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The RNA binding protein Arrest (Bruno) regulates alternative splicing to enable myofibril maturation in *Drosophila* flight muscle

Maria L. Spletter, Christiane Barz, Assa Yeroslaviz, Cornelia Schönbauer, Irene R.S. Ferreira, Mihail Sarov, Daniel Gerlach, Alexander Stark, Bianca H. Habermann and Frank Schnorrer

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Editorial Decision:	07 November 2014
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Transaction Report:

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

Editor: Esther Schnapp

1st Editorial Decision

07 November 2014

Thank you for the submission of your manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, all referees remark that this study, despite overlaps with the recent paper by Oas et al, is significantly more systematic and comprehensive and also provides some new insight. We can therefore offer to publish your manuscript following minor revisions. Please address all referee concerns that can be addressed within 2 weeks (and address all of them in a point-by-point response), and send us a revised, final manuscript before the 25th of November in order to enable online publication of your manuscript this year.

You do not need to shorten the manuscript, as we will publish it as a long article. However, you do need to add a materials and methods section to the main manuscript file. Commonly used materials and methods can be moved to the supplementary information, however, materials and methods essential for the understanding of the experiments described in the main text must remain in the main manuscript file.

Please also make sure that all microscopy images have scale bars, and please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used

to calculate p-values in the respective figure legends. This information must be provided in the figure legends.

I look forward to seeing a revised version of your manuscript as soon as possible. Please let me know if you have questions or comments regarding the revision.

REFeree REPORTS:

Referee #1:

In this study, Spletter and colleagues investigated the roles of Salm and Aret during indirect flight muscle (IFM) development. Muscle-specific knock-down or deletion of the transcriptional regulator salm resulted in tubular conversion of IFM. RNA-Seq analysis of salm mutant IFM identified a large number of salm-dependent splicing events, some of which were validated by isoform-specific reporters. Since expression of the RNA binding protein aret was lost in salm-IR IFM, the authors reasoned that aberrant splicing in salm mutants was mainly caused by aret. To prove the hypothesis that aret acts as a splicing regulator by which salm controls IFM-specific splicing, an aret knock-down experiment was performed. Loss of aret led to a "flightless" phenotype with severe defects in myofibril formation. RNA-Seq analysis of aret loss-of-function IFM revealed that some but not all salm-depending splicing events were similarly affected in aret mutant IFM.

This is a quite interesting and well-conducted study with detailed phenotypic analyses. However, more detailed molecular insights into the regulation of Aret-dependent splicing are lacking. It also remains unclear how salm regulates aret. Despite substantial overlapping with the work from Oas et al (J. Cell Bio, 2014), this study significantly deepens the understanding of the regulatory network controlling muscle-specific splicing in flies. In particular the transcriptome-wide identification of salm and aret-regulated genes and splicing events gives the reader a more comprehensive overview of salm/aret-regulated events. Furthermore, this study also revealed that Aret undergoes dynamic subcellular trafficking, which might contribute to IFM development.

Specific points:

A more detailed analysis of already existing RNA-Seq data from salm and aret should be performed in order to disclose how many splicing events regulated by salm do indeed depend on aret. This would help to support the claim that aret is a major downstream target employed by salm to direct IFM-specific splicing.

Obviously, transgenic expression of aret in salm mutants would provide additional insights about the relative contribution of aret to the salm phenotype and identify splicing events that (indirectly) depend on salm but not on aret. In addition, the authors did barely touch on the regulation of aret by salm referring to a previous study, which is slightly disappointing. Finally, it remains enigmatic HOW aret regulates splicing and whether aret acts as bona fide splicing factor or exerts its functions by different means. Although the study would gain a lot by the latter mentioned experiments I do not consider them mandatory.

In summary, this is nice and very well done study, which goes significantly beyond the previously published results by Oas et al. In my view the genetic analysis and the phenotype characterization is top of the class while the mechanistic part is less well developed. Although the latter shortcomings can be addressed to improve the manuscript and make it more comprehensive the resulting workload is probably not appropriate given the specific circumstances.

