THE EMBO JOURNAL

Manuscript EMBO-2010-75754

RITA, a novel modulator of Notch signaling, acts via nuclear export of RBP-J

Stephan A. Wacker, Cristobal Alvarado, Goetz von Wichert, Uwe Knippschild, Joerg Wiedenmann, Karen Clauss, G. Ulrich Nienhaus, Horst Hameister, Bernd Baumann, Tilman Borggrefe, Walter Knoechel and Franz Oswald

Corresponding author: Franz Oswald, University of Ulm

Review timeline:

Submission date:	23 August 2010
Editorial Decision:	27 September 2010
Revision received:	23 October 2010
Additional Correspondence:	26 October 2010
Accepted:	26 October 2010

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 27 September 2010

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are enclosed. As you will see, all three reviewers express significant interest in your work and are broadly in favour of publication, pending satisfactory revision. The major issue expressed to varying degrees by all the referees concerns the characterisation of the Xenopus phenotype. This is most clearly expressed by referee 2 point 1, who states that further quantitative analysis of the Xenopus phenotypes (both gain and loss of function) would be important. This also relates to the concern of referee 1 regarding the physiological significance of RITA as a Notch regulator: better data on the morphant phenotype would help to strengthen the argument that RITA is important for Notch regulation in vivo. Referee 3 also makes the point that more detailed analysis of the early neurogenesis phenotype, as opposed to the broader but more superficial characterisation of the morphants, would be valuable. Finally, this referee also requests that you provide data on the expression pattern of RITA in Xenopus, which would clearly be valuable here.

In the light of the referees' positive recommendations, I would therefore like to invite you to submit a revised version of the manuscript, addressing all the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses included in the next, final version of the manuscript. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

In addition, we are currently implementing a policy of requesting an Author Contributions statement in all papers; please can I ask you to include such a statement in your revised manuscript?

We generally allow three months as a standard revision time, and as a matter of policy, we do not consider any competing manuscripts published during this period as negatively impacting on the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REVIEWS

Referee #1 (Remarks to the Author):

The authors present a tremendous amount of careful biochemistry, over expression and loss of function data to try and position RITA as a novel Notch regulator. My main concern is the lack of correlation between the strength of the data demonstrating the physiological relevance of this observation and the strength of the claims made by the authors. This is because the strength of the paper is in demonstrating what RITA CAN do, not in demonstrating what it actually does.
The main deficiency is that one has to take it as an article of faith that when RITA is lost, adding a small amount of RBPjk to the nucleus (Fig. 7) translates to a meaningful change in N activity. This is not shown in the paper, and the Notch the literature never established biochemically or genetically that RBPjk is limiting. Therefore, the authors should back off the claim that RIOTA is a negative regulator and instead suggest it might be one. With a few simple linguistic changes, the paper will be suitable for publication in the EMBO journal. A case in point the authors should consider is KyoT2: it can inhibit Notch, but its knockout did not result in Notch phenotypes.

For example, here is how the abstract can be improved:
Notch proteins are receptors of an evolutionary highly conserved signaling pathway that regulates numerous cell fate decisions during development. Signal transduction involves the presenilin-dependent intracellular processing of Notch and nuclear translocation of the intracellular domain, NICD. NICD associates with the DNA binding protein RBP-J/CBF-1 to activate transcription of Notch target genes. Here we report the identification and functional characterization of RITA (C12ORF52) as a novel RBPJ/CBF-1 interacting protein. RITA is a highly conserved 36kDa protein that, most interestingly, binds to tubulin in the cytoplasm and is shuttles rapidly between cytoplasm and nucleus. This shuttling RITA exports RBP-J/CBF-1 from the nucleus. Functionally, we show that RITA CAN reverse a Notch-induced loss of primary neurogenesis in Xenopus laevis. Furthermore, RITA is able to down regulate Notch-mediated transcription. Thus, we PROPOSE that RITA acts as a negative modulator of the Notch signaling pathway, controlling the level of nuclear RBP-J/CBF-1 WHERE ITS AMOUNTS ARE LIMITING.

