Drosophila Activin signaling promotes muscle growth through InR/TORC1-dependent and -independent processes

Myung-Jun Kim and Michael O'Connor
DOI: 10.1242/dev.190868

Editor: Thomas Lecuit

Review timeline
Original submission: 29 March 2020
Editorial decision: 19 May 2020
First revision received: 19 August 2020
Editorial decision: 16 October 2020
Second revision received: 2 November 2020
Accepted: 16 November 2020

Original submission

First decision letter

MS ID#: DEVELOP/2020/190868

MS TITLE: Drosophila Activin signaling promotes muscle growth through InR/dTORC1 dependent and independent processes

AUTHORS: Myung-Jun Kim and Michael O'Connor

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Reviewer 1

Advance summary and potential significance to field

The manuscript by Kim and O'Connor reports new findings related to mode of action of Activin/TGF-beta signaling and its downstream targets in growing Drosophila larvae and in particular how it regulates the size of body wall muscles. This paper complements published last year work from the

O'Connor lab showing that the motor neuron derived Activin signaling plays a major role in scaling muscle size in growing larvae. In the present paper authors try to understand intrinsic muscle response to TGF-beta signals. They show that components of InR/dTORC1 pathway are activated by Activin leading to an increase in MHC levels and positive regulation of muscle width. They also observe that Activin positively influences muscle length regulated by the addition of new sarcomeres (this has already been shown in 2019 paper) and that this Activin function is independent of InR/dTORC1. Overall, presented data are convincing (even if in opposition to negative regulation of Insulin/TOR by Myostatin in vertebrates) and provide new clues for dissecting multiple functions of TGF-beta in developing vertebrate muscles. Below are my suggestions to authors:

Comments for the author

1. Authors previously demonstrated that size of nuclei changes in Activin mutant muscles, and size of myonuclei is related to endoreplication. Thus, one possibility is that endoreplication is also regulated by TGF-beta and could have impact on muscle size. Is there any evidence on that from the RNAseq of Smad2 mutant and babo GOF larvae ? Influence on nuclei size/endoreplication needs at least to be discuss.

2. Authors perform their analyses on muscle 6, which as shown in Fig. 1 is indeed reduced in length and width in both babo and Smad2 mutant context. However, from Fig.1A one could see that the adjacent muscle 7 seems not to differ in width but only looks shorter. To better document changes in muscle size and in particular muscle width, which is differentially regulated, authors should provide measures of more than one larval muscle.

3. Activin is distributed via heamolymph (systemic supply) and is also supplied to muscle via cell-cell contact (motor neurons). Regarding author's previous work it would be interesting to test whether InR/dTORC1 dependent versus independent effects on muscle size are both downstream of motor neuron derived Activin signals.

Minor points:
1. In Fig. 1A dorsal seems down which is unusual for muscle views.
2. Evidence for no change in sarcomere size is lacking.
3. Documentation of effects of PDK1 and AKT levels (GOF and LOF) on muscle size via immunostainings would support their functions.

Reviewer 2

Advance summary and potential significance to field

Myung-Ju Kimi and Michael O'Connor describe an interesting observation regarding the function of Activin signaling in muscle growth. In contrast to the activity of this pathway in mammals, where the myostatin-dependent activin signaling was shown to inhibit muscle growth, in Drosophila this pathway is shown to be a positive element in promoting muscle growth, and in addition the authors show that the major ligand that triggers this activity is activin beta (Act-b). The authors further show that Act-b signaling regulates positively the InR/dTORC1 pathway, and that it acts through promoting the transcription of Pdk1 and Akt1. In addition, they further show that downstream of this pathway is the regulation of Myosin Heavy Chain (MHC) protein levels. However, this alone could not explain muscle growth.
Interestingly the authors demonstrate a differential regulation of muscle width and muscle length by the two TGF-b pathways. Enhancing Ins/dTORC1 led to an increase in muscle width, whereas enhancing the Act-b pathway elevated both muscle width and muscle length. I find this manuscript interesting and novel. The evidence for the authors model is often convincing, however several important controls are missing (see below).