Referee #2:

In different muscle types some proteins are present as different isoforms that arise from alternative splicing. In adult *Drosophila* there are two broad categories of muscle type, fibrillar and tubular. This paper builds on previous elegant work from the same group (Schonbauer et al 2011) that showed that the Salm transcription factor "is required and sufficient for fibrillar" muscle fate choice. In Schonbauer et al they said "the salm-induced switch is largely transcriptional, but also changes alternative splicing". Thus, part of the salm effect is on alternative splicing, but the question is how

is this achieved. They had also previously shown that the RNA binding protein Aret was salm-dependent.

In the submitted paper the single key finding is they identify aret as acting downstream of salm to regulate alternative splicing. They find that aret also affects gene transcription and also that some muscle alternative splicing does not require aret. Thus, Aret is not the "whole story", but the authors convincingly show it an important player in muscle type identity. The paper also demonstrates that salm controls expression and alternative splicing of many differentially expressed sarcomeric genes and documents the requirement for aret in fibrillar muscle development.

A great deal of information is presented (9 Figs plus all the Supplemental information). This includes "RNA seq" of 3 different muscles, salm-mutant muscle and finally Aret-mutant muscle. This is high quality experimental work and the Figures are impressive (including helpful overview of their working model (Fig9)). Another strength of the study is that it is in vivo in a classic developmental system and is thus demonstrably of functional relevance. Generally, there is good evidence for the conclusions drawn - however, please see the points below for occasions where the presentation could be more precise.

The authors refer to a recently published paper from another group on the Aret protein, which shows "that Aret regulates splicing of sls, wupA and ZASP52 in" *Drosophila* fibrillar flight muscles. Clearly there is overlap with these published results, but the submitted paper from the Schnorrer lab is based on a different approach and presents significant other information.

The submitted work is important to the field of muscle development in the classic model organism *Drosophila melanogaster*. This is valuable in itself, but is also a valuable paradigm for alternative splicing in muscle in other organisms (see the authors' pertinent Discussion). The significance extends beyond muscle though, because there is still much to learn about the regulation of alternative splicing in physiologically relevant systems.

Evidence to support the conclusions drawn?

p6 They say: "To confirm our RNA-Seq data and to investigate the expression and localisation". Strictly, their experiments do not confirm the RNA-seq data, rather they confirm a very small subset (which is not picked at random).

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P10 They say: "However, correct expression of a significant fraction of the sarcomeric genes in fibrillar muscle does require aret function." I couldn't see the evidence for this "significant fraction" presented. Rather they describe 3 examples of such genes, albeit nicely documented (and also two not aret-dependent).

In the Discussion they say: "A role for Aret in directly regulating splicing in *Drosophila* was not known prior to this work." However, I cannot see a demonstration of a direct effect in this paper.

The description of the timing of Aret nuclear expression and of muscle isoform alternative splicing could be clearer. There does not seem to be a compelling correlation. For example Fig6-associated text says: "This suggests that Aret is required for myofiber maturation and sarcomere growth happening after 48h APF, potentially correlating with its nuclear localisation from 72h APF onwards." The Timing doesn't seem quite right. In the Discussion they say "until 48h APF [Aret] is found largely in the cytoplasm of the developing IFMs". In contrast, the Fig5-associated text says the majority of Aret is cytoplasmic from 24-60h. In the Discussion they also say: "Interestingly, a number of these splicing events occur between 48h and 90h APF". This questions which are due to Aret, in particular those at 60h or before.

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Other

They could comment on the fibrillar alternative splicing not regulated by Aret. Are there other candidate RNA-binding proteins down regulated in Salm mutants?

Define "RRM".

Referee #3:

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page 10 should say: "..., expression of the tubular muscle-specific Mlp84B is strongly upregulated in..." instead of "gained" Also, this should be better explained. I can see that there is no alternative splicing of Mlp84B, but it is still spliced, and in adults, Aret is in the nucleus and could affect Mlp84B splicing, therefore it is not obvious to me why it should be an indirect effect. Especially the last sentence of this paragraph is confusing: "This regulation happens both at a transcriptional and a splicing level." What is the evidence for transcriptional regulation through Aret? Even if Aret activates a transcription factor that upregulates Mlp84B, the transcription factor activation could be on the level of splicing.

