally compares kdcontrol with RhoG-kd1 and EGFP with EGFP+RhoG. The new Figure 7B (old Figure 6D) now compares miR-124 + EGFP with miR-124 + RhoG G12V.

7) In the abstract, the last statement is a "discernible developmental phenotype". It might be more clear to say "to control axon and dendritic branching."
We corrected this.

8) On p. 5, the mutations abolishing the 3' UTR binding sites should be referenced in the methods here, or described here.
We corrected this.

9) *On p. 7, "To finally proof the importance..." should be changed to something like "To further demonstrate the importance of..."*
We corrected this.

All full citations of the literature are given in the References.

1st Editorial Decision 30 March 2012

Thank you for submitting your study to the EMBO Journal. This is a resubmission of manuscript #79528 that was rejected post review last year. Your study has now been seen by the same set of referees and their comments are provided below. The referees are listed in the same order as on the previous submission. As you can see the referees find the analysis significantly improved and support publication in the EMBO Journal. Referee #3 has a few minor text issues that should fixed before publication here. Once we get the revised manuscript back we will proceed with its acceptance for publication here.

Yours sincerely

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

the authors have satisfactorily addressed all my concerns and I can now recommend publication

Referee #2

This is a resubmission of a manuscript that was previously reviewed by this referee. The authors have satisfactorily answered to the criticisms raised, by improving some of the data and the quality of presentation as previously indicated.

Referee #3

The authors did a good job of responding to the reviewer comments. The organization of the manuscript is much improved, and critical controls have been added. In sum, the authors present a thorough and convincing case that RhoG inhibits axonal and dendritic branching via distinct mechanisms (DOCK/ELMO/Rac1 and Cdc42, respectively). They also show that the micro RNA mir-124 specifically inhibits RhoG expression and function in dendrites.

The results about expression of RhoG-F37A and Rac knockdown synergizing in dendrites is still a bit confusing, but the authors present plausible explanations.

I would note one use of the word "proof" on p. 7. The authors might want to consider revising it to something like "To demonstrate the in vivo relevance...."

Is mir-124 regulation of RhoG conserved in flies and worms? Do the Mtl genes have mir-124-family binding sites?

1st Revision - authors' response 03 April 2012

Manuscript EMBOJ-2012-81218R - Reply to referees´ comments

Minor concerns of Referee #3

1) *I would note one use of the word "proof" on p. 7. The authors might want to consider revising it to something like "To demonstrate the in vivo relevance...."*

We revised the word “proof” to the word “demonstrate” on page 7 (now “To demonstrate the in vivo relevance of these results,…”).

2) *Is mir-124 regulation of RhoG conserved in flies and worms? Do the Mtl genes have mir-124-family binding sites?*

We examined whether the miR-124-dependent regulation of RhoG may be conserved in flies and worms by using the algorithms PicTar, miRanda, and TargetScan from the public domain (the algorithm MiRtarget2 cannot be used for scanning *Drosophila* or *C. elegans* genes). We found that the 3´UTRs derived from the genes coding for Mtl and Mig-2 (the functional equivalents of RhoG in *Drosophila* and *Caenorhabditis elegans*, respecitively) do not contain miR-124-binding sites.
We revised the wording in paragraph 2 on page 10 to “According to the algorithms PicTar (Krek *et al*, 2005), miRanda (John *et al*, 2004), TargetScan (Lewis *et al*, 2005), and MiRtarget2 (Wang and El Naqa, 2008) for microRNA target prediction, the 3´UTR of the RhoG gene comprises two miR-124 binding sites (Figure 5A and Supplementary Figure S7). These sites are highly conserved in mice, rats, and humans, while the 3´UTRs derived from the genes coding for Mtl and Mig-2 (the functional equivalents of RhoG in *Drosophila* and *Caenorhabditis elegans*, respecitively) do not contain miR-124-binding sites.