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Major comments:
1. Fig 1: Muscle length as well as width should be normalized to larval length and width. It has been demonstrated that the size of somatic muscles is highly sensitive to larval size and vice versa. If the mutant larvae are smaller in size it would reflect muscle length, as well as muscle width. If the authors are unable to differentiate between larval size and muscle size, they should at least show that muscle length or width are reduced in baboo and smad knockdown specifically in muscles.
2. Counting the Z-discs: I was unable to see in the manuscript the method of Z-discs counting. Was it unbiased and automated? Since the sarcomeres are not in a single plane, it would be extremely difficult to count individual z discs.
3. How the surface area was measured? Did the authors performed analysis at 3D?
4. Fig 1C: Overexpression of dSmad2 in dsmad2 mutant should be compared to both dsmad2 mutants and to muscles overexpressing dsmad2.
5. Fig2: the authors show the changes in phosphorylation intensity, but they should show that the protein levels are the same, or alternatively normalize it to the protein level. Also, what are the two bands shown for pAKT? I assume that the authors summed the intensity of both?
6. Page 17: “In contrast, the total amount of Actinin (Actn)” . Did the authors mean alpha-actinin?

Reviewer 3

Advance summary and potential significance to field

This manuscript investigates the role of TGF-beta signalling, specifically Activin signalling, in regulating larval muscle fiber size in Drosophila. This is interesting because it is well established that Activin/Myostatin negatively regulates muscle fiber size in mammals by down-regulating insulin signalling. However, the authors show here in mechanistic detail that Activin signalling rather promotes muscle fiber growth in the Drosophila larval model.
The authors investigate this effect in mechanistic detail using an extensive collection of genetic tools. They show convincingly that Activin signalling differentially impacts TORC1 and TORC2 activity leading to differential S6K and Akt phosphorylation. More Activin signalling boosts insulin signalling by transcriptionally inducing Akt and PDK1, this stimulates TORC1 and thus S6K phosphorylation.
The authors provide evidence that the enhanced S6K activity results in more efficient translation of a key sarcomeric protein, Myosin heavy chain, and thus may provide a mechanism how sarcomeres and thus muscle fibers grow: fibers expand in diameter by lateral sarcomere growth or in length by assembly of additional sarcomeres. Most of these data appear solid and the experiments are well controlled and documented.

Comments for the author

The authors observe that muscle autonomous direct up-regulation of PDK1 or S6K activity results largely in muscle fiber diameter but not length increase, whereas increased Activin signalling (baboon gain of function) rather promotes muscle fiber length growth. Thus, the authors speculate that the Activin signalling pathway somehow sets muscle fiber geometry by differentially regulating the transcription of sarcomeric components. While this is certainly an interesting hypothesis, the current version of the papers does not take into account that length of every muscle fiber is

determined by its attachment sites, here the distance between the tendons cells in the epidermis. Thus, the here studied VL muscles can only grow in length if the length of the larval segment increases as well. It is well established that the number of sarcomeres is not determined genetically, but biomechanically to match optimal sarcomere length with mechanical (passive) tension at the relaxed muscle state. A too long fiber with too many sarcomeres would not be able to contract efficiently, thus mechanical feedback adjusts the amount of sarcomeres. This fits to the here reported diameter growth but length growth in case of direct S6K manipulation.
In the discussion the authors speculate on a differential role of the Zasp proteins for length versus width growth of the larval muscle fibers. It is important to remember that currently there is no evidence for differential localisation of any sarcomeric component to certain sarcomeres but not others in a muscle fiber. González-Morales et al., 2019 provide interesting evidence that the isoform balance between growing and blocking Zasp isoforms regulates myofibril size in fibrillar flight muscles, in which each myofibril has stereotyped diameter. In larval muscles instead myofibrils are less well defined and all align laterally resulting in the observed cross-striated pattern, not present in flight muscles.
Hence, it would be informative to discuss a combination of muscle autonomous and non autonomous effects that Activin signalling may induce, including mechanical feedbacks, that likely contribute to fiber geometry. E.g. intense exercise in adult humans largely expands muscle fiber diameter but not length, as the skeleton cannot grow anymore.

Additional points.
1. It would be nice to show representative muscle fiber stainings for all the genotypes shown at least for figures 1 and 6, potentially also for some that are currently only used for western blotting.