1st Revision - authors' response

25 November 2014

Referee #1:

In this study, Spletter and colleagues investigated the roles of Salm and Aret during indirect flight muscle (IFM) development. Muscle-specific knock-down or deletion of the transcriptional regulator salm resulted in tubular conversion of IFM. RNA-Seq analysis of salm mutant IFM identified a large number of salm-dependent splicing events, some of which were validated by isoform-specific reporters. Since expression of the RNA binding protein aret was lost in salm-IR IFM, the authors reasoned that aberrant splicing in salm mutants was mainly caused by aret. To prove the hypothesis that aret acts as a splicing regulator by which salm controls IFM-specific splicing, an aret knock-down experiment was performed. Loss of aret led to a "flightless" phenotype with severe defects in myofibril formation. RNA-Seq analysis of aret loss-of-function IFM revealed that some but not all salm-depending splicing events were similarly affected in aret mutant IFM.

This is a quite interesting and well-conducted study with detailed phenotypic analyses. However, more detailed molecular insights into the regulation of Aret-dependent splicing are lacking. It also remains unclear how salm regulates aret. Despite substantial overlapping with the work from Oas et al (J. Cell Bio, 2014), this study significantly deepens the understanding of the regulatory network controlling muscle-specific splicing in flies. In particular the transcriptome-wide identification of salm and aret-regulated genes and splicing events gives the reader a more comprehensive overview of salm/aret-regulated events. Furthermore, this study also revealed that Aret undergoes dynamic subcellular trafficking, which might contribute to IFM development.

We thank the reviewer for appreciating the importance and significance of our study.

Specific points:

A more detailed analysis of already existing RNA-Seq data from *salm* and *aret* should be performed in order to disclose how many splicing events regulated by *salm* do indeed depend on *aret*. This would help to support the claim that *aret* is a major downstream target employed by *salm* to direct IFM-specific splicing.

We thank the reviewer for this interesting point. Following the reviewer's suggestion, we have redone the entire bioinformatic analysis. We have added duplicates for all mRNA-Seq samples, which now allowed a much more thorough analysis using the DESeq2 and DEXSeq programs to identify regulated genes and regulated exons, respectively. Our earlier conclusions regarding exons or isoforms regulated by *Salm* all hold true. In a fibrillar core set of more than 700 genes, at least one exon is up regulated in flight muscles in a *salm* dependent way. These genes are also highly enriched for sarcomeric genes, thus providing a direct link to functional properties of flight muscles. These results are presented in Figure 2.

Additionally, we have now also analysed the *aret-IR* IFM Seq data using the same DESeq2 and DEXSeq analysis. We can now demonstrate that nearly 80 % of the *salm* regulated exons are also regulated by *aret*, strongly supporting our point that *Aret* regulates a large subset of the *salm* targets by regulating their splicing. This analysis also showed that the overlap at the level of transcription regulation is rather small (14 %). We have included this new analysis in a new figure (now Figure 7). As our data supporting *Aret* function to regulate IFM-specific splicing is strongly expanded, we also added "splicing" to the title of our manuscript.

Obviously, transgenic expression of *aret* in *salm* mutants would provide additional insights about the relative contribution of *aret* to the *salm* phenotype and identify splicing events that (indirectly) depend on *salm* but not on *aret*.

We have tried to rescue the *salm* phenotype by re-expressing *aret*; however that experiment was not successful. The reason for that is that over-expression of *aret* with Mef2-GAL4 using *UAS-aret* already leads to severe muscle defects, resulting in early fiber degeneration. However, we need to use Mef2-GAL4 for efficient knock-down of *salm* or for flip-out of our conditional allele, and thus we cannot use a weaker or later expressed GAL4. Hence, this experiment is currently not possible and needs to wait for a GAL4 independent re-expression of *aret* at close to physiological levels.

In addition, the authors did barely touch on the regulation of *aret* by *salm* referring to a previous study, which is slightly disappointing.

We do show in this study that *aret* expression in IFMs, both on the mRNA and protein level, is dependent on *salm* function (Figure 3A, B, E). We have also confirmed the *salm* requirement for *Aret* protein expression during development, however did not present that in manuscript, as it does not really add an additional point. We could provide these data to the reviewer if necessary.