Referee #2 (Remarks to the Author):

This is a very interesting study that identifies a novel regulator of Notch signaling termed RITA. RITA was recovered based on its ability to bind the downstream transcriptional effector of Notch signaling (RBPJk) and the study presents a reasonably thorough initial analysis of the molecular/biochemical properties of RITA. In addition, experiments in Xenopus oocytes reveal the loss-of-function and positive activation phenotypes of RITA on Notch-dependent neurogenesis. In general, most of the experiments are performed in enough detail and with appropriate controls to

support the main conclusions, and the molecular, biochemical, and genetic results all fit very well together.

The study is of relatively high significance in the Notch field, because it shows convincingly that RITA acts as a nucleo-cytoplasmic shuttling factor that exports RBPJk out of the nucleus, thereby downregulating the duration and/or intensity of Notch signaling. Although much attention has focused on the upstream ligand-activated steps of Notch signaling, relatively less work has explored the types of regulation that might influence the downstream nuclear events in the pathway, so this study reveals an important, potentially major regulatory mode of Notch signaling that has not been appreciated previously.

Additional comments:

1. The only substantive criticism is that for the Xenopus studies, there are no numbers, percentages, or statistics of any kind provided for the morpholino and overexpression phenotypic classes. How many injected oocytes show the reported defects in neurogenesis, eye, skeletal and intestinal development for each experiments? Perhaps in earlier days it was acceptable to show "representative" photos as in Figure 8 with no quantitative data, but these Xenopus studies would be much more convincing with proper statistical documentation.

2. It would be very helpful for the general reader if the authors would include a diagram of the RITA protein showing the locations of the various motifs defined by their deletion and mutagenesis studies, e.g. the RBPJk binding domain, the NLS, NES etc. In addition it would be useful to how well the RBJk binding domain of RITA aligns to those of other RBPJk-binding proteins, such as NICD.

3. In the text on p. 11, it is stated that overexpression of RITA (wt or DeltaTub) causes increased cytoplasmic and decreased nuclear RBPJk, but these effects seem rather weak in Figure 7B (not Figure 7C as erroneously written on p. 11). It is important to explain how many replicates of this experiment were done and perform quantitative scanning densitometry to ascertain the percent reduction/increase +/- standard error.

4. The authors note (p. 12) that RITA proteins are found in "the complete metazoan phylum" yet then go on to mention that no Drosophila RITA could be found. It is probably better to not say "complete" phylum in this case. Also, the same paragraph has a typographical error, "functional homologous" instead of "functional homologue".

5. One of the last sentences of the Results section is confusing, as it states that "transcriptional activation of several Notch targets....was rescued after coinjection of RITA(wt)". To more accurately reflect the actual experiment, this should be reworded to something like: "transcriptional activation of several Notch targets caused by dominant activated Notch1DeltaE.....was suppressed/reversed after coinjection of RITA(wt)".

Referee #3 (Remarks to the Author):

The paper by Wacker et al is a very comprehensive study describing the function of RITA, a new component of the Notch signaling pathway. Their study describes the discovery of a previously uncharacterized protein C12ORF52 in an yeast two hybrid screen designed to identify RBP-J interacting proteins. Their study goes on to show that it is a RBP-J Interacting and Tubulin Associated protein (RITA). They show that by binding RBP-J, RITA has the potential to inhibit Notch signaling in cell culture using luciferase based Notch activity assays. They also do gain and loss of function experiments with RITA in the Xenopus system to show it inhibits Notch signaling by demonstrating that it alters primary neurogenesis in a manner that is consistent with the role of Notch signaling in Lateral inhibition. Then in a series of elegant experiments using engineered fragments of RITA and in vitro studies they show that RITA has a key role in shuttling in and out of the nucleus. They go on to show that it is likely to compete with NICD in binding RBP-J, and once bound it shuttles RBP-J out of the nucleus leaving NICD behind, limiting the number of active RBP-J NICD complexes available to active Notch target genes. Additional experiments identify components of RITA required for binding to tubulin. Though very interesting and nicely

demonstrated, the physiological significance of the interaction with tubulin remains enigmatic at this time.