2. How was larval age controlled? As size of the larva should determine muscle fiber length of the VL muscles this is critical and currently not well explained in the methods.
At the end of Figure 1, the authors state that Act signalling regulates muscle length independently of larval body size. On which evidence is this conclusion based? I did not find larval body length measurements. The sum of the length of all abdominal VL1 muscles should directly determine the length of the larvae.

3. Would it be possible to measure the 3D volume of VL fibers by scanning 3D stacks? My feeling is that the widely reported 50 fold increase of muscle volume from L1 to L3 is rather an underestimate as the not been carefully quantified. L1 VL muscles are about 50µm long and L3 > 500. This is a suggestion for the future rather than an essential point for this paper.

4. The current model figure is very complicated and difficult to understand. It is hard to grasp the impact of TGF beta on transcription, with the resulting consequence in signalling which then impinges on translation of sarcomeric components (translation is not mentioned). The signalling part is very complex and potentially unnecessarily detailed. In particular, the link from Pdk1 to T342 in Akt1 is never mentioned in the paper and thus potentially confusing, as the paper stresses the effect of Pdk1 on a reduction of P-Akt1 levels.

First revision

Author response to reviewers' comments

Reviewer 1 Advance summary and potential significance to field
The manuscript by Kim and O'Connor reports new findings related to mode of action of Activin/TGF-beta signaling and its downstream targets in growing Drosophila larvae and in particular how it regulates the size of body wall muscles. This paper complements published last year work from the O'Connor lab showing that the motor neuron derived Activin signaling plays a major role in scaling muscle size in growing larvae. In the present paper authors try to understand intrinsic muscle response to TGF-beta signals. They show that components of InR/dTORC1 pathway are activated by Activin leading to an increase in MHC levels and positive regulation of muscle width. They also observe that Activin positively influences muscle length regulated by the addition of new

sarcomeres (this has already been shown in 2019 paper) and that this Activin function is independent of InR/dTORC1. Overall, presented data are convincing (even if in opposition to negative regulation of Insulin/TOR by Myostatin in vertebrates) and provide new clues for dissecting multiple functions of TGF-beta in developing vertebrate muscles. Below are my suggestions to authors:

Reviewer 1 Comments for the author
1. Authors previously demonstrated that size of nuclei changes in Activin mutant muscles, and size of myonuclei is related to endoreplication. Thus, one possibility is that endoreplication is also regulated by TGF-beta and could have impact on muscle size. Is there any evidence on that from the RNAseq of Smad2 mutant and babo GOF larvae ? Influence on nuclei size/endoreplication needs at least to be discuss.

This is a good point. We've looked the expression of Myc, a well-known stimulator of endoreplication, in RNAseq results and found no change in transcript level. The RNAseq result was verified by qPCR. To determine if endoreplication is involved in the smaller muscle phenotype in a more direct way, we overexpressed Myc in the muscle of dSmad2 mutant and found that it does not rescue the muscle size even though it greatly increases the nuclear size. Therefore, we think Activin pathway promotes muscle growth in a way that does not involve an effect on endoreplication. The new results are presented in Fig. S4.

2. Authors perform their analyses on muscle 6, which as shown in Fig. 1 is indeed reduced in length and width in both babo and Smad2 mutant context. However, from Fig.1A one could see that the adjacent muscle 7 seems not to differ in width but only looks shorter. To better document changes in muscle size and in particular muscle width, which is differentially regulated, authors should provide measures of more than one larval muscle.

We performed the same assay on muscle 7 of abdominal segment 2 as well as on muscle 6 and 7 of segment 3 and obtained similar results: that is, reductions in Z-disc number and width in babo and dSmad2 mutant muscles, leading us to conclude that the effect of Activin pathway on muscle growth is not limited to certain muscle(s) but is a general effect. The results from muscle 7 of abdominal segment 2 are presented in Fig. S1B. The decrease in the width of muscle 7 seems to be unimpressive probably because the width of muscle 7 is much narrower and any change will be smaller in real length making it hard to appreciate by eye. However it is apparent upon quantification.

3. Activin is distributed via heamolymph (systemic supply) and is also supplied to muscle via cell-cell contact (motor neurons). Regarding author's previous work it would be interesting to test whether InR/dTORC1 dependent versus independent effects on muscle size are both downstream of motor neuron derived Activin signals.