Finally, it remains enigmatic HOW *aret* regulates splicing and whether *aret* acts as bona fide splicing factor or exerts its functions by different means. Although the study would gain a lot by the latter mentioned experiments I do not consider them mandatory.

We absolutely agree with the reviewer that these will be the next exciting challenges to address in the near future.

In summary, this is nice and very well done study, which goes significantly beyond the previously published results by Oas et al. In my view the genetic analysis and the phenotype characterization is top of the class while the mechanistic part is less well developed. Although the latter shortcomings can be addressed to improve the manuscript and make it more comprehensive the resulting workload is probably not appropriate given the specific circumstances.

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In the submitted paper the single key finding is they identify aret as acting downstream of salm to regulate alternative splicing. They find that aret also affects gene transcription and also that some muscle alternative splicing does not require aret. Thus, Aret is not the "whole story", but the authors convincingly show it an important player in muscle type identity. The paper also demonstrates that salm controls expression and alternative splicing of many differentially expressed sarcomeric genes and documents the requirement for aret in fibrillar muscle development.

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The submitted work is important to the field of muscle development in the classic model organism *Drosophila melanogaster*. This is valuable in itself, but is also a valuable paradigm for alternative splicing in muscle in other organisms (see the authors' pertinent Discussion). The significance extends beyond muscle though, because there is still much to learn about the regulation of alternative splicing in physiologically relevant systems.

We thank the reviewer for highlighting the high quality of our data and their importance for the field even beyond muscle biology.

Evidence to support the conclusions drawn?

p6 They say: "To confirm our RNA-Seq data and to investigate the expression and localisation". Strictly, their experiments do not confirm the RNA-seq data, rather they confirm a very small subset (which is not picked at random).

We have reworded that statement to be more precise.

P7 They say: "Together, these systematic data demonstrate that salm indeed determines fibrillar muscle physiology by controlling expression and alternative splicing of many differentially expressed sarcomeric genes." Is it known that these isoforms do control physiology in this system? Their discussion comment on p10 is more circumspect: "These unique isoform combinations potentially determine the specific physiological and biophysical features of the different muscle types."

We have toned down our statement on P7 a bit to avoid a too strong claim.

P10 They say: "However, correct expression of a significant fraction of the sarcomeric genes in fibrillar muscle does require aret function." I couldn't see the evidence for this "significant fraction" presented. Rather they describe 3 examples of such genes, albeit nicely documented (and also two not aret-dependent).

We have now included a much more thorough and systematic analysis of the *aret-IR* IFMs by RNA-Seq (see response to reviewer one above), now providing very strong evidence that

many exons of the fibrillar gene set are indeed regulated by Aret, a good portion of which are sarcomeric genes (Figure 2E and new Figure 7).

In the Discussion they say: "A role for Aret in directly regulating splicing in *Drosophila* was not known prior to this work." However, I cannot see a demonstration of a direct effect in this paper.

We have deleted "directly" from this sentence.

The description of the timing of Aret nuclear expression and of muscle isoform alternative splicing could be clearer. There does not seem to be a compelling correlation. For example Fig6-associated text says: "This suggests that Aret is required for myofiber maturation and sarcomere growth happening after 48h APF, potentially correlating with its nuclear localization from 72h APF onwards." The Timing doesn't seem quite right. In the Discussion they say "until 48h APF [Aret] is found largely in the cytoplasm of the developing IFMs". In contrast, the Fig5-associated text says the majority of Aret is cytoplasmic from 24-60h. In the Discussion they also say: "Interestingly, a number of these splicing events occur between 48h and 90h APF".

This questions which are due to Aret, in particular those at 60h or before.

We have not investigated the sarcomere length at 60h APF to address a potential sarcomere growth between 48h and 60h APF. However, we now provide systematic mRNA-Seq data of wild-type and *aret-IR* IFMs showing that a significant fraction of exons is already regulated at 30h and 72h, however that fraction more than doubles between 72h APF and 1 day adulthood. This shows that even before the detected Aret nuclear concentration at 72h, Aret regulates splicing of many proteins. We tried to make this clearer in the revised version.