Over all this is a very nice study and makes an important contribution to our understanding of yet another level at which the Notch signaling pathway is dynamically regulated. The only part of the study that I did not find as compelling is the description of changes in the Xenopus related to the size of the eye, jaw, branchial arches and the intestine. I see the advantage of not limiting oneself to changes in early neurogenesis, and in showing that changes in RITA function affect other structures in a manner consistent with a broad effect on Notch signaling. However, its not made clear how Notch signaling affects these other structures organs and therefore I felt it detracts from the simple story that can be told using the pattern of neurogenesis as an example. In addition, very little was said about the expression pattern of RITA. Is it broadly expressed in Xenopus, is it in both Notch and Delta expressing cells or is its expression typically restricted to Notch expressing cells and perhaps regulated by Notch activity? Is the protein symmetrically inherited by daughter cells during neurogenesis? Perhaps many of these interesting questions await future studies.

I apologize to the editors and authors for taking so long on this review.

1st Revision - authors' response 23 October 2010

We would like to thank all the reviewers for their positive reviews with helpful suggestions and criticisms that in our opinion have greatly improved the manuscript.

Referee #1 (Remarks to the Author):

The authors present a tremendous amount of careful biochemistry, over expression and loss of function data to try and position RITA as a novel Notch regulator. My main concern is the lack of correlation between the strength of the data demonstrating the physiological relevance of this observation and the strength of the claims made by the authors. This is because the strength of the paper is in demonstrating what RITA CAN do, not in demonstrating what it actually does.
The main deficiency is that one has to take it as an article of faith that when RITA is lost, adding a small amount of RBPjk to the nucleus (Fig. 7) translates to a meaningful change in N activity. This is not shown in the paper, and the Notch the literature never established biochemically or genetically that RBPjk is limiting. Therefore, the authors should back off the claim that RITA is a negative regulator and instead suggest it might be one. With a few simple linguistic changes, the paper will be suitable for publication in the EMBO journal. A case in point the authors should consider is KyoT2: it can inhibit Notch, but its knockout did not result in Notch phenotypes.

For example, here is how the abstract can be improved:
Notch proteins are receptors of an evolutionary highly conserved signaling pathway that regulates numerous cell fate decisions during development. Signal transduction involves the presenilin-dependent intracellular processing of Notch and nuclear translocation of the intracellular domain, NICD. NICD associates with the DNA binding protein RBP-J/CBF-1 to activate transcription of Notch target genes. Here we report the identification and functional characterization of RITA (C12ORF52) as a novel RBPJ/CBF-1 interacting protein. RITA is a highly conserved 36kDa protein that, most interestingly, binds to tubulin in the cytoplasm and is shuttles rapidly between cytoplasm and nucleus. This shuttling RITA exports RBP-J/CBF-1 from the nucleus. Functionally, we show that RITA CAN reverse a Notch-induced loss of primary neurogenesis in Xenopus laevis. Furthermore, RITA is able to down regulate Notch-mediated transcription. Thus, we PROPOSE that RITA acts as a negative modulator of the Notch signaling pathway, controlling the level of nuclear RBP-J/CBF-1 WHERE ITS AMOUNTS ARE LIMITING.

We changed the statements in accordance with the suggestions of reviewer #1 in the abstract, the introduction and the discussion.
page 2, paragraph 1, Abstract
eg. „RITA reverses" > „RITA can reverse", „we conclude" > „we propose"
page 4 paragraph 3, „that modulates" > „that may modulate", „thereby functions as" > „thereby may function as"

Page 16 paragraph 1, „thereby limiting" > „We propose that it thereby limits", „we conclude" > „ we postulate"
Page 19/20 last paragraph of discussion, „RITA does so" > „RITA may do so", „identified" > „indicated"

Referee #2 (Remarks to the Author):

This is a very interesting study that identifies a novel regulator of Notch signaling termed RITA. RITA was recovered based on its ability to bind the downstream transcriptional effector of Notch signaling (RBPJk) and the study presents a reasonably thorough initial analysis of the molecular/biochemical properties of RITA. In addition, experiments in Xenopus oocytes reveal the loss-of-function and positive activation phenotypes of RITA on Notch-dependent neurogenesis. In general, most of the experiments are performed in enough detail and with appropriate controls to support the main conclusions, and the molecular, biochemical, and genetic results all fit very well together.