We measured muscle width, thickness and Z-disc number from larvae expressing Actbeta in the motor neurons of Actβ mutant as well as controls and found that Actbeta expression rescues the reductions in growth along the three axes. From these, we proposed that the motor neuron-derived Actβ is the major ligand responsible for inducing Activin signaling necessary for InR/dTORC1-dependent and independent muscle growth. The results are presented in Fig. 1.

Minor points:
1. In Fig. 1A dorsal seems down which is unusual for muscle views.

We flipped the images, so the dorsal side is now up.

2. Evidence for no change in sarcomere size is lacking.

We measured the Z-disc intervals as a way to assess the sarcomere size and found that it is decreased in babo but not in dSmad2 mutant. So it appears that there is an additional decreasing effect on muscle length in babo mutant. The result is presented in Fig. S1C and D.

3. Documentation of effects of PDK1 and AKT levels (GOF and LOF) on muscle size via immunostainings would support their functions.

We measured muscle width and Z-disc number from the larvae expressing Pdk1-RNAi. The result is presented in Fig. 6C and is in line with the idea that PDK1 activity is necessary to increase the width but not the length of the muscle. We tried to perform the same assay using Akt1-RNAi larvae but they are rather too small to do that.

Reviewer 2 Advance summary and potential significance to field
Myung-Ju Kimi and Michael O'Connor describe an interesting observation regarding the function of Activin signaling in muscle growth. In contrast to the activity of this pathway in mammals, where the myostatin-dependent activin signaling was shown to inhibit muscle growth, in Drosophila this pathway is shown to be a positive element in promoting muscle growth, and in addition the authors show that the major ligand that triggers this activity is activin beta (Act-b). The authors further show that Act-b signaling regulates positively the InR/dTORC1 pathway, and that it acts through promoting the transcription of Pdk1 and Akt1. In addition, they further show that downstream of this pathway is the regulation of Myosin Heavy Chain (MHC) protein levels. However, this alone could not explain muscle growth. Interestingly the authors demonstrate a differential regulation of muscle width and muscle length by the two TGF-b pathways. Enhancing Ins/dTORC1 led to an increase in muscle width, whereas enhancing the Act-b pathway elevated both muscle width and muscle length. I find this manuscript interesting and novel. The evidence for the authors model is often convincing, however several important controls are missing (see below).

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We agree with this point, but based on the findings that dSmad2 expression in the muscle of dSmad2 mutant rescues all the aspects regarding muscle size, we think that the reduction in muscle dimesions is the origin of the size problem. In addition, expression of Actbeta in the motor neurons of Act beta mutant which is shown to rescue the muscle size (Fig. 1G and H in revised figure) rescues the pupal volume (Fig. 7 in Moss-Taylor et al., 2019). Because the pupal volume reflects the larval size, we propose the possibility that it is the change in muscle size that initiates the change in the whole body in Activin pathway mutants. That will warrant the worth of muscle measurement on its own.

2.Counting the Z-discs: I was unable to see in the manuscript the method of Z-discs counting. Was it unbiased and automated? Since the sarcomeres are not in a single plane, it would be extremely difficult to count individual z discs.

We used PeakFinder macro of ImageJ to first detect the Z-discs from anti-Actinin staining and manually corrected some errors. We newly stated this in the methods section and a representative image is presented in Fig. S1C. Because the Z-discs seem continuous from top to the bottom of the muscle (Fig. 1D), we think that counting the Z-discs on the surface of the muscle would not introduce a significant error in the number.

3.How the surface area was measured? Did the authors performed analysis at 3D?

We added new results from assays to measure the muscle thickness (Fig. 1D, E, G, H; Fig. 6B). Together with the measurements of muscle width and Z-disc number (length), this provides the 3D measurement.

4.Fig 1C: Overexpression of dSmad2 in dsmad2 mutant should be compared to both dsmad2 mutants and to muscles overexpressing dsmad2.

We compared it to dSmad2 mutant because we were interested in if it rescues the muscle size or not. But as you pointed out, it will be interesting if dSmad2 overexpression in dSmad2 mutant

promotes the muscle growth to the level of dSmad2 overexpression in otherwise wild-type muscle. Because of the time limit, we would like to do it in the future.