Figures

Can the GO information be more clearly explained/presented (Fig 2B)?

We now provide the full GO component list using REVIGO (Figure 2C) and a selected tree highlighting the important enrichments for contractile fiber and mitochondrial parts (Figure 2D). We hope this is now clearer.

Table E1 is not user-friendly, nor is Fig 2C.

We generated a new heatmap for Figure 2C, now called Fig 2E, that highlights exons on the right-hand side from some important sarcomeric genes analysed in this study to make it more user friendly. We have also reorganized the supplemental tables, colour coding important data columns, cutting non-essential data and adding guide tabs that explain the data included in the tables. We have additionally added raw data files of the significantly differentially expressed genes/exons for any reader wanting to get into the details of the mRNA-Seq data, in addition to the GEO uploaded data. Hopefully these materials are more user-friendly.

From the figure legends etc it is not always apparent how the images were obtained, e.g. Figs 4B and 4H.

We tried to make the Figure legends as clear as possible. We do provide a reference for the details how hemi-thoraces, as shown in Figure 4B and 4H, were generated (by cutting with a razor blade after fixation) in the extended Methods section.

Arrows on some images would help, e.g. in Fig5A to show "Aret protein is localised to substructures of the large larval nuclei in the muscle templates, but not in the nuclei of the fusing adult myoblasts".

We have added arrowheads to Figure 5A.

Other

They could comment on the fibrillar alternative splicing not regulated by Aret. Are there other candidate RNA-binding proteins down regulated in *Salm* mutants?

We now provide data that most of the *salm* regulated alternative splicing acts via Aret. At the moment we are not aware of additional RNA binding proteins that are functionally involved.

Define "RRM".

We have included "RNA recognition motif" to define the abbreviation.

Referee #3:

This is a solid manuscript identifying downstream factors of spalt in IFM specification. Compared to Cripps, it is much more extensive and quantitative in the screening part, but less extensive in detailing the molecular mechanism of arrest function.

We thank the reviewer for appreciating the importance of our work.

page 10 should say: "..., expression of the tubular muscle-specific Mlp84B is strongly upregulated in..." instead of "gained" Also, this should be better explained. I can see that there is no alternative splicing of Mlp84B, but it is still spliced, and in adults, Aret is in the nucleus and could affect Mlp84B splicing, therefore it is not obvious to me why it should be an indirect effect. Especially the last sentence of this paragraph is confusing: "This regulation happens both at a transcriptional and a splicing level." What is the evidence for transcriptional regulation through Aret? Even if Aret activates a transcription factor that upregulates Mlp84B, the transcription factor activation could be on the level of splicing.

We thank the reviewer for this important point. Our newly included systematic analysis of the *aret* loss of function IFMs now quantifies the splicing vs. transcriptional role much better (Figure 7), and we have adjusted our manuscript accordingly.

2nd Editorial Decision

02 December 2014

We have now received the referee report (copied below) on your revised manuscript, and I am very pleased to accept it for publication in the next available issue of EMBO reports. Please remember to send us the final manuscript file with 8 main figures today, as discussed.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

REFeree REPORT:

Referee #1:

Spletter et al have submitted a revised version of their manuscript addressing the role of Salm and Aret in indirect flight muscle (IFM) development. The authors have significantly improved the bioinformatics analysis and conducted additional RNA-Seq studies demonstrating that a large set of exon splicing events depends on Sam. In addition, they now show that nearly 80 % of Salm dependent exons are also regulated by Aret further strengthening the argument that Aret regulates numerous Salm targets by regulation of splicing.

The authors also explained convincingly why a rescue of the Salm phenotype by Aret is currently not possible (developmental defects caused by strong overexpression of Aret via Mef2-GAL4/UAS-Aret).

Although I would have welcomed more detailed studies about the regulation of splicing by Aret I agree that such studies would be beyond the scope of the current manuscript.

In conclusion, the revision improved an already strong manuscript, which now unambiguously demonstrates that Aret-dependent splicing defects are a major cause of the Salm phenotype in flies.