The study is of relatively high significance in the Notch field, because it shows convincingly that RITA acts as a nucleo-cytoplasmic shuttling factor that exports RBPJk out of the nucleus, thereby downregulating the duration and/or intensity of Notch signaling. Although much attention has focused on the upstream ligand-activated steps of Notch signaling, relatively less work has explored the types of regulation that might influence the downstream nuclear events in the pathway, so this study reveals an important, potentially major regulatory mode of Notch signaling that has not been appreciated previously.

Additional comments:

1. The only substantive criticism is that for the Xenopus studies, there are no numbers, percentages, or statistics of any kind provided for the morpholino and overexpression phenotypic classes. How many injected oocytes show the reported defects in neurogenesis, eye, skeletal and intestinal development for each experiments? Perhaps in earlier days it was acceptable to show "representative" photos as in Figure 8 with no quantitative data, but these Xenopus studies would be much more convincing with proper statistical documentation.

A table with numbers and percentages of analyzed embryos and phenotypes is included in the Supplement (Supplemental table S3). Cross references can be found in Materials and Methods (page 24 paragraph 2) and in the results section corresponding to figure 8 (page 12 paragraph 2).

2. It would be very helpful for the general reader if the authors would include a diagram of the RITA protein showing the locations of the various motifs defined by their deletion and mutagenesis studies, e.g. the RBPJk binding domain, the NLS, NES etc. In addition it would be useful to how well the RBJk binding domain of RITA aligns to those of other RBPJk-binding proteins, such as NICD.

A schematic drawing of RITA containing the different identified domains has been added as figure 8H.
From our mapping experiments, we conclude that RITA binds to the BTD of RBP-J (Figure S2). Since the RAM domain of Notch also binds to the BTD, we searched for sequence similarities within the RITA RBP-J binding domain and the RAM domain of Notch proteins from various species. We identified a conserved W-X-P motif within the RBP-J binding domain of RITA (see figure S5A). Although not yet analyzed the W-X-P motif within RITA might contribute to the RBP-J interaction. This fact is described in the discussion section (page 16).

3. In the text on p. 11, it is stated that overexpression of RITA (wt or DeltaTub) causes increased cytoplasmic and decreased nuclear RBPJk, but these effects seem rather weak in Figure 7B (not Figure 7C as erroneously written on p. 11). It is important to explain how many replicates of this experiment were done and perform quantitative scanning densitometry to ascertain the percent reduction/increase ± standard error.

The error in the references to figure 7 on page 11 has been corrected.
Densitometric measurements of results from three experiments were added (mean and standard

error, figure 7 C, E).

4. The authors note (p. 12) that RITA proteins are found in "the complete metazoan phylum" yet then go on to mention that no Drosophila RITA could be found. It is probably better to not say "complete" phylum in this case. Also, the same paragraph has a typographical error, "functional homologous" instead of "functional homologue".

„Complete metazoan phylum" has been changed to „a broad range of the metazoan phylum" on page 12. „homologous" has been changed to „homologue" on page 12.

5. One of the last sentences of the Results section is confusing, as it states that "transcriptional activation of several Notch targets....was rescued after coinjection of RITA(wt)". To more accurately reflect the actual experiment, this should be reworded to something like: "transcriptional activation of several Notch targets caused by dominant activated Notch1DeltaE.....was suppressed/reversed after coinjection of RITA(wt)".

This has been changed accordingly on page 14/15.