5.Fig2: the authors show the changes in phosphorylation intensity, but they should show that the protein levels are the same, or alternatively normalize it to the protein level. Also, what are the two bands shown for pAKT? I assume that the authors summed the intensity of both?

We showed that pan AKT level is decreased in babo and dSmad2 mutants (Fig. 4C), so if we normalize the pAKT to pan AKT level, there will be even greater increases in these mutants. The two bands of pAKT are from two isoforms and I summed them. Most papers show the two bands together and in most cases, the two bands behavior similarly. Unfortunately, there is no antibody available for S6K so we couldn't investigate if the total protein level of S6K is changed or not in Activin pathway mutants. The RNAseq (Fig. 4A) and qPCR (data not shown) results show that S6k transcript is not changed in babo and dSmad2 mutants, and thus we assume that the total protein level is not changed.

6.Page 17: "In contrast, the total amount of Actinin (Actn)" . Did the authors mean alpha-actinin?

Yes, it is anti-Actinin. We modified the figures and text accordingly.

Reviewer 3 Advance summary and potential significance to field
This manuscript investigates the role of TGF-beta signalling, specifically Activin signalling, in regulating larval muscle fiber size in Drosophila. This is interesting because it is well established that Activin/Myostatin negatively regulates muscle fiber size in mammals by down-regulating insulin signalling. However, the authors show here in mechanistic detail that Activin signalling rather promotes muscle fiber growth in the Drosophila larval model.
The authors investigate this effect in mechanistic detail using an extensive collection of genetic tools. They show convincingly that Activin signalling differentially impacts TORC1 and TORC2 activity leading to differential S6K and Akt phosphorylation. More Activin signalling boosts insulin signalling by transcriptionally inducing Akt and PDK1, this stimulates TORC1 and thus S6K phosphorylation. The authors provide evidence that the enhanced S6K activity results in more efficient
translation of a key sarcomeric protein, Myosin heavy chain, and thus may provide a mechanism how sarcomeres and thus muscle fibers grow: fibers expand in diameter by lateral sarcomere growth or in length by assembly of additional sarcomeres. Most of these data appear solid and the experiments are well controlled and documented.

Reviewer 3 Comments for the author
The authors observe that muscle autonomous direct up-regulation of PDK1 or S6K activity results largely in muscle fiber diameter but not length increase, whereas increased Activin signalling (baboon gain of function) rather promotes muscle fiber length growth. Thus, the authors speculate that the Activin signalling pathway somehow sets muscle fiber geometry by differentially regulating the transcription of sarcomeric components. While this is certainly an interesting hypothesis, the current version of the papers does not take into account that length of every muscle fiber is determined by its attachment sites, here the distance between the tendons cells in the epidermis. Thus, the here studied VL muscles can only grow in length if the length of the larval segment increases as well. It is well established that the number of sarcomeres is not determined genetically, but biomechanically to match optimal sarcomere length with mechanical (passive) tension at the relaxed muscle state. A too long fiber with too many sarcomeres would not be able to contract efficiently, thus mechanical feedback adjusts the amount of sarcomeres. This fits to the here reported diameter growth but length growth in case of direct S6K manipulation. In the discussion the authors speculate on a differential role of the Zasp proteins for length versus width growth of the larval muscle fibers. It is important to remember that currently there is no evidence for differential localisation of any sarcomeric component to certain sarcomeres but not others in a muscle fiber. González-Morales et al., 2019 provide interesting evidence that the isoform balance between growing and blocking Zasp isoforms regulates myofibril size in fibrillar flight muscles, in which each myofibril has stereotyped diameter. In larval muscles instead myofibrils are less well defined and all align laterally resulting in the observed cross-striated pattern, not present in flight muscles. Hence, it would be informative to discuss a combination of muscle autonomous and non

autonomous effects that Activin signalling may induce, including mechanical feedbacks, that likely contribute to fiber geometry.
E.g. intense exercise in adult humans largely expands muscle fiber diameter but not length, as the skeleton cannot grow anymore.

This is a good point and we added a paragraph about this in the discussion section.

Additional points.
1. It would be nice to show representative muscle fiber stainings for all the genotypes shown, at least for figures 1 and 6, potentially also for some that are currently only used for western blotting.

We added new images about side view (Fig. 1D) and enlarged view (Fig. S1C) of the muscle staining.

2. How was larval age controlled? As size of the larva should determine muscle fiber length of the VL muscles this is critical and currently not well explained in the methods.

We used wandering stage larvae and that is stated in the method section.

At the end of Figure 1, the authors state that Act signalling regulates muscle length independently of larval body size. On which evidence is this conclusion based? I did not find larval body length measurements. The sum of the length of all abdominal VL1 muscles should directly determine the length of the larvae.

We have proposed this based on the findings that dSmad2 expression rescues the decrease in the size of dSmad2 muscle, and expression of Actbeta in the motor neurons rescues the pupal volume of the Actβ mutant (Fig. 7 in Moss-Taylor et al., 2019). But, to avoid any confusion, we toned down the statement.

3. Would it be possible to measure the 3D volume of VL fibers by scanning 3D stacks? My feeling is that the widely reported 50 fold increase of muscle volume from L1 to L3 is rather an underestimate as the not been carefully quantified. L1 VL muscles are about 50µm long and L3 > 500. This is a suggestion for the future rather than an essential point for this paper.

We added new measurements of muscle thickness, so together with the width and z-disc (length) measurements, this provides the 3D measurement.

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We removed some components in the model figure and added graphics to intuitively explain how the Activin and InR/dTORC1 pathways influence the different aspects of muscle growth.

Second decision letter

MS ID#: DEVELOP/2020/190868

MS TITLE: Drosophila Activin signaling promotes muscle growth through InR/dTORC1 dependent and independent processes

AUTHORS: Myung-Jun Kim and Michael O'Connor

I am very sorry and apologise for the long delay before coming back to you.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that you address the remaining very pertinent comments from Reviewer 3. I think that text edit to consider the alternative interpretations mentionned by this referee will be sufficient.The paper will not be sent back to reviewers but please detail in your response the exact text changes you have included to address these comments. I will look at this directly and make sure to take a decision as soon as possible.

Reviewer 1

Advance summary and potential significance to field

Authors responded convincingly to all my comments. They generated new data which enriched further this well designed and nicely documented study. One modification I would suggest is related to the outcome from the performed rescue experiment (major point 3 in my comment). These new data allow now to say that motor neuron derived Activin beta stimulates muscle growth via InR/dTORC1 dependent and independent mechanisms. This important information is actually lacking in the abstract. Authors could also envisage to provide it in the title.

Comments for the author

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Interestingly the authors demonstrate a differential regulation of muscle width and muscle length by the two TGF-b pathways. Enhancing Ins/dTORC1 led to an increase in muscle width, whereas enhancing the Act-b pathway elevated both muscle width and muscle length.

Comments for the author

The authors addressed most of my comments. I think the paper is interesting and convincing and should be accepted.

Reviewer 3

Advance summary and potential significance to field

I have detailed in my former review why I find this paper, which investigates the effect Activin signalling in regulating Drosophila larval muscle growth, an interesting paper.
However, I also pointed out why I do not agree with the strong statements that are still in the abstract and throughout the paper that larval muscle geometry is purely determined by autonomous signaling in the muscle; in particular the model with sarcomere ‘expansion factors’ and ‘initiation factors’ is misleading.