Referee #3 (Remarks to the Author):

The paper by Wacker et al is a very comprehensive study describing the function of RITA, a new component of the Notch signaling pathway. Their study describes the discovery of a previously uncharacterized protein C12ORF52 in an yeast two hybrid screen designed to identify RBP-J interacting proteins. Their study goes on to show that it is a RBP-J Interacting and Tubulin Associated protein (RITA). They show that by binding RBP-J, RITA has the potential to inhibit Notch signaling in cell culture using luciferase based Notch activity assays. They also do gain and loss of function experiments with RITA in the Xenopus system to show it inhibits Notch signaling by demonstrating that it alters primary neurogenesis in a manner that is consistent with the role of Notch signaling in Lateral inhibition. Then in a series of elegant experiments using engineered fragments of RITA and in vitro studies they show that RITA has a key role in shuttling in and out of the nucleus. They go on to show that it is likely to compete with NICD in binding RBP-J, and once bound it shuttles RBP-J out of the nucleus leaving NICD behind, limiting the number of active RBP-J NICD complexes available to active Notch target genes. Additional experiments identify components of RITA required for binding to tubulin. Though very interesting and nicely demonstrated, the physiological significance of the interaction with tubulin remains enigmatic at this time.

Over all this is a very nice study and makes an important contribution to our understanding of yet another level at which the Notch signaling pathway is dynamically regulated. The only part of the study that I did not find as compelling is the description of changes in the Xenopus related to the size of the eye, jaw, branchial arches and the intestine. I see the advantage of not limiting oneself to changes in early neurogenesis, and in showing that changes in RITA function affect other structures in a manner consistent with a broad effect on Notch signaling. However, its not made clear how Notch signaling affects these other structures organs and therefore I felt it detracts from the simple story that can be told using the pattern of neurogenesis as an example.

We decided to turn our main attention to the neurogenesis phenotype, because this delivers a direct and well defined readout of Notch signalling. However, since we also observed very consistently the late phenotypes as described, we felt the necessity to include these, even though their causation remains to be analyzed. Especially the fact that the late phenotype caused by N1 E can be reverted with RITA coinjection, is intriguing and therefore might be interesting for the broad readership of the EMBO Journal.

In addition, very little was said about the expression pattern of RITA. Is it broadly expressed in Xenopus, is it in both Notch and Delta expressing cells or is its expression typically restricted to Notch expressing cells and perhaps regulated by Notch activity? Is the protein symmetrically inherited by daughter cells during neurogenesis? Perhaps many of these interesting questions await future studies.

In addition to the presented temporal expression pattern data (supplemental figure 6) new experiments were performed to analyze the spatial expression in Xenopus embryos. The temporal expression pattern shows a maternal component and an increase of zygotic expression at neurula stages (figure S6C). Experiments with embryonic fragments from different regions of such neurula stage embryos indicate a uniform distribution of RITA at that stage (figure S6D). Whole mount in situ hybridizations support this, except for the fact that in vegetal cells low levels are detected (due to the small number and the big size of these cells or to the high amount of yolk?) (figure S6E). So, we find a broad RITA expression in Xenopus, without restriction to either Notch or Delta expressing cells during the analyzed stages (neurula stage17, tailbud stage 23 (not shown), tadpole stage 35). Therefor a transcriptional control by an activated Notch signalling pathway appears to be improbable.

Since we dealt with almost all of your helpful comments, we hope that you are satisfied with our new experiments and detailed responses.

Additional Correspondence 26 October 2010

Many thanks for submitting the revised version of your manuscript EMBOJ-2010- 75754R. I have now had the chance to read through it as well as your point-by- point response, and I am satisfied that you have addressed the concerns of the referees well. Therefore, I am pleased to be able to tell you that we can go ahead and accept the study for publication without the need to go back to the reviewers for further input.

Before we formally accept, I noticed one minor typo in the Abstract, and I'd like to check that you're happy for me to correct this before proceeding: the first line currently reads "Notch proteins are receptors of an evolutionary highly conserved signalling pathway...", but I think it should be "... evolutionarily highly conserved". If you agree, we can just make this small change in the text file, so you don't need to do anything. Once I have your confirmation on this, we will be able to accept the manuscript formally.

Many thanks for choosing the EMBO Journal for publication of this study, and congratulations on a great piece of work!

Best wishes,

Editor