Comments for the author

1. Larval size. In my initial review I have discussed that the length of the muscle 6 and 7 (VL1, VL2) used here should be set at least in part by larval size. This was also pointed out be reviewer 2. Unfortunately, this revised version does not address this critical point. Growth in muscle length requires total larval length increase whereas growth in width does not necessarily require that (Tubby mutants!). The former data from the authors have shown that muscle length is regulated by secreted Activin-beta from the motor neurons. Hence, non autonomous effects are important, thus larval size (or segment size) needs to be controlled in these experiments. A simple explanation of the results from Fig1 A and B are that baboon and dSmad2 mutant larvae are simply shorter, hence, their muscles are shorter and smaller in diameter. Conversely, baboCA larvae might be longer, which is suppressed by dSmad-RNAi.
A correlation between muscle 6, 7 (VL1-2) muscle length and larval length does not make the results less interesting.
2. Missing muscle images. In my initial review I asked to 'show representative muscle fiber stainings for all the genotypes shown, at least for figures 1 and 6'.
Unfortunately, the authors did not provide any additional muscle images for the other genotypes. Specifically the ones used in Fig1C, E, F and G, and in Figure 6 in particular the ones that strongly increase or decrease Mhc protein levels are interesting (baboon gain of function vs. suppression by Tor dominant negative).
These could also include a Mhc protein stainings to visualise Mhc at the sarcomeres. If space is limited, these could be partially placed in the supplement.
3. Muscle geometry. As stated previously, I find the hypothesis of the authors that differential incorporation of hypothetical sarcomeric components laterally but not longitudinally that trigger lateral but not length increase of the muscle highly unlikely and not supported by the data.
In this revised version, the authors have also measured 'thickness' of the muscle in Z and find that width and thickness do NOT correlate, in fact they seem to anti-correlate in the different genotypes (Fig. 6A and B). Why do the authors still conclude that these muscles laterally expand using a differential factor?
They expand in Y but shrink in Z.
For me, all these data further support of a strong non autonomous mechanical contribution to control of muscle geometry: if the larvae grows in length, the muscle will do so. If the larva does not grow, but sarcomere protein production is up-regulated artificially in muscle autonomously, then the muscle will grow laterally. It will use available space. Apparently, there is more space in the Y instead of the Z axis ('thickness').
If larvae are short, for example in Tubby mutants (supposedly a component of the cuticle), these short larvae are fat, and likely muscles 6 and 7 are short and fat as well.
The revised model suggests a 'vertical expansion factor' and a 'sarcomere initiation factor', which appear highly unlikely to exist. Non of ZASP proteins would match either of the two definitions. How would these factors differentiate Y and Z?

Minor points.
1. Figure 1D needs labelling. Is this an X-Z view?
2. Typo in Figure and Legend of the genotype in FigS4A; at least if this reviewer has understood the logic of the argument the genotype should be Mef2>Myc and not Mef2>Smad2.
Why appears FigS4 in the text before FigS2 and S3?
3. Typos, e.g.: p 5: stimulus for muscle growth. (Egerman and Glass, 2014).
p 11: segment 3 (data not shown) and obtained the similar results.
p 12: Finally, was previously shown that myonuclei of Actβ mutant are smaller p 17: this appears to be the.
4. Flybase nomenclature: Mhc, not MHC 5. p26 Weitkunat et al., 2014 does not show that 'sarcomeres <form> in a head-to-tail manner during metamorphosis'. They rather assemble simultaneously. Later, the muscle grows and new sarcomeres are added somewhere along the long axis. Where, is not clear to date (Spletter et at. eLife 2018).

Second revision

Author response to reviewers' comments

In response to reviewer 3's comments. We have significantly rewritten the portion of the discussion that considers how Activin signaling affects muscle fiber growth. We believe that we give a fair and balanced discussion of several possible models. We completely agree with reviewer 3 that muscle tension and larval body size are likely to be key components of the regulatory process. However, we do not think he/she gives enough weight to the fact that larval cuticle is different from that of pupa and adults and that there is likely a different type of communication between the muscle and the epidermis during larval stages to coordinate the growth of these functional interconnected tissues compared to pupal development. During pupal development the maximal size that the adult cuticle can assume is set by the pupal case cuticle. Therefore, the mechanism which he/she proposes for indirect flight muscle development does not have to be exactly the same for larva. We have also included a new schematic in Fig. 1A, additional images of larval muscle staining as he/she suggested in Fig 6E, images of larval body size after Activin signal modulation in muscle (Fig. 6G) and a revised summary Fig 7 that better illustrates the various models. In addition we corrected the various typos and formatting errors.

Reviewer 1 suggested we change the title and abstract to include mention of motoneurons as the source of Activin signals that regulate muscle size control. Since that was the major point of a previous published paper, and since in the present manuscript we provided little new data on this point, I am not sure that is appropriate in the title. I did, however, reference the involvement of motoneurons in the abstract.

Third decision letter

MS ID#: DEVELOP/2020/190868

MS TITLE: Drosophila Activin signaling promotes muscle growth through InR/dTORC1 dependent and independent processes

AUTHORS: Myung-Jun Kim and Michael O'Connor
ARTICLE TYPE: Research Article

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